UNDERSULFATED β-D-XYLOSIDE GLYCOSAMINOGLYCANS ARE SECRETED IN THE PRESENCE OF THE IONOPHORE MONENSIN

Toshihide KAJIWARA and Marvin L. TANZER
Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06032, USA

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1. Introduction

The monovalent ionophore monensin inhibits secretion of macromolecules from a wide variety of eukaryotic cells [1–7]. Monensin exerts its effects upon intracellular translocation at the Golgi region but its mechanism of action is unknown. Its action is reversible and monensin has minimal effects upon protein synthesis in contrast to colchicine and vinblastine.

Diminished secretion of some macromolecules, when caused by monensin, is accompanied by changes in post-translational modifications of such molecules. For example, qualitative studies of immunoglobulin M synthesis have shown that glycosylation by galactose and fucose seemed to be impaired by monensin [8]. The ionophore decreases proteolytic processing of Sindbis viral glycoprotein [9] and also interferes with sulfation of the proteoglycans produced by chondrocytes in culture [7]. To focus more closely upon this latter phenomenon we have used a β-D-xyloside acceptor of glycosaminoglycan chain synthesis, obviating the need for endogenous core protein formation. Chondroitin sulfate chains are synthesized on exogenously supplied β-D-xylosides at the expense of synthesis on the normal core protein acceptor [10]. The results obtained here, using 4-methyl umbelliferyl-β-D-xylopyranoside as an acceptor, show that monensin effectively inhibits sulfation of β-D-xyloside glycosaminoglycans.

2. Materials and methods

2.1. *Chondrocyte preparation*

The sterna were dissected from 72, 17-day-old chick embryos and placed in sterile Krebs-Ringer solution containing glucose (2 mg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (0.25 μg/ml). The sternal perichondrium was stripped away by means of fine forceps while viewing through a dissecting microscope. The cartilage was minced into small pieces and placed in a flask containing 0.1% clostridial collagenase and 0.25% trypsin. The suspension was gassed with air containing 5% CO_2. The contents of each flask were stirred very gently at 37°C for 2 h, then spun at 500 × *g* for 2 min. The pellet was resuspended and digestion was repeated for 30 min. The cells were harvested by spinning at 500 × *g* for 2 min and washed thrice in fresh Dulbecco's modified Eagle medium. The final suspension was passed through 2 layers of lens paper to remove debris. The cells were sedimented again, resuspended, and counted. Viability was checked using trypan blue. The cell suspension was divided among plastic culture tubes with 1×10^7 cells contained in 3 ml Dulbecco's modified Eagle medium.

2.2. *Incubation conditions*

Monensin was added at 10^{-8}–10^{-7} M. 4-Methyl umbelliferyl-β-D-xylopyranoside was dissolved at 0.5 M in dimethyl sulfoxide and added to each culture medium to give 1 mM xyloside final conc. and 0.2% dimethyl sulfoxide. Preincubation was carried out at 37°C in a ratory shaker for 2 h after gassing with air, 5% CO_2. The chondrocytes were harvested by centrifugation, fresh medium was added, containing $Na_2{}^{35}SO_4$ (10 μCi/ml) and [^{3}H]glucosamine (10 μCi/ml), and the previous monensin and β-D-xyloside concentrations were maintained. Gassing was done as before and the chondrocyte suspensions were incubated for another 4 h. The incubation was terminated by chilling the culture tube at 4°C, and then separating the cells and medium by centrifugation.

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2.3. *Glycosaminoglycan analysis*

The culture medium was made 4 M in guanidine–HCl and passed through a column (1 X 47 cm) of Sephadex G-25 in 4 M guanidine–HCl, 0.1 M Tris–HCl (pH 7.0). The chondrocytes were washed thrice in phosphate-buffered saline, then stirred in 4 M guanidine–HCl in 1 M Tris–HCl (pH 7.0) for 5 h, following which centrifugation was carried out at 3000 rev./min for 15 min. The supernatant was passed through the Sephadex G-25 column. The excluded material was then passed through a column (1.5 X 96 cm) of Sepharose C1-6B in 4 M guanidine–HCl, 0.1 M Tris–HCl (pH 7.0). Fractions of 3 ml were collected and analyzed for radioactive content.

2.4. *Protein assay*

In every experiment, an aliquot of chondrocytes was dissolved in SDS and the protein content was measured [11].

2.5. *Chondroitinase ABC digestion and chromatography*

The xyloside-linked glycosaminoglycan fraction obtained by gel filtration on a column of Sepharose CL-6B was dialyzed against distilled water, following which the solution was concentrated by pervaporation across a dialysis membrane, and then lyophilized. The chondroitinase ABC digestion and subsequent paper chromatography were performed according to [12].

3. Results and discussion

Incubation of the chondrocytes in the presence of monensin was evaluated by separately analyzing the cells and culture medium. Fig.1 shows for the macromolecules of culture medium, that both glucosamine and SO_4 incorporation diminished at the highest concentration of monensin, falling to 77% and 36% of control values, respectively. At 3×10^{-8} M monensin, glucosamine incorporation was close to normal while SO_4 incorporation was 73% of control values. At 10^{-8} M, monensin had only slight effects upon incorporation of both precursors. In contrast to the data found for the culture medium, fig.1 shows that monensin produced less effect upon the cells. Only SO_4 incorporation was impaired over 10^{-8}–10^{-7} M.

Gel filtration patterns of the culture medium show (fig.2) that the elution positions of the xyloside-initiated glycosaminoglycans were similar for control and ionophore-treated cells. The figure also shows the ratio of $^{35}SO_4$ to [^{3}H]glucosamine. There is a progressive decrease in the ratio with increasing concentration of monensin, indicating that the ionophore has caused undersulfation of the xyloside-initiated carbohydrate chains. In order to directly show that diminished sulfation was occurring, the samples were digested by chondroitinase ABC followed by analysis of the digestion products (fig.3). Comparison of the samples shows that there was a progressive increase in non-sul-

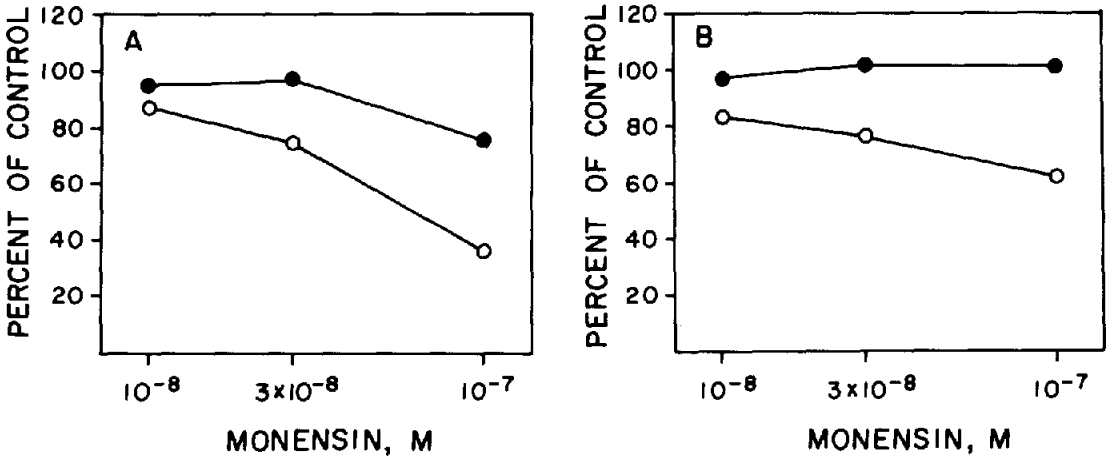

Fig.1. Effects of monensin on the synthesis and secretion of xyloside-initiated glycosaminoglycans which were excluded by Sephadex G-25: (A) culture medium, control values were 3.2×10^5 cpm/mg ^{35}S and 5.9×10^4 cpm/mg ^3H; (B) chondrocytes control values were 1.6×10^4 cpm/mg ^{35}S and 9.5×10^3 cpm/mg ^3H; (●—●) [^{3}H]glucosamine; (○—○) $^{35}SO_4$.

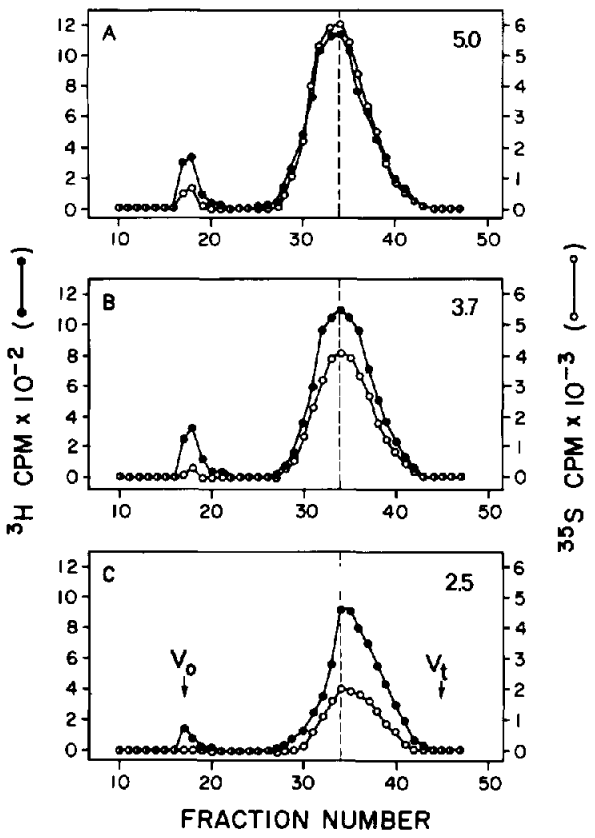

Fig.2. Effects of monensin on the gel filtration profiles of $^{35}SO_4$ and [^{3}H]glucosamine-labeled glycosaminoglycans initiated with a β-D-xyloside. The samples which had been excluded by a Sephadex G-25 column (fig.1) were applied to a column of Sepharose CL-6B as described in the text: (A) control; (B) monensin 3 × 10^{-8} M; (C) monensin 1 × 10^{-7} M; (●——●) [^{3}H]glucosamine; (○——○) $^{35}SO_4$. The number in the upper right-hand corner of each panel is the $^{35}SO_4$/[^{3}H]-glucosamine ratio of the major peak.

fated dissaccharide with a corresponding decrease in the 6-sulfated disaccharide as a function of monensin concentration. The inhibition was less than seen in fig.1, due to variable response of the embryonic chondrocytes from experiment to experiment.

There is considerable interest in the relationship between intracellular translocation of macromolecules destined for secretion and the structural modifications of such molecules while they are en route to the cell surface. Monovalent ionophores such as monensin have served as useful probes for studying this process since they disrupt the intracellular flow without permanently damaging cells and they also have minimal effects upon energy production [13] and protein synthesis [4]. Monensin does cause substantial changes in cellular ultrastructure, affecting primarily mitochondrial and Golgi architecture [1,6]. Although fibroblasts are also affected in this way [6], we have found that, in the chondrocytes used here, both the Golgi elements and rough endoplasmic reticulum are altered by the presence of monensin (unpublished). The endoplasmic reticulum becomes markedly dilated and filled with electron-dense granular material while the Golgi architecture becomes distorted as seen in fibroblasts [6].

The normal path of chondroitin sulfate biosynthesis is incompletely understood although the general outlines are known [14]. A coordinated process of

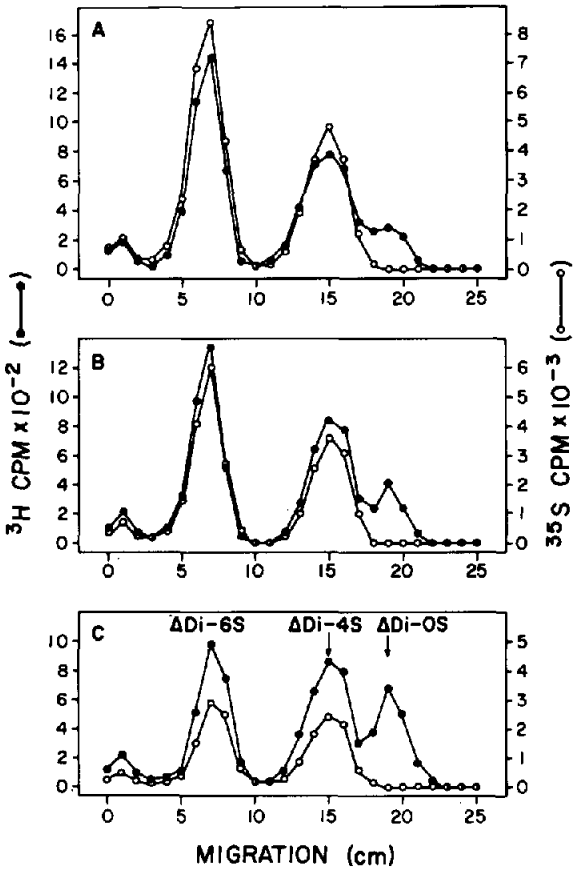

Fig.3. Effects of monensin on the separated digestion products following sequential incubation with chondroitinase ABC and paper chromatography: (A) control; (B) monensin 3 × 10^{-8} M; (C) monensin 1 × 10^{-7} M; (●——●) [^{3}H]glucosamine; (○——○) $^{35}SO_4$. Disaccharide standards were chromatographed simultaneously and migrated at the positions indicated.

carbohydrate chain growth upon an acceptor (core) protein occurs as the protein traverses the cisternal membrane of the endoplasmic reticulum and passes on to the Golgi elements. Sulfation occurs in the Golgi zone [15] but whether complete or incomplete carbohydrate chains are substrates in the reaction is not clear [14]. These results, using monensin to impair sulfation of xyloside-initiated chains, indicate that the ionophore exerts its effects after core protein synthesis and that such synthesis is not required for ionophore inhibition of sulfation. This result is reminiscent of the brachymorphic mutation in which sulfation is impaired due to inadequate levels of active sulfate [16].

There are some differences, comparing these results and those seen when proteoglycan synthesis, secretion and sulfation were studied [7]. Although undersulfation occurs in both cases, it is more pronounced for proteoglycans found in both the culture medium and chondrocytes.

Since the monensin effects are a phenotypic copy of the brachymorphic genotype we measured $^{35}SO_4$ incorporation into [^{35}S]PAPS in the presence of the ionophore. No differences were found compared to controls (unpublished). Measurement of sulfotransferase activity, sulfatase activity, free sulfate and glucosamine uptake into cells and ATP levels in the cells also showed no change when monensin was present (unpublished). Since fig.2,3 indicate a decrease in $^{35}SO_4/^3H$ ratio due to monensin, the intracellular precursor pool specific activities may have been altered. If so, this must occur in some localized regions since, as noted above, we did not find total incorporation of either precursor into whole chondrocytes to be affected by monensin. Since the Golgi components are markedly distorted, it may be that monensin causes undersulfation by impairing access of the proteoglycans to the sulfotransferases in the luminal walls of the Golgi structures. At present this seems to be the most tenable hypothesis which accounts for all of the data. Most interestingly, the undersulfated proteoglycans continue to appear in the culture medium at near normal levels, while the type II procollagen accumulates in the same chondrocytes [7]. The manner in which this dichotomy of monensin effects may occur is currently being studied.

Acknowledgements

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