Monensin-induced accumulation of β-D-xyloside-initiated glycosaminoglycans

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Received 21 September 1982

Secretion *Translocation* *Proteoglycan* *Chondrocyte* *Golgi complex* *Endoplasmic reticulum*

1. INTRODUCTION

The carboxylic ionophore, monensin, has profound inhibitory effects upon secretion of macromolecules by eukaryotic cells [1–6]. In certain instances monensin has also been shown to alter post-translational events such as correct glycosylation of proteins [7,8] and the sulfation of proteoglycans [9,10]. When monensin impairs the secretion of proteoglycans from chick embryo chondrocytes [11], the bulk of the accumulated intracellular material is an undersulfated, underglycosylated core protein [12]. If a β-D-xyloside acceptor is used to initiate glycosaminoglycan synthesis in the presence of monensin, undersulfated carbohydrate chains appear in the culture medium [13]. Here, we sought to determine whether the secretion of xyloside-initiated chains is also impaired by monensin, similar to the effect upon proteoglycan molecules. The results indicate that even the xyloside-initiated glycosaminoglycans accumulate intracellularly when monensin is present in the culture medium.

2. MATERIALS AND METHODS

2.1. *Chondrocyte preparation and incubation conditions*

The sterna were dissected from 17-day-old chick embryos and chondrocytes were prepared as in [13]. Monensin was added at 10^{-6} M and 4-methyl umbelliferyl-β-D-xylopyranoside was dissolved in dimethyl sulfoxide and added to the culture medium to give 1 mM xyloside and 0.2% dimethyl sulfoxide as final concentrations. Preincubation was for 1 h at 37°C on a rotary shaker bath, after which the chondrocytes were harvested and resuspended in fresh medium containing $Na_2{}^{35}SO_4$ (10 μCi/ml) and [^{3}H]glucosamine (10 μCi/ml), or [^{14}C]glucosamine (10 μCi/ml) and [^{3}H]serine (50 μCi/ml); the previous monensin and β-D-xyloside concentrations were maintained. After gassing with air containing 5% CO_2, chondrocyte suspensions were incubated for another 4 h until the experiment was terminated by chilling the cultures at 4°C and then separating the cells and medium by centrifugation.

The medium was frozen after addition of the protease inhibitors phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, EDTA, 6-amino caproic acid and benzamidine at final concentrations of 1, 2, 10, 100 and 5 mM, respectively. Cell pellets were washed twice in phosphate-buffered saline and then lysed by suspension for 5 min in 1 ml 1% NP-40, 0.02 M Tris, 0.15 M NaCl (pH 7.4) with the above concentrations of protease inhibitors. Nuclei were pelleted by centrifugation at 15000 rev./min for 2 min. The supernatant was removed and aliquoted for direct analysis and for immunoprecipitation with anti-proteoglycan antiserum.

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Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

2.2. *Immunoprecipitation of cell lysate and culture medium*

Immunoprecipitation was performed on samples labeled with [^{3}H]serine and [^{14}C]glucosamine, using antiserum from rabbits innoculated with purified PGS-1 proteoglycan which was kindly provided by Dr P. Goetinck (University of Connecticut). Serum (20 μl) was added to 500 μl cell lysate or 3 ml culture medium. Incubation was at 4°C for 15 h at which time 8 units of Calbiochem goat-antirabbit gammaglobulin were added. After 2 h at 4°C, the precipitated radioactivity was pelleted by centrifugation at 3000 × *g* for 5 min. The pellets were washed twice with buffer containing protease inhibitors, 0.5% NP-40, 0.15 NaCl, 0.02 M Tris (pH 7.4) then resuspended in 6 M guanidium chloride with protease inhibitors for gel filtration on Sepharose 2BCL.

2.3. *Analytical procedures*

Gel filtration was performed on a Sepharose 2BCL column (1.5 × 96 cm) or Sepharose 6BCL (1.5 × 97 cm) equilibrated with 4 M guanidium chloride, 0.05 M Tris (pH 7.0) including protease inhibitors. Fractions (3 ml) were collected and analyzed for radioactive content.

3. RESULTS AND DISCUSSION

3.1. *Gel filtration of secreted xyloside-initiated glycosaminoglycans*

Cultures labeled with [^{3}H]glucosamine and $^{35}SO_4$ were examined by gel filtration on Sepharose 6BCL. The culture medium was passed through the gel filtration column, providing a profile of the labelled macromolecules (fig. 1). A small peak is seen at the exclusion volume (fractions 18–21), followed by a major peak centered about fraction 35. We have shown the major peak to be xyloside-initiated glycosaminoglycans [13]. The initial peak probably contains some residual proteoglycans synthesized in the presence of xyloside [14]. As noted in [13], the major peaks do not coincide exactly, for control and monensin samples, probably as a consequence of undersulfation due to monensin. The ^{35}S:^{3}H ratio in fig. 1, for monensin and control, were 3.3 and 4.5, respectively. The diminished ^{3}H radioactivity in monensin was at ~20% of control levels. This decrease was greater than reported, most likely due to the 10-fold higher

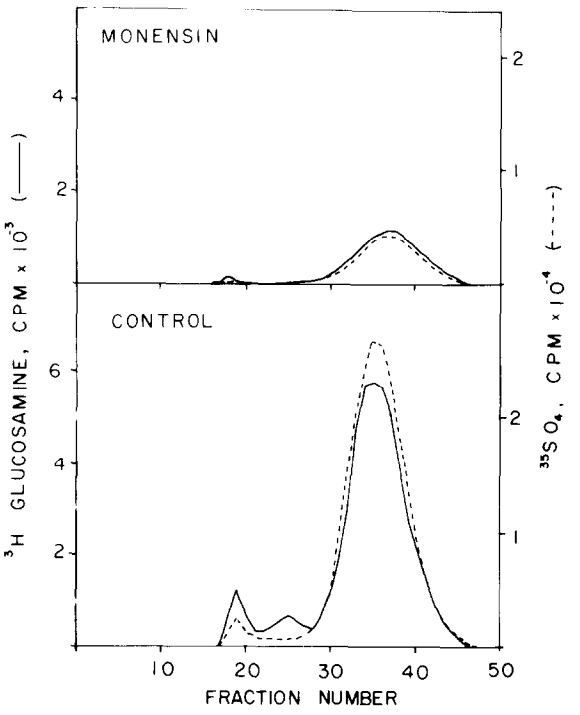

Fig. 1. Effects of monensin on the gel filtration profiles of $^{35}SO_4$ and [^{3}H]glucosamine-labeled glycosaminoglycans initiated with a β-D-xyloside. The samples of culture medium were applied to a column of Sepharose 6BCL as in the text. Monensin was 1 μM during the experiments: (——) [^{3}H]glucosamine; (----) $^{35}SO_4$.

concentration of monensin used in the present studies.

3.2. *Gel filtration of secreted proteoglycans*

In another series of experiments, the xyloside was omitted but monensin was maintained at 1 μM. The proteoglycans were labeled with [^{14}C]glucosamine and [^{3}H]serine, isolated by immunoprecipitation and passed through a Sepharose 2BCL column. Fig. 2 shows the elution profiles for monensin and control samples. The peak at the void volume (fractions 18–20) is probably hyaluronic acid, which is known to co-precipitate with proteoglycan and its antiserum [12,15]. The major peak, which is double-labeled proteoglycan [12], shows an elution differential between control and monensin samples, with the monensin peak eluting later. This result is consistent with our earlier demonstra-

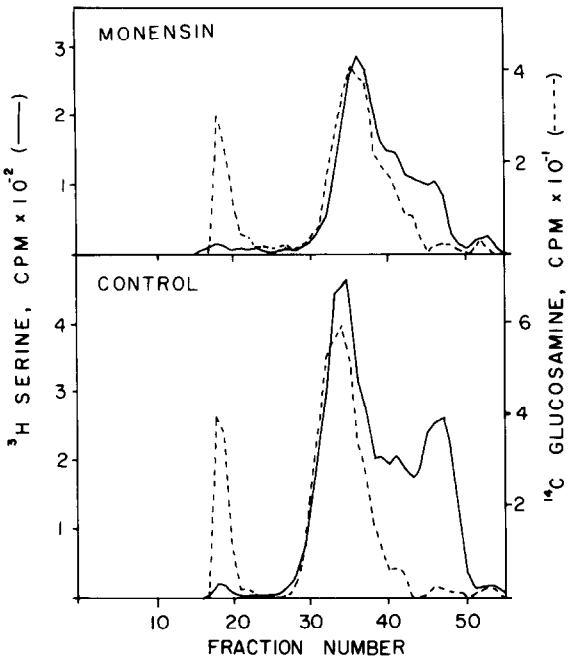

Fig. 2. Effects of monensin on the gel filtration profiles of labeled molecules immunoprecipitated from the culture medium with anti-proteoglycan antiserum. The precipitates were dissolved and applied to a column of Sepharose 2BCL as in the text: (——) [^{3}H]serine; (----) [^{14}C]glucosamine.

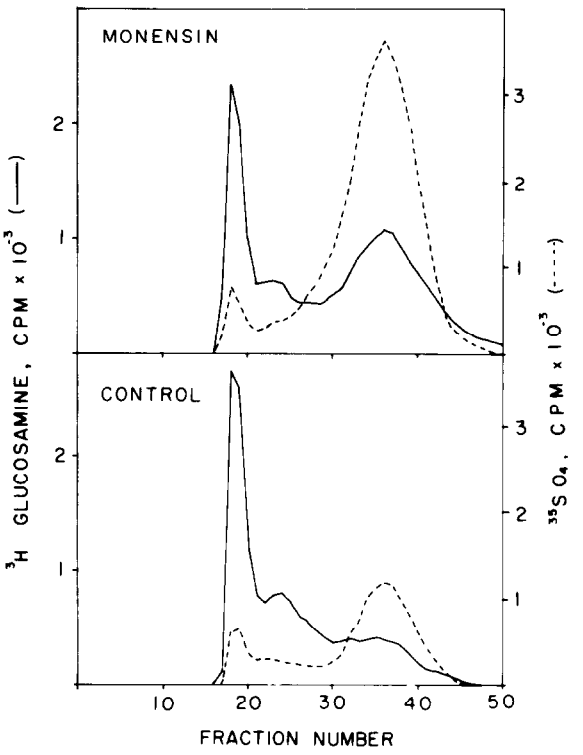

Fig. 3. Effects of monensin on the gel filtration profiles of $^3SO_4$ and [^{3}H]glucosamine-labeled macromolecules obtained from chondrocytes incubated in the presence of a β-D-xyloside. Cell lysates were applied to a column of Sepharose 6BCL as in the text: (——) [^{3}H]glucosamine; (----) $^{35}SO_4$.

tion that the pronase-derived glycosaminoglycans from such samples showed that the monensin ones eluted later than the controls [9]. The serine-labeled material eluting at fractions 45–48 in both samples is probably link protein, as indicated [12]. Surprisingly, the degree of monensin-induced inhibition of proteoglycan secretion is not as pronounced as seen when xyloside is used to initiate carbohydrate chain growth (fig. 1).

3.3. *Gel filtration of chondrocyte lysates*

The chondrocytes which had been incubated in the presence of xyloside were lysed and the clarified lysates were passed through the Sepharose 6BCL column. Fig. 3 shows the elution profiles for these samples, which had been labeled with [^{3}H]-glucosamine and $^{35}SO_4$. The peak appearing at the void region probably contains hyaluronic acid, some proteoglycans and other macromolecules such as procollagen while the peak at fractions 35–40 is probably similar to that seen in fig. 1, since it elutes at the same location and contains both ^{3}H and $^{35}SO_4$ labels. This peak is much more abundant in the monensin cell lysates than in the control cell lysates and we believe it contains xyloside-initiated glycosaminoglycans. Thus, the data in this figure are reciprocal to those in fig. 1 and suggest that the xyloside-initiated carbohydrate chains are accumulating in the monensin-treated chondrocytes.

When xyloiside is omitted and the proteoglycans are immunoprecipitated from the clarified cell lysates, followed by their gel filtration on Sepharose 2BCL, the patterns in fig. 4 are obtained. The peak appearing at the void volume, analogous to that seen in fig. 2, is most likely hyaluronic acid which has co-precipitated with proteoglycan [12,15]. The components eluting in fractions 30–42 show much less correspondence between the ^{3}H

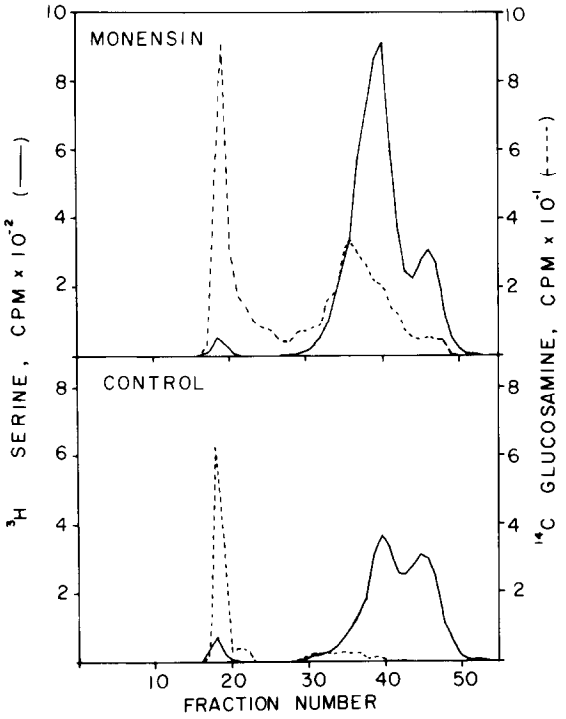

Fig. 4. Effects of monensin on the gel filtration profiles of labeled macromolecules immunoprecipitated from chondrocyte lysates (see text). The precipitates were dissolved and applied to a column of Sepharose 2BCL as in the text: (——) [^{3}H]serine; (----) [^{14}C]glucosamine.

and ^{14}C labels than seen in fig. 2, suggesting a more heterogeneous population of proteoglycans. Both labels in this region are more abundant in the monensin-treated samples than in the controls, consistent with the data of fig. 3 and with a secretory blockade due to the ionophore. The last peak in fig. 4, centered about fraction 45, is most likely to be link protein. It is also seen in fig. 2 and is known to co-precipitate with proteoglycan and its anti-serum [12,15].

Monensin has multiple effects upon chondrocytes, as detected by structural, biochemical and immunological methods. The cells show ultrastructural changes including a distorted Golgi apparatus and distended rough endoplasmic reticulum [11]. Both procollagen (type II) and proteoglycan accumulate in the affected cells, as shown by radioimmunoassay [11]. The accumulated proteoglycan antigen contains a large proportion of undersulfated and underglycosylated species [12]. The results reported here are consistent with these earlier data and also demonstrate a new phenomenon, namely that xyloside-initiated glycosaminoglycans also tend to accumulate in chondrocytes when monensin is present in the culture medium. The exact mechanism(s) by which monensin causes undersulfation, and in the case of proteoglycans, underglycosylation, is not clear. Detailed studies of the sulfation defect did not elicit any specific cause [16] and it may be that impaired transit of core protein through the chondrocyte, in conjunction with distorted Golgi structures, is sufficient to limit access to luminal transferases as well as to cause an increase in the proportion of underglycosylated species. Similar considerations may apply to the effects of monensin upon the glycosylation of fibronectin (in human fibroblasts); a large proportion of the secreted fibronectin contains oligosaccharides of the high mannose variety [8]. Whatever the specific mechanisms may be, it is clear that monensin is a most useful agent for perturbing the secretory pathway and affecting post-translational modifications of macromolecules destined for release from the cell.

ACKNOWLEDGEMENTS

This research was supported in part by NIH grants AM-17220 and AM-12683 to M.L.T.

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