Monensin inhibits synthesis of proteoglycan, but not of hyaluronate, in chondrocytes

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Monensin (10 nM–1 μ M) inhibited the incorporation of [35 S]sulphate and [3 H]glucosamine into proteoglycans by rat chondrosarcoma cells, but the incorporation of [3 H]glucosamine into hyaluronate was unaffected. The results suggest that hyaluronate synthesis occurs in a cell compartment separate from chondroitin sulphate synthesis.

Cartilage proteoglycans are complex macromolecules in which many glycosaminoglycan chains are attached to a protein core. Chondrocytes isolated from the Swarm rat chondrosarcoma have been shown to produce cartilage-type proteoglycans in culture. The proteoglycans are secreted from the cells as monomers, but form aggregates in the culture medium by binding to hyaluronate and a specific link protein (Kimura *et al.*, 1979). All three components of the aggregate are synthesized by the chondrocytes, but it remains to be determined how aggregation is delayed until the components have been secreted from the cells (Hardingham, 1981; Kimura *et al.*, 1981b). Experiments suggest that link protein may be secreted already bound to the proteoglycan (Kimura *et al.*, 1980), but the pathways of synthesis and secretion of hyaluronate remain unknown. The post-translational modifications of the proteoglycan protein core, which involve the initiation, elongation and sulphation of the chondroitin sulphate chains, occur primarily in the Golgi region of the cell, and with chick chondrocytes it has been shown that monensin is a potent inhibitor of chondroitin sulphate synthesis and sulphation (Tajiri *et al.*, 1980). Monensin is a carboxylic ionophore that interferes with the intracellular pathways of secretory proteins in many different cell types (Tartakoff & Vassalli, 1977, 1978; Uchida *et al.*, 1979). It causes a rapid dilation of the Golgi elements, which may result from partial Na⁺/K⁺ equilibration across the membranes (Tartakoff & Vassalli, 1978). The present study examines further the effects of monensin on proteoglycan and hyaluronate synthesis in chondrocytes.

Materials and methods

Materials were obtained as previously described (Mitchell & Hardingham, 1981). In addition, monensin, Zwittergent 3-14 (*N*-tetradecyl-*NN*-dimethyl-3-ammonio-1-propanesulphonate) and *Streptomyces* hyaluronidase (EC 4.2.2.1) were obtained from Calbiochem–Behring Co. (Bishop's Stortford, Herts., U.K.). [35 S]Sulphate (25–40 Ci/mg), D-[6- 3 H]-glucosamine hydrochloride (20 Ci/mmol) and L-[3- 3 H]serine (11 Ci/mmol) were from Amersham International (Amersham, Bucks., U.K.).

Chondrocyte cultures

Primary cultures of chondrocytes from Swarm rat chondrosarcomas were prepared after digestion with trypsin and collagenase as described by Kimura *et al.* (1979). Cells were plated at a density of 1.5×10^6 cells per 35 mm culture plate in 2 ml of Dulbecco's modified Eagle's medium containing 15 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], 10 mM-Bes {2-[bis-(2-hydroxyethyl)amino]-ethanesulphonic acid}, 10 mM-Tes (2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino)ethanesulphonic acid) and 20% (v/v) foetal calf serum. After 24 h, the medium was changed to 2 ml of a modified form containing 2% foetal calf serum, insulin (0.016 unit/ml) and kanamycin (10 μ g/ml). The modified medium was changed daily. Experimental incubations were performed in 1 ml of medium with cells after 2 or 3 days in culture. Monensin was dissolved in ethanol to give a 0.5 M solution. Control cultures containing ethanol at the highest concentration used were included in all experiments. Ethanol gave up to 14% inhibition of [35 S]sulphate incorporation, but negligible inhibition of [3 H]glucosamine or [3 H]serine incorporation. The onset of monensin effects appeared rapid, e.g. 90% of the maximum effect on [35 S]sulphate incor-

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puration occurred within 20 min, and inhibition continued without evidence of further toxicity for at least 6 h. Cells were routinely preincubated with monensin for 120–180 min before the addition of radioactive precursors.

The effect of *p*-nitrophenyl β -D-xyloside on monensin-inhibited cultures was determined by incubating cultures with 0.5 μ M-monensin, with and without 100 μ M-*p*-nitrophenyl β -D-xyloside for 150 min and then adding 20 μ Ci of [35 S]sulphate/ml or 5 μ Ci of [3 H]glucosamine/ml for 300 min. [35 S]Sulphate-labelled macromolecules were determined by extraction in 1% (w/v) Zwittergent/2 M-guanidine hydrochloride and chromatography on columns of Sephadex G-50, as described in Fig. 1. The incorporation into [3 H]glucosamine-labelled macromolecules was determined after alkali elimination and Sephadex G-50 chromatography, as described in Fig. 4.

Assay methods

Hexuronic acid was determined by an automated procedure (Heinegård, 1973) of the modified (Bitter & Muir, 1962) carbazole reaction, with glucurono-lactone as standard. Radioactivity was measured by mixing samples with water and adding Lumagel (Lumac Systems, Basle, Switzerland); d.p.m. were measured in a Searle mark III liquid-scintillation spectrometer with automatic quench correction.

Results

Effect of monensin on the synthesis and secretion of [35 S]sulphate- or [3 H]glucosamine-labelled macromolecules

Initial experiments were designed to determine the concentrations at which monensin affected protein synthesis, polysaccharide synthesis and sulphation in monolayer cultures of the chondrocytes. Cells were thus incubated with monensin (1 μ M–10 nM) and the incorporation of [35 S]sulphate, [3 H]glucosamine and [3 H]serine into total macromolecules was determined (Fig. 1). Monensin inhibited the incorporation of all three precursors, but at sharply different concentrations (Fig. 1). The total incorporation of each precursor was 50% of control values with 30 nM for [35 S]sulphate, 0.2 μ M for [3 H]glucosamine and 1 μ M for [3 H]serine. Sulphation was thus much more sensitive to monensin than was polysaccharide synthesis, and protein synthesis was only inhibited at higher concentrations of monensin. These results thus supported the proposal that the major site of monensin action was in the Golgi elements. It was noticeable that the inhibition of [3 H]glucosamine incorporation did not fall below 15–20% of control. This may represent the fraction of total [3 H]glucosamine incorporation that occurs outside monensin-sensitive compartments in the cell.

Fig. 1. *Effect of monensin on the incorporation of [35 S]sulphate, [3 H]glucosamine and [3 H]serine into macromolecules*

After preincubation in monensin (0–1 μ M), duplicate cultures were incubated with [35 S]sulphate (40 μ Ci/ml) for 180 min (●) or with [3 H]glucosamine (20 μ Ci/ml) for 300 min (■). The culture medium was removed and the cell layer extracted for 1 h at 4°C with 2 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 5.8, and proteinase inhibitors (0.1 M-6-aminohexanoic acid, 5 mM-benzamide hydrochloride, 10 mM- Na_2EDTA and 1 mM-phenylmethanesulphonyl fluoride). The extract was removed and the remaining cell residue extracted with 1% (w/v) Zwittergent 3–14 in 2 M-guanidine hydrochloride/0.05 M-sodium acetate, pH 5.8, at 4°C for 1 h. Previous experiments showed that 2 M-guanidine released pericellular material, but Zwittergent was required to release intracellular labelled macromolecules (Kimura *et al.*, 1981a; Mitchell, 1981). The total incorporation was determined after chromatographing the medium, pericellular extract and cell extract on columns (25 cm $\times$ 0.8 cm) of Sephadex G-50 in 4 M-guanidine hydrochloride to remove unincorporated label (Kimura *et al.*, 1979). To measure total protein synthesis, duplicate cultures were incubated with [3 H]serine (10 μ Ci/ml) for 240 min (▲). A single extract was then made of the cells and medium by adding 1 ml of a concentrated ($\times 2$) solution of Zwittergent and guanidine hydrochloride to give final concentrations of 1% (w/v) and 2 M respectively, with proteinase inhibitors as above. After 1 h at 4°C, the extracts were dialysed against several changes of water containing the proteinase inhibitors to remove unincorporated label and then adjusted to 4 M-guanidine hydrochloride to determine the total radioactivity. The total incorporations per plate in the control cultures were: [35 S]sulphate, 7.93×10^5 d.p.m.; [3 H]glucosamine, 1.03×10^6 d.p.m.; [3 H]serine, 11.5×10^6 d.p.m. (approx. 3.5% in proteoglycan). There was excellent agreement of these results with other similar experiments on monensin inhibition with incubation times from 2 to 6 h, some with dual labelling (Mitchell, 1981).

The effect of monensin on secretion was assessed by measuring the distribution of ^{35}S -labelled macromolecules between cell and medium fractions. The secretion of ^{35}S -labelled macromolecules into the

Fig. 2. Effect of monensin on the secretion of (a) [^{35}S]sulphate and (b) [^3H]glucosamine-labelled macromolecules

Incubations and analyses were as in Fig. 1. Secretion was determined from the proportion of the total radioactivity in the medium and pericellular-extract fractions compared with that in the cell extract. The dashed lines show the effect of monensin on total incorporation as in Fig. 1.

medium was inhibited by monensin, but by only 52% when the total incorporation of [^{35}S]sulphate was inhibited by 92.4% (Fig. 2a). Sulphation was thus much more sensitive to monensin than was secretion, as reported by Tajiri *et al.* (1980). With [^3H]glucosamine (Fig. 2b), the results showed a parallel concentration-dependent inhibition of incorporation and secretion of [^3H]glucosamine-labelled macromolecules in the presence of monensin. However, comparison of Figs. 2(a) and 2(b) suggested that secretion of ^{35}S -labelled and [^3H]glucosamine-labelled macromolecules were affected by monensin at similar concentrations, although their total incorporation was affected differently.

Effect of *p*-nitrophenyl β -D-xyloside on monensin inhibition of incorporation of [^{35}S]sulphate and [^3H]glucosamine

p-Nitrophenyl β -D-xyloside stimulates total chondroitin sulphate synthesis in chondrocytes, as it acts as an acceptor for the initiation of chondroitin sulphate chains (see Rodén & Schwartz, 1975). The total rate of chain synthesis is thus enhanced, as it is no longer limited by the availability of acceptor sites on proteoglycan protein core (Mitchell & Hardingham, 1982). The rate of chondroitin sulphate synthesis in the presence of *p*-nitrophenyl β -D-xyloside is thus a measure of capacity of the chondroitin sulphate-synthesizing enzymes (Schwartz, 1977). In the presence of β -D-xyloside (100 μM), the rate of [^{35}S]sulphate incorporation in control cultures was increased to 164%, whereas in monensin-treated cultures it was increased only from 2.3% to 8.2% of the control rate. Similarly, [^3H]glucosamine incorporation was increased to 111% in controls, but only from 14.7% to 24.6% in monensin-treated cultures. These results show that monensin does not inhibit sulphation

and glycosylation merely by limiting the availability of protein acceptor for chondroitin sulphate and glycoprotein synthesis.

Effect of monensin on the size of proteoglycans

The proteoglycans synthesized in the presence of monensin were extracted from the cultures in 2M-guanidine hydrochloride/1% (w/v) Zwittergent and the sizes were compared by gel chromatography on Sepharose CL-2B under dissociative conditions (Fig. 3). At low extents of inhibition the size of the monomer did not appear to change, but with inhibition above 50% the average size became progressively smaller. Fewer proteoglycans were

Fig. 3. Effect of monensin on the size of proteoglycans synthesized by cultures of rat chondrosarcoma cells. After preincubation in (a) control medium, (b) 10nM-monensin, (c) 30nM-monensin, (d) 60nM-monensin and (e) 100nM-monensin, duplicate cultures were incubated with [^{35}S]sulphate (40 $\mu\text{Ci}/\text{ml}$) for 180 min. An extract of the combined medium and cell layer was made in 1% Zwittergent in 2M-guanidine hydrochloride containing proteinase inhibitors as above. Samples adjusted to 4M-guanidine hydrochloride were chromatographed on a column (120 cm $\times$ 1.0 cm) of Sepharose CL-2B eluted in 4M-guanidine hydrochloride/0.05M-sodium acetate, pH 5.8 at 22°C. Fractions (1 ml) were collected and the radioactivity was determined. The void volume (V_0) and included volume (V_i) of the column were determined with hyaluronate of high molecular weight and $^3\text{H}_2\text{O}$ respectively.

Fig. 4. *Effect of monensin on the biosynthesis of hyaluronate and chondroitin sulphate*

After preincubation with or without monensin as indicated, duplicate cultures were incubated with [³H]glucosamine ([³H]GlcN; 10 μCi/ml) for 360 min. Then 10 M-NaOH was added to the cooled culture plate (final concn. 0.5 M) and left for 18 h at 4°C. The extract was neutralized with acetic acid and chromatographed on columns of Sephadex G-50 in 4 M-guanidine hydrochloride as in Fig. 1 to remove unincorporated label and low-molecular-weight oligosaccharides. Carrier chondroitin sulphate (1 mg/extract) was added to the pooled V₀ fractions, which were then dialysed and freeze-dried. Samples dissolved in 0.5 M-sodium acetate, pH 6.8, were chromatographed on a column (143 cm × 0.9 cm) of Sepharose CL-6B in 0.5 M-sodium acetate, pH 6.8, before (a–f) and after (g–l) digestion with *Streptomyces* hyaluronidase. Fractions (1 ml) were collected and the radioactivity was determined. The digestion was carried out in 0.1 M-sodium acetate/0.15 M-NaCl (pH 7.1) for 20 h at 37°C with 2.5 turbidity-reducing units of enzyme/mg of uronic acid in the sample. Columns (f) and (l) were loaded with samples prepared from twice the volume of extract as for those applied to the other columns. For determining [³⁵S]sulphate incorporation, parallel cultures were incubated with [³⁵S]sulphate (10 μCi/ml) for 360 min. The radioactivity excluded from Sephadex G-50 columns was determined in an extract of the medium and cell layer in 1% Zwittergent/2 M-guanidine hydrochloride prepared as in Fig. 3. Values in parentheses show the radioactivity in hyaluronate (hatched area) as a percentage of the total incorporation of [³H]glucosamine in control cultures.

thus being synthesized in the presence of monensin, and they were of smaller average size, and this may have resulted from both decreased sulphation and shorter chondroitin sulphate chains.

Effects of monensin on synthesis of hyaluronate and chondroitin sulphate

Cultures incubated with [³H]glucosamine for 6 h were extracted in 0.5 M-NaOH at 4°C for 18 h to

release by β -elimination all of the chondroitin sulphate chains. The extract from the control culture showed an elution profile of a small excluded peak of hyaluronate (Fig. 4a), that was digested by the *Streptomyces* hyaluronidase (Fig. 4g), and a retarded chondroitin sulphate peak. As the concentration of monensin increased, the proportion of radioactivity in the chondroitin sulphate peak decreased, and it appeared more polydisperse (Figs. 4d, 4e and 4f), but the amount of radioactivity in the hyaluronate peak did not change compared with the control cultures. Hyaluronate synthesis was thus not inhibited even by $1\mu\text{M}$ -monensin, which gave 98% inhibition of [^{35}S]sulphate incorporation, 80% inhibition of [^3H]glucosamine incorporation and 53% inhibition of protein synthesis (Fig. 1).

Discussion

The results showed that the sulphation of chondroitin sulphate was particularly sensitive to inhibition by monensin. The secretion of proteoglycan was also inhibited by monensin, but only at concentrations that severely inhibited its synthesis, and even at higher concentrations inhibition was not complete. These results are comparable with those reported by Tajiri *et al.* (1980) with chick chondrocytes, who also showed much less effect on proteoglycan secretion than on procollagen secretion, which was inhibited and led to its accumulation in the cells. This may imply that the block in secretion observed with other proteins occurs at a stage in intracellular migration before the location of chondroitin sulphate synthesis on proteoglycans is reached. Proteoglycan protein core devoid of chondroitin sulphate chains may thus have accumulated before the site of chondroitin sulphate synthesis, but it would not have been detected by the techniques used in the present study.

Previous results (Mitchell & Hardingham, 1981, 1982; Mitchell, 1981; Kimura *et al.*, 1981a) showed there to be an inverse correlation between the rate of chondroitin sulphate synthesis in these chondrocyte cultures and the length of the chains synthesized. Inhibition of proteoglycan protein-core synthesis with cycloheximide thus led to fewer, longer chondroitin sulphate chains being synthesized, whereas stimulation of synthesis with *p*-nitrophenyl β -D-xyloside led to more, shorter chains being formed. The chain length of chondroitin sulphate thus appeared to be related to the ratio of chain-synthesizing-enzyme activity to acceptor concentration. The failure of added xyloside to relieve inhibition suggests that monensin's major effect is to decrease the chain-synthesizing-enzyme activity, by, for example, disrupting membrane-associated enzymes or their supply of nucleotide sugar substrates, although it may also affect the availability of the

acceptor. The results observed with monensin thus follow a different pattern from those with cycloheximide or xyloside, as the decrease in chondroitin sulphate synthesis is accompanied by a decrease in the average size of the chains.

The synthesis of proteoglycans is believed to follow the pathway of other newly synthesized proteins that are destined for secretion, involving synthesis into the internal compartments of the endoplasmic reticulum, movement within these compartments or within vacuoles to the Golgi lamella, before migration within vacuoles to the outer cell membrane for secretion (Palade, 1975). As carbohydrate groups of many different types are added to the proteins at different stages of this pathway and these additions require high-energy metabolic intermediates, there must be mechanisms for transferring these components from the cytosol into the membrane-bound compartments. One of the effects of monensin may thus be to perturb the mechanisms of this energy transfer. For proteoglycan synthesis it could particularly affect (a) phosphoadenosine 5'-phosphosulphate production, which is required for sulphation, and (b) and supply of nucleotide sugars for chondroitin sulphate chain elongation.

The failure of monensin to inhibit hyaluronate synthesis has several important implications. It suggests that hyaluronate is synthesized within a different compartment from chondroitin sulphate, as previously suggested by Kleine (1978), and at this location it is insensitive to monensin effects on membranes. The continuation of hyaluronate synthesis shows that UDP-glucuronic acid and UDP-N-acetylglucosamine are available at this site of synthesis, which thus may be outside the membranous compartments of the endoplasmic reticulum. It remains unknown how hyaluronate biosynthesis is initiated, but it does not seem to be made as a proteoglycan, as its biosynthesis is not inhibited by cycloheximide at concentrations that inhibit proteoglycan synthesis (Mitchell, 1981). A site of synthesis remote from the pathways followed by normal secretory proteins is thus not ruled out, and the intracellular separation of hyaluronate synthesis from proteoglycan and link-protein synthesis could explain why the interaction between these components to form proteoglycan aggregates is delayed until after secretion from the cell.

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