Selective inhibition of proteoglycan and hyaluronate synthesis in chondrocyte cultures by cyclofenil diphenol, a non-steroidal weak oestrogen

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(1) Cyclofenil diphenol, a weak non-steroidal oestrogen, binds to albumin. In the presence of concentrations of albumin just sufficient to keep cyclofenil diphenol in solution, the compound inhibited the synthesis of [35 S]proteoglycans, [3 H]glycoproteins, [3 H]hyaluronate and [3 H]proteins in primary cultures of chondrocytes from the Swarm rat chondrosarcoma in a dose-dependent manner. When excess albumin was present, conditions were found (90 μ g of cyclofenil diphenol and 4 mg of albumin per ml of culture medium) which completely inhibited [35 S]proteoglycan and [3 H]hyaluronate synthesis but had little effect on [3 H]protein or [3 H]glycoprotein synthesis. (2) The time of onset of inhibition of [35 S]proteoglycan synthesis by cyclofenil diphenol was very rapid ($t_i < 25$ min) and incompatible with an action mediated through suppression of proteoglycan core protein synthesis. Cyclofenil diphenol inhibited the synthesis of [35 S]chondroitin sulphate chains onto *p*-nitrophenyl β -D-xyloside in the cultures. (3) Cyclofenil diphenol had little effect on the secretion from chondrocytes of [35 S]proteoglycans synthesized immediately prior to treatment. (4) Chondrocyte cultures treated with cyclofenil diphenol recovered their biosynthetic activities almost completely within 3 h of removing the compound from the culture medium. (5) Cyclofenil diphenol had a similar inhibitory action on the synthesis of [35 S]proteoglycans in secondary cultures of human dermal fibroblasts from both normal subjects and patients with systemic sclerosis. (6) It is proposed that cyclofenil diphenol inhibits the synthesis of [35 S]proteoglycans by interfering with the formation of the glycosaminoglycan side chains of these molecules in the Golgi apparatus of cells. The action may be due to disturbance of Golgi membrane organization by the compound.

Cyclofenil [F6066; bis(*p*-acetoxyphenyl)cyclohexylidenemethane] is a weak non-steroidal oestrogen (Watnick & Neri, 1968) which has been used to induce ovulation (Persson, 1965), and more recently to treat systemic sclerosis (Blom-Bülow *et*

al., 1980; C. M. Black, M. I. V. Jayson & A. G. White, unpublished work). It is metabolized in rats, rabbits and man to cyclofenil diphenol [F6060; bis(*p*-hydroxyphenyl)cyclohexylidenemethane] (Larsson, 1965) which is structurally similar to α -diethylstilboestrol (Fig. 1). Both F6066 and F6060 bind to oestrogen receptors, although with a rather lower affinity than 17 β -oestradiol (Garg *et al.*, 1983).

Oestradiol and stilboestrol retard body growth (Gaarenstroom & Levie, 1939), but the mechanism for this action is still unclear. Growth retardation also occurs in mice treated with cyclofenil and was correlated with a lowered uptake of [35 S]sulphate into costal cartilage *in vivo* (Herbai, 1971a). The size of the sulphate pool was unaffected by the treatment. This suggests that cyclofenil or its

Abbreviations used: F6066, cyclofenil; F6060, cyclofenil diphenol; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid; Bes, 2-[bis(2-hydroxyethyl)amino]ethanesulphonic acid.

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Fig. 1. Structure of (a) diethylstilboestrol, (b) α -diethylstilboestrol, and (c) cyclofenil diphenol (F6060)

metabolite, may inhibit [35 S]proteoglycan synthesis by the cartilage chondrocytes, either by direct action on the cells, or via a secondary hormonal or metabolic effect.

Proteoglycans, a major component of the extracellular matrix of cartilages, are very large molecules (M_r about 2×10^6) composed of sulphated glycosaminoglycan side chains linked covalently to a core protein. In the matrix they form even larger proteoglycan aggregates by many molecules binding at intervals along the non-sulphated glycosaminoglycan, hyaluronic acid (Hascall, 1977).

Cyclofenil diphenol (F6060) binds to albumin, and we have used this property to maintain the compound in solution in tissue culture medium. We show below that when minimal concentrations of albumin are present, F6060 has a general inhibitory effect on the synthesis of proteins, glycoproteins, proteoglycans and hyaluronate in cultures of chondrocytes. However, when the relative albumin concentration is increased, favouring lower concentrations of unbound F6060 in the culture medium, the inhibitory effect of the oestrogen is primarily directed against synthesis of hyaluronate and the glycosaminoglycan side chains of proteoglycans.

Methods

Cell cultures

Single chondrocytes were obtained from trypsin/collagenase digests of tissue from the transplantable Swarm rat chondrosarcoma, as described by Kimura *et al.* (1979). Chondrocytes (1×10^6

cells/ml) were plated in Dulbecco's modified Eagle's medium (GIBCO Europe) containing 4.5 g of glucose/l, 15% foetal calf serum, 15 mM-Hepes, 10 mM-Tes, 10 mM-Bes, 100 units of penicillin/ml and 100 units of streptomycin/ml; 2 ml of cells were plated per 35 mm dish (day 0). The medium was changed daily.

Human dermal skin fibroblasts derived from biopsy outgrowths were a gift from Ms. Olive Cutting, Clinical Research Centre, Harrow, Middx., U.K. Cells were grown to confluence in 75 cm² Falcon tissue culture flasks in Dulbecco's modified Eagle's medium supplemented with 8% (v/v) foetal calf serum and 0.07 mM-ascorbic acid. The medium was changed twice weekly. On the 7th or 8th passage, cells from trypsin-treated (0.25% trypsin in phosphate-buffered saline, 10 min, 37°C) confluent cultures were reseeded on 35 mm dishes. These were used for experiments when they had grown almost to confluence.

Preparation of treatment media

Bovine serum albumin, Cohn fraction V (Sigma Chemical Co.), either containing bound fatty acids (product no. A4503), or essentially free of fatty acids (A6003), was dissolved in Dulbecco's modified Eagle's medium (5 g/100 ml). Cyclofenil diphenol (F6060, a gift from AB Ferrosan, Malmö, Sweden) was dissolved in distilled/deionized water (25 mg/1.25 ml) by addition of 1 M-NaOH (0.28 ml). The solution was diluted with water to give a concentration of 2.5 mg of F6060/ml. Portions were mixed with the albumin to give a stock solution and the pH adjusted to 7.4. The stock solution was diluted further with the albumin solution and Dulbecco's modified Eagle medium to give treatment media of specific F6060 and protein concentration. All treatment media also contained 100 ng of insulin/ml (ox pancreas; Wellcome Reagents, Beckenham, Kent, U.K.), 15 mM-Hepes, 10 mM-Tes, 10 mM-Bes, 100 units of penicillin/ml and 100 units of streptomycin/ml. Control media had an identical composition except for the absence of F6060.

Treatment and labelling of cultures

Chondrocytes and fibroblast cultures were maintained in Dulbecco's modified Eagle's medium supplemented with foetal-calf serum. On day 2, the medium of chondrocyte cultures was replaced with 2 ml of Dulbecco's medium containing 100 ng of insulin/ml, organic buffers, and antibiotics. Cultures were incubated (37°C) for 1 h. The medium was replaced with 2 ml of treatment or control medium. During the latter part (usually 1 h) of the treatment period (usually 3–4 h) the medium was replaced with 1 ml of labelling medium. The composition of labelling media was identical to

that of treatment or control media, but contained in addition either $\text{Na}_2^{35}\text{SO}_4$ (20–40 $\mu\text{Ci/ml}$), D-[6- ^3H]glucosamine hydrochloride (40 $\mu\text{Ci/ml}$), L-[4,5- ^3H]lysine (20–40 $\mu\text{Ci/ml}$) or L-[5- ^3H]proline (40 $\mu\text{Ci/ml}$). Some cultures were dual-labelled with $\text{Na}_2^{35}\text{SO}_4$ and one of the other compounds. In some experiments Dulbecco's modified Eagle medium from which Na_2SO_4 had been omitted was used for the labelling medium. All radiochemicals were from Amersham International. Fibroblast cultures were treated in the same way, after passage of cells, as described above.

Extraction of cell layers

Labelling media were removed and solid guanidine hydrochloride and concentrated proteinase inhibitors added to give final concentrations of 4M-salt, 1mM-N-ethylmaleimide, 1mM-phenylmethanesulphonyl fluoride, 5mM-benzamidine hydrochloride, 10mM-disodium EDTA and 0.1M-6-aminohexanoic acid. The cell layer was solubilized with ZwittergentTM₃₋₁₂ (Calbiochem-Behring Corp.), and guanidine hydrochloride, in the presence of the same inhibitors, as described previously (Mason *et al.*, 1982a).

Analysis of labelled macromolecules in cell cultures

The medium and solubilized cell layer were combined in most experiments. Incorporation of radioactive precursors into [^3H , ^{35}S]proteoglycans, [^3H]proteins and [^3H]glycoproteins was measured by gel filtration on prepacked Sephadex G-25 (PD10) columns (Pharmacia Fine Chemicals) as described previously (Mason *et al.*, 1982b). Incorporation of [^3H]glucosamine into [^3H]chondroitin sulphate isomers and [^3H]hyaluronate was estimated by isolating their specific chondroitinase ABC lyase (Miles Biochemicals) disaccharide products on cellulose t.l.c. plates (Mason *et al.*, 1982b). Dissociative Sepharose CL2B chromatography of proteoglycans (Mason *et al.*, 1982a) in combined media and cell layer fractions of cultures and associative Sepharose CL2B chromatography (Venn & Mason, 1983) of culture media was carried out by previously published methods. Proteinase inhibitors, but not guanidine hydrochloride, were added to the media used for associative chromatography.

Pulse-chase protocol

Chondrocyte cultures (2×10^6 cells/35mm dish) were incubated for 1 h in sulphate-free Dulbecco's modified Eagle medium containing 100ng of insulin/ml, organic buffers and antibiotics, as above. The medium was replaced with 1 ml of Dulbecco's medium (from which Na_2SO_4 had been omitted), containing 100 μCi of $\text{Na}_2^{35}\text{SO}_4$ /ml, 0.3mg of fatty-acid-free albumin/ml, 100ng

of insulin/ml, buffers and antibiotics, and the cultures were labelled for 5min. The labelling medium was removed, the cell layer twice washed rapidly with 2ml of cold Dulbecco's modified Eagle medium (with Na_2SO_4 , insulin, buffers, antibiotics), containing F6060 (10 $\mu\text{g/ml}$) and albumin (fatty-acid-free, 0.3mg/ml), and the washes were discarded. Cultures were then chased for various times from 5–60min with 2ml of fresh medium of the same composition. Control cultures were treated in the same way but F6060 was omitted. After removing the chase medium, the cell layer was extracted with 4M-guanidine hydrochloride/50mM-sodium acetate buffer, pH 5.8, containing proteinase inhibitors, with gentle agitation (1h, 4°C). The extract was removed and the cell residue dissolved in 0.5M-NaOH (18h, 22°C). The extract contains [^{35}S]proteoglycans which are predominantly extracellular, whilst the cell residue fraction contains those which are predominantly intracellular (Kimura *et al.*, 1979; Mason *et al.*, 1982b).

Results

F6060 is only slightly soluble in water (saturation 140ng/ml at 22°C). To obtain higher concentrations, F6060 was prepared in solution with bovine serum albumin, to which it binds. Albumin itself had a stimulatory effect on the incorporation of [^{35}S]sulphate, [^3H]glucosamine and [^3H]lysine into macromolecules synthesized by the chondrocyte cultures (Table 1). In all subsequent experiments where the concentration of albumin was varied appropriate controls were done.

The minimum concentration of fatty-acid-free albumin required to maintain F6060 in solution was 30 μg of protein/ μg of compound. Under these conditions concentrations of F6060 greater than 1 $\mu\text{g/ml}$ had an inhibitory effect on the incorporation of [^{35}S]sulphate into proteoglycans, [^3H]glucosamine into macromolecules and [^3H]lysine into protein in chondrocyte cultures (Fig. 2). Above 10 μg of F6060/ml, [^{35}S]proteoglycan synthesis was almost abolished. Although incorporation of [^3H]glucosamine into macromolecules and [^3H]lysine into proteins was inhibited by F6060, the degree of inhibition at high concentrations was rather less than that found for [^{35}S]proteoglycans.

The [^3H]glycosaminoglycan fraction was isolated from the cultures and analysed for its content of [^3H]chondroitin-6-sulphate, [^3H]chondroitin-4-sulphate and [^3H]hyaluronate. Chondroitin-4-sulphate constitutes about 90% of the chondroitin sulphate isomers synthesized by these cells (Mason *et al.*, 1982a). About 12–15% of the total [^3H]glycosaminoglycans synthesized is hyaluronic acid, although in some sets of cultures this may be

Table 1. *Effect of albumin on incorporation of radioactive precursors into macromolecules by chondrocyte cultures*
Replicate cultures were maintained with daily changes in Dulbecco's modified Eagle's medium containing 15% (v/v) foetal calf serum. On day 2 the medium was changed to Dulbecco's modified Eagle's medium containing 100 ng of insulin/ml for 1 h and then to fresh medium supplemented with insulin (100 ng/ml) and albumin (fatty-acid-free) for 4 h. Cultures were labelled with [³⁵S]sulphate, [³H]glucosamine or [³H]lysine for the last 1 h of the albumin treatment. Each incorporation is the mean for duplicate cultures ± the range.

[Albumin] (g/ml)	10 ⁻⁵ × Incorporation (d.p.m./culture)		
	[³⁵ S]Sulphate into proteoglycans	[³ H]Glucosamine into macromolecules	[³ H]Lysine into protein
0	3.23 ± 0.04	9.70 ± 0.28	1.54 ± 0.00
1.5	2.97 ± 0.03	8.01 ± 0.02	1.57 ± 0.00
30	3.48 ± 0.11	8.74 ± 0.16	1.59 ± 0.01
300	4.25 ± 0.08	11.77 ± 0.07	1.73 ± 0.13
750	4.59 ± 0.14	12.40 ± 0.19	2.04 ± 0.11

Fig. 2. *Effect of F6060 on the synthesis of proteoglycans and other macromolecules by chondrocyte cultures in the presence of minimal albumin concentrations*

Cultures were treated on day 2 with medium containing F6060 and fatty-acid-free albumin (1:30, w/w) for 3 h. Replicate cultures were labelled with [³⁵S]sulphate, [³H]glucosamine or [³H]lysine during the final 1 h of treatment. Control cultures treated with the same concentrations of albumin incorporated (2.5–2.8) × 10⁵ d.p.m. of ³⁵S into proteoglycans (○), (4.2–5.0) × 10⁵ d.p.m. of ³H from [³H]glucosamine into macromolecules (●), and (1.5–2.0) × 10⁵ d.p.m. of ³H from [³H]lysine into proteins (▲). Each point shown represents the mean for duplicate cultures (maximum variation of individual cultures, ±4% of mean). Inset, [³H]chondroitin-6-sulphate (C6S), [³H]-chondroitin-4-sulphate (C4S) and [³H]hyaluronate (HA) synthesis in the same cultures, as determined by chondroitinase ABC disaccharide analysis. For other details, see the text.

as high as 20% (Mason *et al.*, 1982*b*). Incorporation of [³H]glucosamine into all three glycosaminoglycans was inhibited by F6060 (Fig. 2, inset).

Effect of albumin concentration on F6060 inhibition of [³⁵S]proteoglycan and [³H]protein biosynthesis

The effects of F6060 could be modulated by varying the albumin concentration in the culture

medium. Maximum inhibition was achieved when the concentration of fatty-acid-free albumin (0.3 mg/ml) was the minimum required to keep 10 μg of F6060/ml in solution. Excess albumin antagonized the inhibitory effect of F6060 on [³⁵S]-proteoglycan synthesis in a dose-dependent manner (Fig. 3*a*). With albumin containing bound fatty acids, the minimum concentration required to

Fig. 3. Effect of albumin concentration on F6060 action

(a) Effect on proteoglycan synthesis of increasing the concentration of fatty-acid-free albumin in culture medium containing 10 μg of F6060/ml. Bars show the range from the mean (●) for duplicate cultures. Other conditions are as for Fig. 2. (b) Comparison of the effect of F6060 on $[^{35}\text{S}]$ proteoglycan synthesis with varying concentrations of fatty acid free albumin (FA⁻) or albumin containing bound fatty acid (FA⁺). The medium contained 10 μg of F6060/ml with (1) 0.3 mg of FA⁻ albumin/ml, (2) 0.4 mg of FA⁻ albumin/ml and (3) 0.4 mg of FA⁺ albumin/ml, or 100 μg of F6060/ml with (4) 4 mg of FA⁺ albumin/ml and (5) 10 mg of FA⁺ albumin/ml. (c) Effect of various concentrations of F6060 with constant concentration of 4 mg of FA⁺ albumin/ml on incorporation of $[^{35}\text{S}]$ sulphate into proteoglycans (hatched bars) and $[^3\text{H}]$ lysine into proteins (open bars).

maintain 10 μg of F6060 in solution was higher (0.4 mg/ml) than for the fatty-acid-free protein. The inhibitory effect of the F6060 on $[^{35}\text{S}]$ -proteoglycan synthesis was similar, irrespective of the type of albumin used (Fig. 3b, columns 2 and 3). Large excesses of albumin completely eliminated the inhibition of $[^{35}\text{S}]$ proteoglycan synthesis by

even very high concentrations of F6060 (Fig. 3b, columns 4 and 5).

In other experiments the concentration of albumin containing fatty acids was kept constant at 4 mg/ml, and the F6060 concentration was varied between 25 μg /ml and 100 μg /ml. Treatment was continued for 4 h and cultures labelled with

[^3H]lysine and [^{35}S]sulphate during the last 1 h. [^3H]Protein and [^{35}S]proteoglycan synthesis were affected differently under these conditions (Fig. 3c). At high concentrations of F6060 proteoglycan synthesis was profoundly inhibited, whereas protein synthesis was only partly inhibited. In several similar experiments when cultures were treated with 90 μg of F6060/ml and 4 mg of fatty acid-containing/ml, [^{35}S]proteoglycan synthesis was inhibited by 85–95%, but [^3H]protein synthesis by only 19–23%.

The inhibitory effects of F6060 were independent of the insulin concentration in the medium. Increasing the insulin concentration from 100 ng/ml (the concentration used normally) to 100 000 ng/ml had no effect on either the incorporation of [^{35}S]sulphate and [^3H]lysine into proteoglycans and proteins synthesized by control cultures, nor did it reverse the inhibitory effect of F6060 on these pathways (results not shown).

Differential effect of F6060 on incorporation of [^3H]glucosamine into proteoglycans and glycoproteins

When the chondrocyte cultures are labelled with [^3H]glucosamine about 60% of the ^3H radioactivity

incorporated into macromolecules is found in [^3H]glycosaminoglycans and 40% in [^3H]glycoproteins of a non-proteoglycan character (Mason *et al.*, 1982b). Cultures were treated with 90 μg of F6060/ml and 4 mg of fatty-acid containing albumin/ml for 4 h and dual labelled with [^3H]glucosamine and [^{35}S]sulphate during the last 1 h. [^{35}S]Proteoglycan synthesis was almost completely inhibited (<5% of controls), but incorporation of [^3H]glucosamine into macromolecules was only reduced to 30% of controls. Dissociative Sepharose CL2B chromatography of the combined media and cell layer fractions of control cultures resolved three main components, in agreement with previous results (Fig. 2; Mason *et al.*, 1982a). These were: a ^3H -labelled peak eluting in the void volume, previously identified as [^3H]hyaluronate (Mason *et al.*, 1982a), a major ^3H , ^{35}S -labelled peak ($K_{\text{av}} = 0.24$) corresponding to proteoglycans, and a ^3H -labelled peak ($K_{\text{av}} = 0.85$) representing smaller non-sulphated glycoproteins. After F6060 treatment the [^3H]hyaluronate and [^3H , ^{35}S]proteoglycan peaks were undetectable in chromatograms of the culture fractions, but radioactivity in the [^3H]glycoprotein peak ($K_{\text{av}} = 0.85$) was only reduced by 30% (chromatograms not shown). Thus these conditions (90 μg of F6060 and 4 mg of fatty-acid-containing albumin/ml) result in profound inhibition of proteoglycan and hyaluronate synthesis, but have relatively little effect on the synthesis of other glycoproteins.

Time of onset of F6060 inhibition of [^{35}S]proteoglycan synthesis

F6060 effects proteoglycan synthesis very rapidly (Fig. 4). The time taken to achieve 50% inhibition of [^{35}S]proteoglycan synthesis in Expt. (a) was 24 min. The almost immediate effect on proteoglycan synthesis and the observation that F6060 can, under certain conditions, completely block formation of proteoglycans whilst having little effect on protein synthesis, suggests it may act on the biosynthesis pathway at a point other than proteoglycan core protein synthesis.

Hydrodynamic properties of proteoglycans from inhibited cultures

The size of [^{35}S]proteoglycans synthesized by partially inhibited cultures was compared with that of control cultures by chromatographing portions of the combined medium and cell layer extract on a dissociative Sepharose CL2B chromatography column (Fig. 5). In the experiments shown, total [^{35}S]proteoglycan synthesis was reduced in the F6060-treated culture to 32% of the control culture. Incorporation of [^3H]lysine into proteins in similarly treated cultures was about 90% of the control. Chromatography profiles show

Fig. 4. Time of onset of inhibitory effect of F6060 on [^{35}S]proteoglycan synthesis

Cultures were treated for various times with (a) 25 μg of F6060/ml and 1 mg of fatty-acid-containing albumin/ml and labelled in the last 20 min of treatment, or (b) with 100 μg of F6060/ml and 4 mg of fatty-acid-containing albumin/ml and labelled in the last 45 min of treatment.

two peaks. Peak 1 has a K_{av} of 0.27 and corresponds to the large high-density chondroitin-4-sulphate proteoglycans synthesized by the chondrocytes (Mason *et al.*, 1982a; Kimura *et al.*, 1979). Peak 2 is a much smaller proteoglycan (K_{av} 0.72), and may be the low-density chondroitin-6-sulphate proteoglycan previously identified in dissociative CsCl equilibrium density gradient centrifugation of cell layer extracts (Mason *et al.*, 1982b). F6060 treatment reduced synthesis of the major [35 S]-proteoglycan (peak 1) to 10%, but had less effect on the synthesis of the small [35 S]proteoglycan (peak 2, Fig. 5b). It is of interest to note that the synthesis of [3 H]chondroitin-6-sulphate is inhibited to a lesser degree by F6060 than the synthesis of [3 H]chondroitin-4-sulphate (Fig. 2). The K_{av} of both

proteoglycan peaks remains unchanged in the treated culture.

Culture medium from treated and control cultures was chromatographed directly on an associative Sepharose CL2B column to test for the presence of natural proteoglycan aggregates (Kimura *et al.*, 1979). In both cases about 65% of the macromolecular 35 S activity recovered from the column was eluted in the void volume (V_0) (results not shown). Thus those proteoglycans that are synthesized in F6060-inhibited cultures are fully functional and form proteoglycan aggregates to the same extent as found for control culture proteoglycans.

Effect of F6060 on proteoglycan secretion

Chondrocyte cultures were pulsed with [35 S]-sulphate for 5 min. The pulse medium was removed and the cultures chased with fresh medium with or without F6060, as described in the Methods section. The [35 S]proteoglycan present in the chase medium, a 4M-guanidine hydrochloride extract of the cell layer, and in the cell residue, was established at various chase times. The pulse medium contained less than 1% of the total [35 S]proteoglycans synthesized. Newly synthesized proteoglycans move from the intracellular compartment (cell residue) into the extracellular matrix (4M-guanidine hydrochloride extract) and finally are lost from the matrix into the chase medium (Kimura *et al.*, 1979; Mason *et al.*, 1982b). The effects of F6060 on this secretory process are shown in Fig. 6. Similar amounts of [35 S]proteoglycans were recovered from treated and control cultures for each chase time. The variation between duplicate cultures was small (Fig. 6a). When F6060 was present in the chase medium a small delay occurred in the passage of newly synthesized [35 S]proteoglycan from the cell residue fraction into the 4M-guanidine hydrochloride extract fraction (Fig. 6b). This was best seen 10 min into the chase, when the proportion of [35 S]proteoglycan remaining in the cell residue of treated cultures (open triangle) was 35% of the total proteoglycans present, compared with only 27% in the control cultures. The delay in secretion of proteoglycans from chondrocytes treated with F6060 did not affect the later distribution of [35 S]proteoglycan between the residue, extract and chase medium compartments of the culture (Fig. 6b).

Inhibition of chondroitin sulphate chain synthesis by F6060

Inhibition of [35 S]proteoglycan synthesis by F6060 could occur through inhibition of core protein synthesis, thereby depriving the cell of a suitable substrate for glycosylation, or through a

Fig. 5. Dissociative Sepharose CL2B chromatography of [35 S]proteoglycans after treatment of chondrocyte cultures with F6060

Cultures were treated with medium containing either 6 mg of fatty-acid-containing albumin/ml (a), or 100 μ g of F6060/ml and 6 mg of fatty-acid-containing albumin/ml (b), for 24 h and labelled with [35 S]sulphate during the last 4 h of treatment. Portions of the combined medium and cell layer extract were chromatographed on a Sepharose CL2B column under dissociative conditions and fractions were counted for 35 S radioactivity. The shaded area corresponds to the elution of unincorporated [35 S]sulphate. Percentages show the 35 S activity under peak 1 and 2 in the treated culture (b) expressed as a proportion of those in the control (a).

Fig. 6. F6060 and $[^{35}\text{S}]$ proteoglycan secretion. Cultures were pulsed with $[^{35}\text{S}]$ sulphate for 5 min, washed rapidly and chased with medium containing albumin (fatty-acid-free, 0.3 mg/ml) with or without F6060 (10 $\mu\text{g}/\text{ml}$) (see the Methods section for details). $[^{35}\text{S}]$ Proteoglycan was estimated in the chase medium ($\bullet$, $\circ$), cell layer extract ($\blacksquare$, $\square$) and cell residue ($\blacktriangle$, $\triangle$) in control (filled symbols) and treated (open symbols) cultures. (a) Total $[^{35}\text{S}]$ proteoglycan recovered in the chase medium, extract and residue at various chase times. Bars represent the range, where present, of duplicate cultures for each time. (b) $[^{35}\text{S}]$ Proteoglycan distribution between the chase medium, extract and residue at various chase times. Solid lines, control cultures; broken line, cell residue fraction, treated cultures.

failure to synthesize glycosaminoglycan chains, even though a core protein was present. To test whether F6060 inhibited chondroitin sulphate chain synthesis, experiments were carried out using *p*-nitrophenyl β -D-xyloside as an artificial initiator for chondroitin sulphate synthesis. This compound acts as an acceptor for galactosyl transferase-1 in the polymerization of chondroitin sulphate chains (Rodén, 1980). Chondroitin sulphate is synthesized by the chondrosarcoma chondrocytes in the presence of *p*-nitrophenyl β -D-xyloside, even when protein synthesis has been blocked by cycloheximide treatment (Kimura *et al.*, 1981). The effect of F6060 on the incorporation of $[^{35}\text{S}]$ sulphate into chondroitin sulphate and

$[^3\text{H}]$ lysine into proteins in the presence of the xyloside is shown in Table 2(a). For comparison the effect of blocking protein synthesis with cycloheximide in the presence of the xyloside is shown in Table 2(b). The addition of 0.3 mM-xyloside to the cultures causes a 5.7-fold stimulation of chondroitin $[^{35}\text{S}]$ sulphate synthesis. The concomitant reduction in $[^3\text{H}]$ protein synthesis in the presence of the xyloside may be due to the energy resources of the cell being diverted into glycosaminoglycan synthesis. At the concentration of F6060 used for this experiment chondroitin $[^{35}\text{S}]$ sulphate synthesis was inhibited by over 98% compared with the control culture, whereas protein synthesis was only reduced by about 60%. Addition of 0.3 mM-xyloside to the F6060-treated cultures did not bring about any increase in the incorporation of $[^{35}\text{S}]$ sulphate into macromolecules, and $[^3\text{H}]$ protein synthesis also remained unchanged. In cultures treated with cycloheximide (Table 2b), $[^3\text{H}]$ protein synthesis was reduced by 75%, and chondroitin $[^{35}\text{S}]$ sulphate synthesis by about 80% compared with the control. However, if xyloside was incorporated into the medium of cycloheximide-blocked cultures, the synthesis of ^{35}S -labelled macromolecules was increased 4.3-fold, in accordance with previously published data (Kimura *et al.*, 1981). These results suggest that the failure of chondrocytes to incorporate $[^{35}\text{S}]$ sulphate into macromolecules when treated with F6060 is attributable to an absence of chondroitin sulphate chain synthesis due either to the inhibition of polymerizing enzyme, or to a failure in supply of UDP-sugar substrates.

Recovery of chondrocyte cultures from F6060 treatment

The biosynthetic activities of the chondrocyte cultures recovered almost completely after withdrawing F6060 treatment (Fig. 7a). During the first 3 h after removing F6060 $[^3\text{H}]$ protein synthesis recovered completely, and $[^{35}\text{S}]$ proteoglycan synthesis to about 80% of that in control cultures. Thereafter there was a slight decline in both $[^3\text{H}]$ -protein and $[^{35}\text{S}]$ proteoglycan synthesis, and even 21 h after exposure to F6060 the synthesis of these two types of macromolecule remained depressed by about 10 and 15% respectively, compared with controls. The reason for the biphasic response during the recovery period is unknown. Similar results were obtained in two separate experiments.

p-Nitrophenyl β -D-xyloside was added to cultures that had been allowed to recover for 21 h after exposure to F6060 to test their capacity to synthesize large amounts of chondroitin $[^{35}\text{S}]$ -sulphate (Fig. 7b). Cultures treated with F6060 and then allowed to recover for 21 h achieved 90% of the incorporation of $[^{35}\text{S}]$ sulphate into macro-

Table 2. F6060 inhibition of chondroitin sulphate synthesis onto *p*-nitrophenyl β -D-xyloside

Cultures were treated with F6060 for 4 h, exposed to *p*-nitrophenyl β -D-xyloside and cycloheximide during the last 2 h, and labelled with [35 S]sulphate and [3 H]lysine during the last 1 h of treatment. Results show the mean $\pm$ the range for duplicate cultures. The xyloside was dissolved in dimethyl sulphoxide (final concn. 0.002%). Control medium in (a) contained dimethyl sulphoxide and fatty-acid-free albumin (0.75 mg/ml). Control medium in (b) contained dimethyl sulphoxide, but no albumin. In further controls (not shown) in which dimethyl sulphoxide was omitted, [35 S]proteoglycan and [3 H]protein synthesis were both 15% lower than in (a) and 1% and 11% lower, respectively, than in (b).

Additions to culture	$10^{-4} \times$ Radioactivity (d.p.m./culture)	
	Chondroitin [35 S]sulphate	[3 H]Protein
(a) F6060 treatment		
None (control)	7.41 ± 0.36	12.13 ± 0.13
Xyloside (0.3 mM)	42.30 ± 0.90	6.05 ± 0.37
F6060 (25 μ g/ml)	0.12 ± 0.03	4.90 ± 0.05
Xyloside + F6060	0.09 ± 0.01	4.57 ± 0.05
(b) Cycloheximide treatment		
None (control)	6.79 ± 0.43	9.09 ± 0.38
Xyloside (0.3 mM)	52.18 ± 0.37	3.59 ± 0.53
Cycloheximide (10 μ g/ml)	1.44 ± 0.01	2.32 ± 0.03
Xyloside + cycloheximide	29.27 ± 0.39	0.31 ± 0.09

Fig. 7. Recovery from F6060 treatment

Chondrocyte cultures were treated with F6060 (25 μ g/ml) and fatty-acid-free albumin (0.75 mg/ml) for 3 h, allowed to recover in fresh medium without the inhibitor and labelled during the last 1 h of treatment or recovery with [35 S]-sulphate and [3 H]lysine. (a) Incorporation of radioactivity into [35 S]proteoglycan (●) and [3 H]protein (▲) at various recovery times. Results are for the mean incorporations in duplicate cultures expressed as a percentage of appropriate control cultures. (b) [35 S]Glycosaminoglycan synthesis in control cultures (1, 2) and F6060-treated cultures (3, 4) after recovery for 21 h, with *p*-nitrophenyl β -D-xyloside treatment (2, 4) during the last 2 h, and labelling in the last 1 h of the recovery period. Bars show the range for duplicate cultures.

molecules after xyloside stimulation, compared with control cultures. Thus the failure of F6060-treated cultures to recover 100% of the [35 S]proteoglycan synthesis of control cultures after 21 h recovery time (Fig. 7a) cannot be due either to limited availability of UDP-sugar precursors for glycosaminoglycan chains or to insufficient polymerizing enzyme activity.

Effect of F6060 on [35 S]proteoglycan and [3 H]protein synthesis in human fibroblast cultures

Cyclofenil has been used to treat the fibrotic disorder systemic sclerosis. We investigated the effect of its metabolite, F6060, on the incorporation of [35 S]sulphate and [3 H]lysine into macromolecules by near confluent cultures of dermal

Table 3. *Effect of F6060 on [³⁵S]proteoglycan and [³H]protein synthesis in human dermal fibroblast cultures from normal and systemic sclerosis patients*

Confluent cultures were treated for 3 h with the additions shown and labelled during the last 1 h of treatment with [³⁵S]sulphate and [³H]lysine. Results are the mean $\pm$ the range for duplicate cultures. Abbreviations used: FA⁺, fatty-acid-containing; FA⁻, fatty-acid-free.

Additions to medium	10 ⁻⁴ $\times$ Total radioactivity incorporated into macromolecules (d.p.m./culture)			
	Normal		Systemic sclerosis	
	³⁵ S	³ H	³⁵ S	³ H
(a) Albumin (FA ⁺) (4.0 mg/ml)	4.42 $\pm$ 0.11	37.10 $\pm$ 0.59	5.90 $\pm$ 0.40	43.14 $\pm$ 2.41
(b) F6060 (0.1 mg/ml) + albumin (FA ⁺) (4.0 mg/ml)	0.09 $\pm$ 0.04	13.77 $\pm$ 0.50	0.09 $\pm$ 0.02	10.61 $\pm$ 0.30
(c) F6060 (0.025 mg/ml) + albumin (FA ⁺) (1.0 mg/ml)	0.28 $\pm$ 0.05	8.21 $\pm$ 0.46	0.32 $\pm$ 0.01	5.69 $\pm$ 0.01
(d) F6060 (0.025 mg/ml) + albumin (FA ⁻) (0.75 mg/ml)	0.09	5.91	0.09 $\pm$ 0.01	4.44 $\pm$ 0.21
(e) F6060 (0.025 mg/ml) + albumin (FA ⁺) (4.00 mg/ml)	5.42 $\pm$ 0.2	40.92 $\pm$ 1.86	6.95 $\pm$ 0.33	43.76 $\pm$ 1.81

fibroblasts from normal subjects and patients (Table 3). The effects on both types of fibroblasts were the same, and were similar to those observed for the chondrocyte cultures. High concentrations of F6060 in the presence of high concentrations of albumin virtually abolished [³⁵S]proteoglycan synthesis, but only inhibited [³H]protein synthesis by between 63 and 75% (Table 3b). With lower concentrations of F6060 and albumin in the same ratio (w/w), inhibition of [³H]protein synthesis increased to 78–87%, whereas [³⁵S]proteoglycan synthesis was inhibited by about 95% (Table 3c). Cultures treated with the same concentration of F6060 but lower concentrations of albumin, achieved by using fatty acid-free protein, showed even greater inhibition of the biosynthetic pathways (Table 3d). Increasing the ratio of albumin to F6060 abolished the inhibitory effect of the compound (Table 3e). These results show that the action of F6060 on the biosynthesis of macromolecules is not confined to a single cell type and suggest that, as in the chondrocyte cultures, it could be used to inhibit specifically proteoglycan synthesis in fibroblast cultures, while leaving protein synthesis intact, by using particular ratios of F6060 to albumin.

Discussion

Cyclofenil diphenol has an inhibitory effect on the synthesis of macromolecules in chondrocytes. All the experiments demonstrating this were carried out in medium supplemented with albumin and insulin. Insulin has previously been shown to be as effective as foetal calf serum in promoting the synthesis of proteins, proteoglycans and hyaluronate in chondrocytes (Stevens *et al.*, 1981; d'Arville & Mason, 1983). By using albumin and insulin to supplement the culture medium rather than foetal calf serum, it was possible to control strictly the

concentration and type of protein present. Excess insulin had no effect on the inhibitory action of F6060 on the cells.

F6060 was maintained in solution by binding to albumin. When the concentration of the latter was the minimum required to solubilize a specific amount of cyclofenil, the compound had an inhibitory effect on the synthesis of proteins, glycoproteins, proteoglycans and hyaluronate. The presence of excess albumin abolished the inhibition. This suggests that the inhibitory effects of F6060 are mediated by free compound entering the cells. Excess albumin in the medium would provide more extracellular binding sites for the F6060, reducing the availability of the free compound.

Savu *et al.* (1979) showed that diethylstilboestrol, a compound of similar structure to cyclofenil diphenol, binds to albumin with an association constant (K_a) of approx. $2 \times 10^5 \text{ M}^{-1}$, and that up to 11 mol are fixed per mol of protein. Binding isotherms between microgram amounts of diethylstilboestrol and 30 μg of albumin/ml showed that about 50% of the oestrogen was in the bound state (Savu *et al.*, 1979). Similar ratios of cyclofenil diphenol and albumin were used in our experiments. Assuming a similar interaction occurs between cyclofenil diphenol and albumin, it is likely that the oestrogen would be present in both bound and unbound forms under these conditions.

The most important finding in our experiments is that by varying the albumin:F6060 ratios and concentrations, conditions can be found that inhibit proteoglycan and hyaluronate biosynthesis in the chondrocytes but have little or no effect on protein or glycoprotein synthesis. The results obtained with F6060-treated fibroblast cultures suggest that similar conditions could be determined for this cell type. An inhibitor that specifically blocks proteoglycan and hyaluronate synthesis in chondrocytes and other cells could find wide

application in the investigation of the role of these molecules in morphogenesis, cell-matrix interactions and extracellular matrix physiology.

It is unlikely that F6060 inhibits proteoglycan synthesis via an oestrogen-receptor-mediated mechanism for switching off core protein synthesis, even though the compound binds to such receptors (Garg *et al.*, 1983) and oestrogen receptors are present in several types of chondrocyte (Rosner *et al.*, 1982; Young & Stack, 1982). The chondrosarcoma chondrocytes have a pool of unglycosylated core protein in their endoplasmic reticulum system which is only half used up by glycosylation some 100 min after blocking protein synthesis (Kimura *et al.*, 1981). Our experiments show that the time of onset of F6060 inhibition of [³⁵S]proteoglycan synthesis is too rapid to be compatible with an action blocking formation of core protein. Secondly, chondroitin sulphate chains can be synthesized on *p*-nitrophenyl β -D-xyloside in cycloheximide-treated cells, but not in F6060-treated cells. These findings, together with the results showing simultaneous inhibition of hyaluronate synthesis, suggest that F6060 blocks the formation of glycosaminoglycan chains. This conclusion is also compatible with the finding that the compound inhibits [³⁵S]proteoglycan synthesis in fibroblasts. The proteoglycans synthesized by fibroblasts and chondrocytes are different, but the pathways leading to their production would share a number of common features. These include a requirement for certain UDP-sugars, transport of the latter from the cytoplasm into the Golgi apparatus, and transfer of the sugars from the UDP donor onto growing glycosaminoglycan chains. Since UDP-glucuronic acid is a common substrate for chondroitin sulphate and hyaluronate polymerization, either its formation, transport, or transfer could be a target for inhibition by F6060. A further possibility is that the compound intercalates itself in the membranes of the Golgi apparatus, disturbing the organization of the UDP-sugar transferases which polymerize glycosaminoglycan chains. This proposal would require that chondroitin sulphate proteoglycans are glycosylated in a different, and F6060-susceptible, part of the Golgi to most other glycoproteins, since the latter can still be synthesized in F6060-treated chondrocytes. It has been proposed that hyaluronate is also synthesized in the Golgi (Barland *et al.*, 1967), but more recent evidence suggests that the inner side of the plasma membrane may be the polymerization site (Prehm, 1984).

There is evidence that naturally occurring oestrogens can inhibit [³⁵S]proteoglycan synthesis (Priest *et al.*, 1960; Herbai, 1971b; Rosner *et al.*, 1979). We have shown that 17 β -oestradiol inhibits

the formation of [³⁵S]proteoglycan synthesis in chondrosarcoma chondrocyte cultures, but at pharmacological rather than physiological concentrations (M. K. Bansal & R. M. Mason, unpublished work). It is not known whether this action operates by a similar mechanism to F6060 inhibition.

The findings that F6060 inhibits proteoglycan synthesis in fibroblast cultures from both normal and systemic sclerosis patients is of interest. Patients with this disorder synthesize excessive amounts of extracellular matrix molecules, including collagen, fibronectin and proteoglycans (LeRoy, 1981). Initial trials with cyclofenil therapy have proved beneficial in ameliorating the dermal effects of the disorder (C. M. Black, M. I. V. Jayson, & A. G. White, unpublished work). Our experiments suggest that F6060, the metabolite of cyclofenil, could, potentially, reduce dermal fibroblast proteoglycan synthesis in systemic sclerosis.

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