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The relationship between proteoglycan synthesis in Swarm chondrocytes and pathways of cellular energy and UDP-sugar metabolism

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ABSTRACT

The effect of anaerobic culture conditions and various metabolic inhibitors on ^{35}S-proteoglycan synthesis, UDP-sugar pools, and the ATP pool were investigated in confluent, primary, day 1 cultures of Swarm chondrosarcoma chondrocytes. (*i*) Incubation under a nitrogen atmosphere for 6 h did not affect ^{35}S-proteoglycan synthesis or the pool size for UDP-glucuronate, other UDP-sugars, or ATP. Incubation with 5 mM sodium azide brought about a 40% reduction of proteoglycan synthesis in the first 30 min but no further change over the subsequent 90 min. UDP-Glucuronate, other UDP-sugar pools, and the ATP level were not affected by azide treatment. The results indicate that proteoglycan synthesis and its energy requirements can be supported entirely by anaerobic metabolism in these cells. (*ii*) ^{35}S-Proteoglycan synthesis, UDP-sugar production, and nucleotide triphosphate pools were inhibited in a concentration-dependent fashion with sodium iodoacetate. A > 70% reduction of the ATP pool after 30 min treatment suggests that glycolysis is a major target for iodoacetate. Lactate production was inhibited by 40% after 3 h treatment with 10^{-4} M iodoacetate. (*iii*) Glutamine deprivation resulted in a 60% contraction in the UDP-*N*-acetylhexosamine pool and markedly inhibited ^{35}S-proteoglycan and ^{3}H-protein synthesis. At the same time the UDP-glucose pool expanded to 200%, but the UDP-glucuronate pool was unchanged. The sum of the UDP-*N*-acetylhexosamine and UDP-hexose pools remained constant. Restoration of glutamine to previously depleted cultures resulted in excessive expansion of the UDP-*N*-acetylhexosamine pool and excessive contraction of the UDP-hexose pool before both adjusted to normal levels. The UDP-xylose pool was very small. No increases were observed during inhibition of proteoglycan synthesis induced by glutamine deprivation. (*iv*) 6-Diazo-5-oxo-L-norleucine (DON), a glutamine analogue and amino transferase inhibitor, induced a further contraction of the UDP-*N*-acetylhexosamine pool and a further decrease in proteoglycan synthesis in glutamine-deprived cultures. Thus cultures use endogenous glutamine during exogenous glutamine deprivation. DON unaccountably stimulated expansion of the UDP-glucuronate pool by 180%, irrespective of whether glutamine was present or not.

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INTRODUCTION

The large aggregating proteoglycan (aggrecan) synthesized by chondrocytes has a core protein of molecular mass of ~ 220 kDa to which may be linked as many as 100 chondroitin sulphate chains, numerous keratan sulphate chains, and O-linked oligosaccharides, and a lesser number of N-linked oligosaccharides. The carbohydrate content of the molecule may account for as much as 90% of its total mass of $2.0–2.5 \times 10^6$ Da[1,2]. Much has been learnt about the synthesis and secretion of proteoglycans from in vitro studies of cultures of chondrocytes from the Swarm chondrosarcoma. These cultures are capable of synthesizing between 1–4 μg of proteoglycan per h per 10^6 cells when maintained in media containing foetal calf serum or insulin[3,4]. Aggrecan accounts for ~ 90% of this output, the remainder being due to small chondroitin 6-sulphate proteoglycans[5,6]. The Swarm chondrocyte aggrecan molecule carries only chondroitin-4-sulphate chains with a repeating unit of 3)-β-D-*N*-acetylgalactosamine 4-sulphate-(1 → 4)-β-D-glucuronate-(1 → . The repeating disaccharide sequence is linked at its reducing end through a series of sugars, glucuronate-galactose-galactose-xylose, to serine residues in the core protein[1].

The biosynthetic assembly of aggrecan is a complex process, the chondroitin sulphate chains being polymerized onto the core protein in the Golgi apparatus by the sequential transfer of carbohydrate units from UDP-sugars. The whole process must depend on active energy metabolism by the chondrocytes to provide an adequate supply of UDP-sugars. Whilst the metabolic pathways leading to the formation of UDP-sugars in cells are well established[7], little is known about how the pools of these molecules relate to the level of proteoglycan synthesis. We have previously described the size and turnover rate of UDP-sugar pools involved in proteoglycan synthesis in cultures of Swarm chondrocytes[3]. In this report we have investigated the dependency of proteoglycan synthesis on specific ATP and UDP-sugar forming pathways in these cells.

EXPERIMENTAL

Details of the materials and methods have been described previously[3]. Briefly, chondrocytes were isolated from the transplantable Swarm rat chondrosarcoma and were set up as confluent primary cultures in Dulbecco's modified Eagle medium (DMEM) containing 100 units/mL penicillin, 100 μg/mL streptomycin, Hepes, Tes, Bes, and 15% foetal bovine serum (day 0). After maintenance overnight in a humidified 95% air, 5% CO_2 incubator at 37°C, the medium was renewed but the foetal bovine serum was replaced by 100 ng insulin/mL (day 1). Experiments were carried out on day 1.

Cultures were either labelled with [^{3}H]serine, [^{35}S]sulphate, or [^{3}H]glucosamine and the incorporation of radioactivity into macromolecules measured by Sephadex G-25 chromatography, as described previously[3,5], or investigated for UDP-sugars

or nucleotide triphosphates. For UDP-sugars, the cell layer was extracted by boiling in distilled water[3]. For nucleotide triphosphate analysis[3], the cell layer was extracted in ice-cold 0.3 M $HClO_3$.

The filtered aqueous extracts and the neutralised, filtered, perchloric acid extracts were analysed by anion exchange-HPLC using a Partisphere SAX column (125 × 4.7 mm i.d.) connected to a Spectrophysics 8700 delivery system. The column effluent was monitored for absorbance at A_{254} using a Spectrophysics LC871 detector and Spectrophysics integrator 4370. The columns were eluted (1 mL/min, 20°C) with three separate isocratic runs: (*i*) 30 mM KH_2PO_4, pH 3.5, which resolved UDP-*N*-acetylglucosamine, UDP-*N*-acetylgalactosamine, UDP-glucose/galactose, and UDP-xylose; (*ii*) 200 mM KH_2PO_4, pH 4.0. which resolved UDP-glucuronate from UDP, CDP, ADP, and GDP; (*iii*) 400 mM KH_2PO_4, pH 4.0, which resolved ATP from 3′-phosphoadenosine 5′-phosphosulphate and UTP/CTP. Full details of these analyses, chromatograms, and quantitation have been published[3].

To investigate the effects of anoxic conditions, the atmosphere in tissue culture flasks containing day 1 chondrocyte cultures was exchanged by passing a stream of oxygen-free nitrogen through the culture medium and over the cells for 3 min. The flasks were then rapidly capped and sealed.

Lactate and alanine in culture media were analysed as described previously[8].

RESULTS AND DISCUSSION

Dependency of proteoglycan synthesis on anaerobic metabolism in Swarm chondrocytes.—Active proteoglycan synthesis requires a plentiful supply of ATP to support protein synthesis, UTP formation for UDP-sugar synthesis, and activation of sulphate in the form of 3′-phosphoadenosine 5′-phosphosulphate for sulphation of glycosaminoglycan chains. When confluent cultures of Swarm chondrocytes were maintained for 6 h under an atmosphere of nitrogen, the level of proteoglycan synthesis was maintained at the same level as in aerobic conditions[9]. These experiments were repeated, but using perchloric acid extraction of the cell layer to maximise ATP recovery whilst UDP-sugars were analysed in aqueous extracts of replicate cultures. There was no change in proteoglycan synthesis, ATP pool size, UDP-glucuronate (Fig. 1), or other UDP-sugar pools (not shown), in anaerobic culture conditions compared to cultures maintained in aerobic conditions. This suggests that the chondrocytes can maintain normal levels of ATP solely from glycolysis, converting glucose to lactate via pyruvate, a totally anaerobic process.

Pyruvate dehydrogenase converts pyruvate to carbon dioxide and acetyl coenzyme A. The latter enters the tricarboxylic acid cycle to generate $NADH + H^+$ and $FADH_2$ which drive the respiratory chain, consuming oxygen and generating ATP by coupled oxidative phosphorylation. We were unable to detect pyruvate dehydrogenase activity in Swarm chondrosarcoma tissue[10]. Moreover, explant cultures released $^{14}CO_2$ from [1-^{14}C]glucose but not from [6-^{14}C]glucose, indicat-

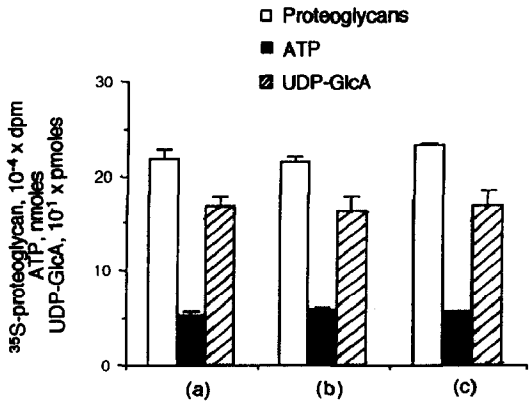

Fig. 1. The effect of anaerobic conditions. Primary cultures were labelled with ^{35}S-sulphate on day 1 for 6 h in flasks which were either (a) loosely capped and equilibrated with the 5% CO_2:95% air atmosphere of the incubator; (b) sealed, containing 100% air atmosphere; or (c) sealed, containing a nitrogen atmosphere. The cultures were then analysed. All results are expressed for 1×10^6 cells. The bars indicate the range for duplicate cultures.

ing that there is decarboxylation via the pentose phosphate pathway but no capacity for mitochondrial pyruvate oxidation[8]. This suggests that energy-dependent processes in the chondrocytes, such as proteoglycan synthesis, depend solely on ATP generated by anaerobic glycolysis. To investigate this, day 1 chondrocyte cultures were incubated for 2 h in medium containing 5 mM sodium azide, an inhibitor of cytochrome oxidase in the respiratory chain. ^{35}S-Proteoglycan synthesis decreased to 60% of control levels within the first 30 min (data not shown), but did not decrease further over the next 1.5 h (Fig. 2). The ATP, UDP-glucuronate (Fig. 2) and other UDP-sugar pools (not shown) did not change at all over the 2 h treatment period, showing that the Swarm chondrocytes maintain their normal energy status and convert UDP-glucose to UDP-glucuronate with no dependence on molecular oxygen. UDP-Glucuronate has a half-life of 12 min in Swarm chondrocytes[3], so inhibition of its synthesis due to anaerobic conditions would have been apparent within the time of the experiment. The initial 40% reduction in ^{35}S-proteoglycan synthesis may be due to azide ion (N_3^-) competition for sulphate transport into the cell. Other monovalent anions have been shown to compete with the sulphate carrier mechanism[11].

UDP-Glucuronate is formed from UDP-glucose, accompanied by a stoichiometric conversion[7] of NAD^+ to $NADH + H^+$. UDP-Glucose dehydrogenase is inhibited by NADH when the NAD/NADH ratio is low[12]. Thus continued formation of UDP-glucuronate depends on the reoxidation of NADH, and in cells with aerobic metabolism this will involve respiratory chain activity. Since Swarm chondrocytes continue to generate UDP-glucuronate and ^{35}S-proteoglycans when oxygen is either not available or cannot be utilized, their redox state must be maintained by other mechanisms. Pyruvate generated from glycolysis must be used to oxidize the NADH generated by glyceraldehyde 3-phosphate dehydrogenase in that pathway,

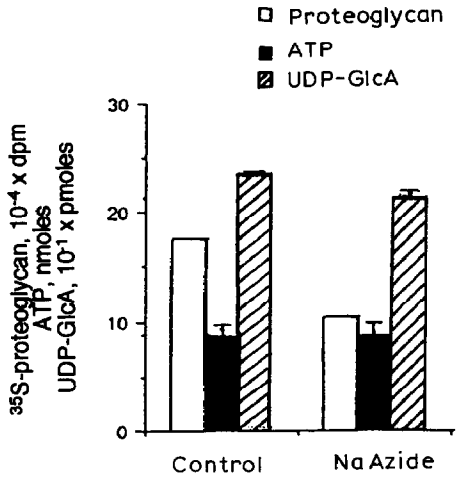

Fig. 2. The effect of sodium azide. Day 1 chondrocyte cultures were incubated in fresh medium (control) or in fresh medium containing 5 mM sodium azide for 2 h. All media contained ^{35}S-sulphate. Replicate cultures were extracted and analysed for ^{35}S-proteoglycans, ATP, and UDP-glucuronate. All results are expressed for 1×10^6 cells. Bars indicate the range for duplicate cultures.

regenerating NAD^+, on which continued anaerobic utilization of glucose in the chondrocyte will depend. One cytosolic mechanism for oxidising NADH generated by UDP-glucose dehydrogenase could involve reduction of dihydroxyacetone phosphate to glycerol 3-phosphate, a reaction catalysed by glycerol 3-phosphate dehydrogenase. Another, in the culture conditions used here, could involve the uptake of exogenous pyruvate from DMEM and its conversion to lactate.

Sodium iodoacetate, inhibits SH-dependent enzymes, including glyceraldehyde 3-phosphate dehydrogenase, and therefore the glycolytic pathway. It inhibited ^{35}S-proteoglycan synthesis, UDP-hexose, and UDP-*N*-acetylhexosamine, UDP-glucuronate, and nucleotide triphosphate synthesis in a concentration dependent manner (Fig. 3). The incorporation of ^{3}H-serine and ^{3}H-glucosamine into macromolecules was also inhibited (data not shown). The concentration dependency and magnitude of the effect were similar to that for ^{35}S-proteoglycans. The ATP pool was greatly reduced after only 30 min of treatment with 10^{-4} M iodoacetate, whereas some other cellular functions were much less affected or unchanged (Table I). This suggests that inhibition of glycolysis is a major target for iodoacetate in these chondrocytes, with loss of other functions secondary to depletion of energy substrates. L-Glutamine fructose 6-phosphate aminotransferase and UDP-glucose dehydrogenase have an SH-group at their active sites[12–14], but UDP-*N*-acetylhexosamines were unaffected and UDP-glucuronate much less affected than the ATP pool after 30 min exposure to iodoacetate.

The accumulation of lactate, the end product of anaerobic glucose metabolism, was decreased by 40% in the culture medium after treating for 3 h with 10^{-4} M iodoacetate (Fig. 4). This degree of inhibition agrees with results obtained when fresh chondrosarcoma explants were cultured with iodoacetate[10] and confirms that

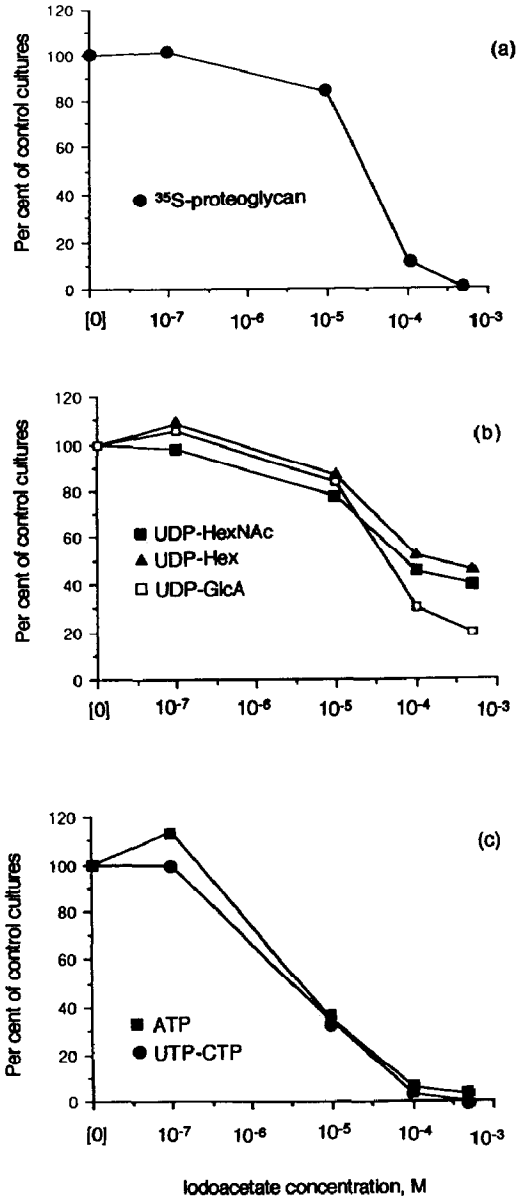

Fig. 3. The effect of sodium iodoacetate. Day 1 chondrocyte cultures were incubated with medium containing increasing concentrations of sodium iodoacetate for 3 h. ^{35}S-Sulphate was added during the last hour, after which cultures were extracted and analysed for (a) ^{35}S-proteoglycans; (b) UDP-*N*-acetylhexosamines, UDP-hexose, and UDP-glucuronate; (c) ATP and UTP-CTP. Results are expressed as a percentage of the control cultures incubated without iodoacetate. Each point is the mean for duplicate cultures. Variation between duplicates was within the range $\pm 5\%$ of the mean.

glycolysis was inhibited by this agent. The apparent degree of inhibition is apparently less than to that observed for ^{35}S-proteoglycan synthesis [$\sim 80\%$ decreased (Fig. 3)]. This may be due to measuring the lactate accumulated over the

TABLE I

Early inhibitory effects of iodoacetate on various chondrocyte functions [a]

Function	% of control
^{35}S-Proteoglycan synthesis	46
^{3}H-Protein synthesis	55
UDP-Hexose pool	100
UDP-*N*-Acetylhexosamine pool	100
UDP-Glucuronate pool	62
ATP Pool	27
UTP-CTP Pool	30

[a] Replicate cultures were incubated in medium containing 10^{-4} *M* sodium iodoacetate, ^{35}S-sulphate and ^{3}H-serine, for 30 min. Cultures were then extracted and analysed for various parameters. Results are expressed as a percentage of control cultures and are the mean of two cultures for each analysis.

whole 3 h period, whereas the ^{35}S-proteoglycan measurements were made during the last hour, at which time the agent would be exerting its maximum effect. Additionally, some lactate may be derived from pyruvate taken up from the DMEM during the period of iodoacetate treatment.

We also measured the accumulation of alanine in the culture medium (Fig. 4). We have shown previously that the Swarm chondrosarcoma is very active in converting glutamine to glutamate, which transaminates pyruvate to form alanine[8]. Alanine production by the Swarm cells was totally unaffected in cultures treated for 3 h with 10^{-4} M or lower concentrations of iodoacetate. The continued operation of this pathway indicates that the cells are not killed outright during the time course of the experiment.

We conclude that the contrasting effects of iodoacetate and azide on the UDP-sugar and ATP pools, and the ability to maintain these pools and ^{35}S-proteoglycan synthesis under a nitrogen atmosphere, are consistent with the operation of these pathways being entirely dependent on anaerobic mechanisms in these chondrocytes.

*Perturbation of UDP-*N*-acetylhexosamine synthesis and its consequences.*—The polymerization of chondroitin sulphate chains must ultimately depend on the availability of both UDP-glucuronate and UDP-*N*-acetylgalactosamine. The latter is derived from fructose 6-phosphate which undergoes amidation by L-glutamine–fructose 6-phosphate aminotransferase[15], yielding glucosamine 6-phosphate. The pathway depends on the availability of glutamine for the amidation[16], and in cell cultures may be perturbed by using glutamine-depleted medium, or by inhibiting the aminotransferase with 6-diazo-5-oxo-L-norleucine (DON), a glutamine analogue[15].

Swarm chondrocyte cultures were changed on day 1 to Dulbecco's MEM, supplemented with, or without, 4 mM glutamine and maintained for various periods. [^{35}S]Sulphate and [^{3}H]serine were added for the last hour of each period to assess proteoglycan and protein synthesis, respectively (Fig. 5). Other cultures were extracted at the end of each period to measure the UDP-sugar pools in the

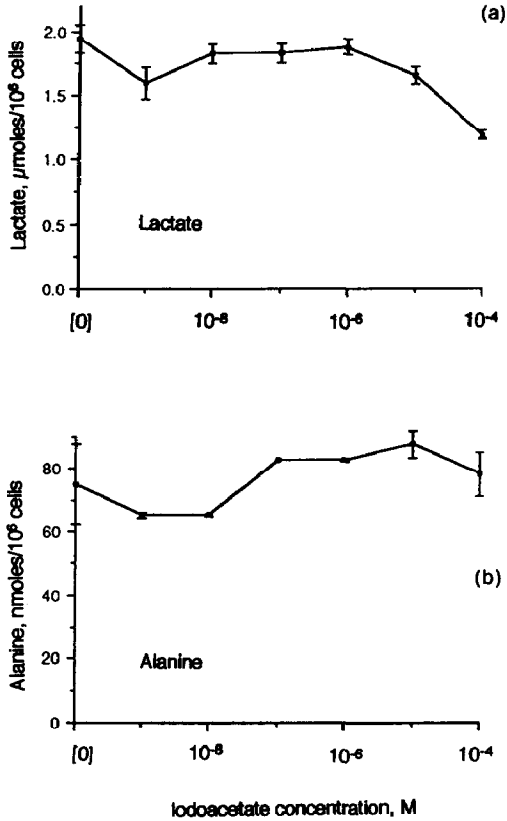

Fig. 4. Lactate (a) and alanine (b) accumulation in media of cultures treated with iodoacetate. Cultures were treated with sodium iodoacetate as in Fig. 3. Lactate and alanine were analysed in the medium at the end of the 3 h treatment period.

chondrocytes (Fig. 6). Both ^{35}S-proteoglycan and ^{3}H-protein synthesis decreased markedly in glutamine-depleted medium, the former taking ~ 9 h but the latter 2 h, or less, to reach a minimum level. The slower decline in ^{35}S-proteoglycan synthesis may be due to a pool of proteoglycan core protein in Swarm chondrocytes which has a half-life in the cell of ~ 60 min prior to its substitution with glycosaminoglycan chains[1]. The changes in UDP-sugar pools were complete within 2 h of glutamine deprivation. UDP-Nacetylhexosamines decreased to 38% of the pool size in control cultures, whilst the UDP-hexose pool increased to 200%. However, the sum of the UDP-*N*-acetylhexosamine and UDP-hexose content of the cells remained the same in the presence or absence of glutamine.

The UDP-glucuronate pool size was unchanged in glutamine-deprived cells (Fig. 6), even though its precursor, UDP-glucose, was elevated and proteoglycan synthesis was greatly decreased. This suggests that synthesis of UDP-glucuronate is tightly regulated and linked to the demand made on the pool for chondroitin sulphate synthesis. UDP-Xylose has been proposed as a negative regulator of UDP-glucose dehydrogenase and therefore of UDP-glucuronate synthesis[17]. Lower

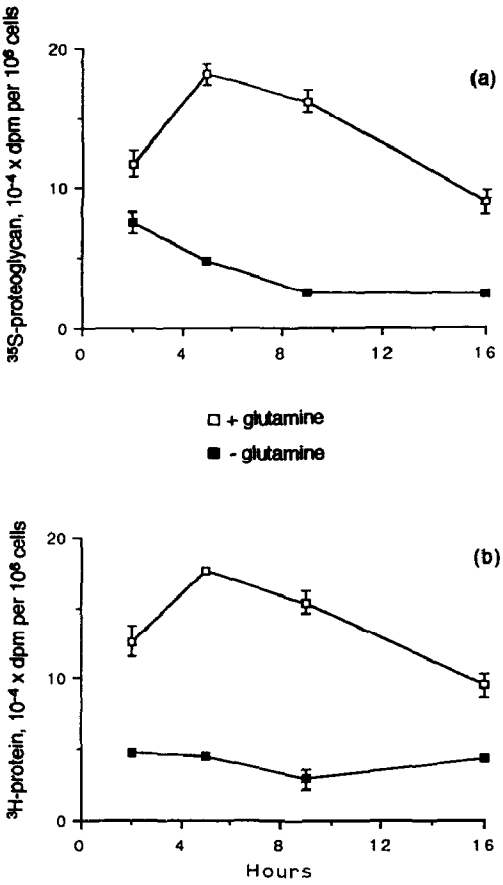

Fig. 5. ^{35}S-Proteoglycan and ^{3}H-protein synthesis. Day 0 chondrocytes were plated overnight in DMEM containing 4 mM glutamine. On day 1 cultures were changed to DMEM supplemented with 4 mM glutamine or without glutamine. The cultures were incubated for various periods and [^{35}S]sulphate and [^{3}H]serine added in the last hour. The cell layers were extracted and ^{35}S-proteoglycan (a) and ^{3}H-protein (b) measured in the combined cell layer and medium. Bars indicate the range for duplicate cultures.

levels of proteoglycan synthesis must decrease demand for xylose, the first sugar of the chondroitin sulphate chain. The amount of UDP-xylose normally present in Swarm chondrocytes is very low[3]. However, we did not detect any increase during depressed proteoglycan synthesis in glutamine-deprived chondrocytes (data not shown). Thus evidence in support of UDP-xylose as a regulator of UDP-glucose dehydrogenase at the cellular level in Swarm chondrocytes is lacking.

When cultures which had been deprived of glutamine for 16 h were given fresh medium containing 4 mM glutamine, ^{35}S-proteoglycan synthesis recovered to control levels within 2–3 h (data not shown). However, the UDP-sugar pools underwent excessive adjustments in size before recovering to control levels (Fig. 7). The *N*-acetylhexosamine pool expanded from its contracted state in glutamine

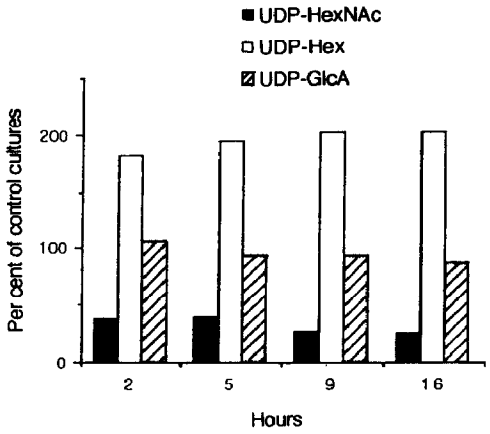

Fig. 6. UDP-Sugar levels during glutamine deprivation. Day 0 chondrocytes were plated overnight in DMEM containing 4 mM glutamine. On day 1 cultures were changed to DMEM without glutamine. The cultures were incubated for various periods after which UDP-sugars were measured. Results for the glutamine-deprived cultures are expressed as a percentage of levels in control cultures supplemented throughout with 4 mM glutamine. The range from the mean for duplicate cultures was less than ±9%.

deprivation to 50% larger than controls, 3 h after restoring glutamine. Conversely, the UDP-hexose pool contracted from its expanded state in glutamine deprivation to only 66% of control levels 3 h after restoring glutamine. Thereafter both UDP-sugar pools gradually adjusted to the levels found in control cultures. The UDP-glucuronate pools did not change during the period of recovery from glutamine deprivation (data not shown). These results and the changes in glutamine

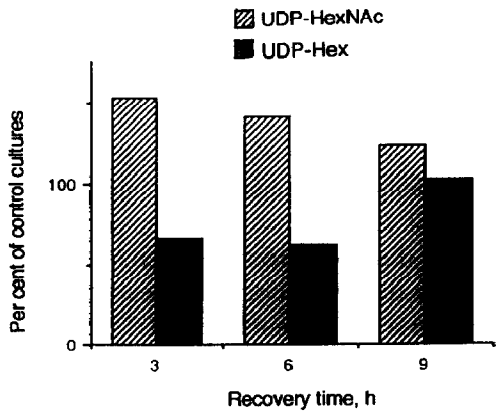

Fig. 7. Recovery of UDP-sugar levels after restoring glutamine to the culture medium. Day 1 chondrocyte cultures were deprived of glutamine for 16 h, as in Fig. 6. Cultures were then incubated for various periods with DMEM containing 4 mM glutamine after which the UDP-sugar levels were measured. Results are expressed as a percentage of levels in control cultures supplemented throughout with 4 mM glutamine. The range from the mean for duplicate cultures was less than ±7%.

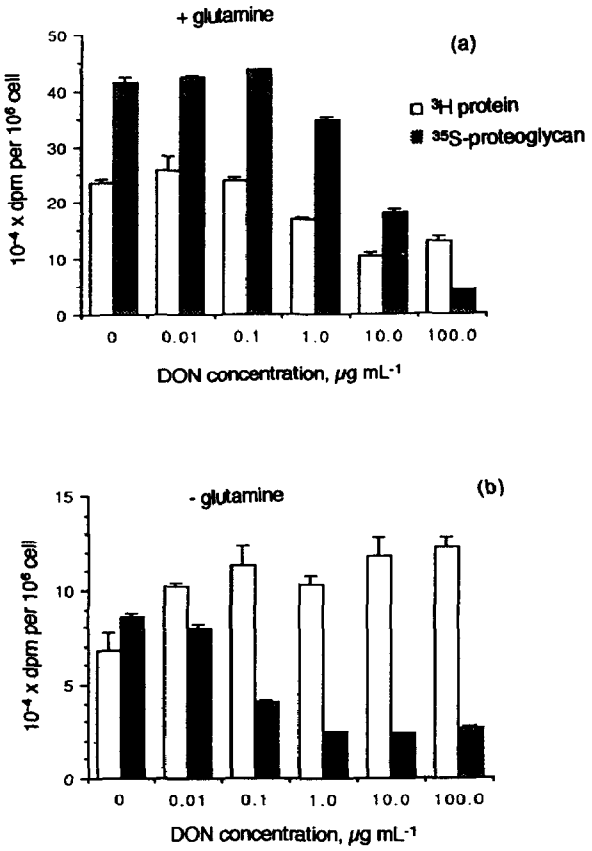

Fig. 8. The effect of 6-diazo-5-oxo-L-norleucine (DON) on ^{3}H-protein and ^{35}S-proteoglycan synthesis in day 1 chondrocyte cultures. Cultures were maintained in either DMEM with 4 mM glutamine (a), or in DMEM without glutamine (b), and increasing concentrations of DON for 6 h. Cultures were labelled with [^{3}H]serine and [^{35}S]sulphate during the last 2 h and the incorporation of radioactivity into macromolecules measured. Bars represent the range from the mean for duplicate cultures.

deprivation indicate that the mechanisms regulating the formation and pool size of UDP-hexoses and UDP-*N*-acetylhexosamine are interdependent.

After 16 h of glutamine deprivation the Swarm chondrocytes still maintained ^{3}H-protein synthesis at about 40% and ^{35}S-proteoglycan synthesis and the UDP-*N*-acetylhexosamine pool at about 25% of control cultures (Figs. 5 and 6). To do this, deprived cultures must utilize endogenous glutamine which they must either synthesize or obtain from catabolism of cell or matrix proteins. To test this we treated glutamine-deprived day 1 cultures with the aminotransferase inhibitor, DON, for 6 h. Cultures were either labelled with [^{3}H]serine and [^{35}S]sulphate during the last 2 h of treatment (Fig. 8), or harvested for UDP-sugar analysis after 6 h (Figs. 9 and 10). Parallel cultures, supplemented with 4 mM glutamine, were also treated with DON for comparison.

In agreement with Fig. 5, ^{35}S-proteoglycan synthesis was reduced in glutamine-deprived cultures to ~ 20% of the level in glutamine-supplemented controls (Fig.

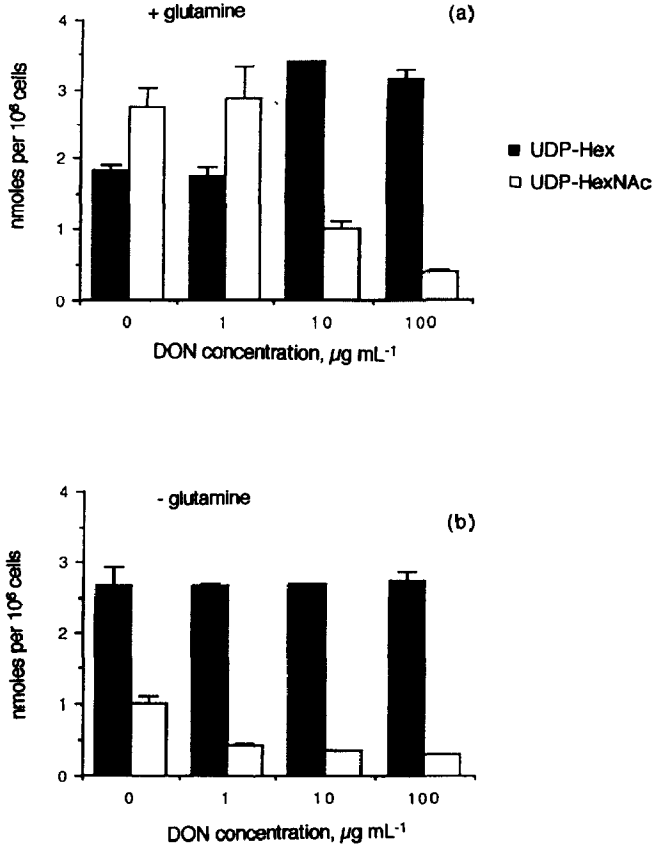

Fig. 9. The effect of 6-diazo-5-oxo-L-norleucine (DON) on UDP-*N*-acetylhexosamine and UDP-hexose levels. Day 1 chondrocytes were maintained for 6 h in the presence (a) or absence (b) of glutamine and increasing concentrations of DON. UDP-Sugar levels were then measured. Bars represent the range from the mean for duplicate cultures.

8). Addition of DON to a concentration of 1 μg mL^{-1} further reduced ^{35}S-proteoglycan synthesis in deprived cultures to 6% of controls (Fig. 8b). This maximum inhibition was accompanied by a further reduction in the size of the UDP-*N*-acetylhexosamine pool compared to that observed with glutamine deprivation alone (Fig. 9b). With 100 μg mL^{-1} DON the pool was reduced to 15% of that in glutamine-supplemented cultures with no drug. This finding confirms that cultures deprived of exogenous glutamine are able to maintain a low level of *N*-acetylhexosamine synthesis using endogenous glutamine as an amide donor.

Glutamine-deprivation reduced ^{3}H-protein synthesis to ~ 30% of the level in glutamine-supplemented cultures (Fig. 8), as expected (Fig. 5). The addition of DON to deprived cultures partially alleviated this, 0.1 mg mL^{-1} of the drug bringing ^{3}H-protein synthesis up to ~ 48% of that in the glutamine-supplemented control. This was unexpected since higher concentrations of DON inhibit ^{3}H-protein synthesis in supplemented cultures. The effect in deprived cultures may be

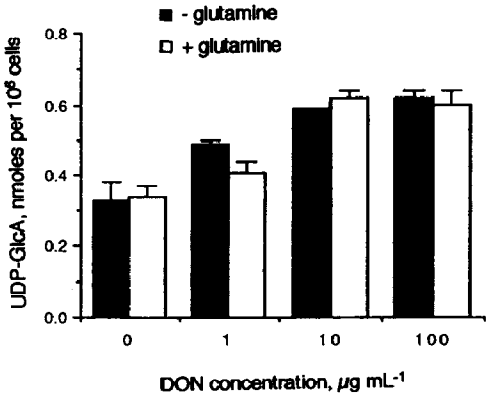

Fig. 10. The effect of 6-diazo-5-oxo-L-norleucine (DON) on UDP-glucuronate levels. Day 1 chondrocytes were maintained for 6 h in the presence or absence of glutamine and increasing concentrations of DON. The UDP-glucuronate level was then measured. Bars represent the range from the mean for duplicate cultures.

due to increased availability of endogenous glutamine for protein synthesis, the drug preventing its alternative utilization in the UDP-*N*-acetylhexosamine synthesis pathway.

DON did not promote any further expansion of the UDP-hexose pool in glutamine-deprived cultures (Fig. 9b). However, when DON was added to glutamine-supplemented cultures in high concentrations (≥ 10 μg mL^{-1}), the UDP-*N*-acetylhexosamine pool contracted whilst the UDP-hexose pool expanded (Fig. 9a). At the same time ^{35}S-proteoglycan synthesis was reduced (Fig. 8a). This is consistent with high concentrations of DON inhibiting the amidation of fructose 6-phosphate to glucosamine 6-phosphate, and thus inducing the same changes as glutamine deprivation, reported above.

The UDP-glucuronate pool size was independent of exogenous glutamine (Fig. 10), in agreement with earlier results. However, addition of DON stimulated an expansion of this UDP-sugar pool in both glutamine deprived and glutamine-supplemented cultures. This was independent of the levels of proteoglycan synthesis under the different conditions. With 10 μg mL^{-1} DON, UDP-glucuronate levels were 180% of controls in either the presence or absence of glutamine whilst ^{35}S-proteoglycan synthesis was 43 and 6% of controls, respectively. The mechanism by which DON expands the UDP-glucuronate pool is not clear.

GENERAL DISCUSSION

Several lines of evidence indicate that Swarm chondrosarcoma chondrocytes maintain normal metabolic function, including a high level of proteoglycan synthesis, using only anaerobic pathways to sustain their energy requirements. These include normal function in anaerobic cell culture conditions, maintenance of ATP pools during azide inhibition of the respiratory chain, absence of detectable

pyruvate dehydrogenase activity[10] and low oxygen consumption of explants of the chondrosarcoma tissue in vitro (Spencer, Palmer and Mason, unpublished work).

Glucuronate is a major component of chondroitin sulphate and, as described above, its synthesis is dependent on the availability of NAD^+, and therefore the redox state of the cell. In some cell types this will depend on aerobic metabolism. Thus UDP-glucuronate pools in rat liver fell to below the level of detection after only 5 min of exposure to anoxic conditions[18]. In contrast, UDP-glucuronate pools were maintained in Swarm chondrocytes during anoxia and azide treatment, indicating that the cells must have an alternative means of oxidising NADH to NAD^+.

It has been proposed that the synthesis of chondroitin sulphate may be restricted in cartilage chondrocytes living in an environment of low oxygen tension whilst the synthesis of keratan sulphate is promoted[19–21]. Galactose effectively replaces the glucuronate unit of chondroitin sulphate in the repeat disaccharide of keratan sulphate[22] and formation of UDP-galactose does not consume NAD^+ (ref 7). Very low oxygen tensions have been measured in cartilage[23,24]. Moreover, there is an increase in the keratan sulphate:chondroitin sulphate ratio in adult and ageing cartilages, which may be expected to be more anoxic[19,20]. Thus, it was reasoned, chondrocytes in older cartilage synthesize proteoglycan with increased keratan sulphate and decreased chondroitin sulphate content in response to a change in redox state accompanying lower tissue oxygen tension[19–21]. Swarm chondrosarcoma chondrocytes, in common with other chondrocytes in rat and mouse cartilage, do not synthesize keratan sulphate[25]. Thus it could be argued that their behaviour is not typical of chondrocytes from other species. Nevertheless, the present results demonstrate that it is possible for these chondrocytes to maintain the same level of proteoglycan synthesis and UDP-glucuronate pool size in anaerobic conditions as found in aerobic environments.

Previous studies suggested that DON perturbed cellular function simply by inhibiting UDP-*N*-acetylhexosamine formation[26,27]. The present results indicate that, additionally, there is an accompanying increase in the UDP-hexose pool size, an expansion of the UDP-glucuronate pool and a reduction in protein synthesis. The latter may be due to decreased availability of L-asparagine, a result of inhibition by DON of γ-amide transfer from glutamine to aspartate during synthesis of the amino acid[28]. The inhibitory effect of DON on proteoglycan synthesis is well established[26,27,29,30]. Depletion of the UDP-*N*-acetylhexosamine pool leads to synthesis of proteoglycans with shortened chondroitin sulphate chains and some entirely unsubstituted serine residues[31]. Our results indicate that inhibition is likely to be due to reduced levels of core protein synthesis as well as to inhibition of UDP-*N*-acetyl-hexosamine synthesis. In contrast to its inhibitory effect on protein synthesis in glutamine-supplemented cultures, DON had a slight stimulatory effect in glutamine-deprived cultures, as described above.

The UDP-hexose pool increased when the UDP-*N*-acetylhexosamine pool decreased during glutamine deprivation or DON treatment and vice versa during

recovery from glutamine deprivation. These pools are the products of the two main branches of UDP-sugar synthesis, being derived from a common glycolytic precursor, glucose 6-phosphate. The results suggest that the commitment of the precursor to each branch is, normally, coordinately regulated. The overall commitment of glucose 6-phosphate to UDP-sugar synthesis can be calculated. The UDP-hexose and UDP-*N*-acetylhexosamine pools are ~ 1.8 and 3.4 nmol per 10^6 cells on day 1, and have turnover rates ($t_{1/2}$) of 12 and 50 min, respectively[3]. Hence approximately 4.5 nmol UDP-hexose and 2.0 nmol UDP-*N*-acetylhexosamine must be synthesized per 10^6 cells per h to maintain these pools. Thus 6.5 nmol of glucose 6-phosphate will be required for this.

The size of the UDP-glucuronate pool was not influenced by either the doubling of its precursor pool, UDP-hexose, or by the decline in proteoglycan synthesis during glutamine deprivation. This infers that UDP-glucuronate synthesis is tightly controlled and probably linked to its utilization for chondroitin sulphate polymerization. In contrast to the other UDP-sugar pools, the UDP-glucuronate pool decreases with time in culture[3]. Measurements for individual cultures from a single cell preparation are highly reproducible but vary within the range 0.15–0.35 nmol per 10^6 cells for different preparations. With a pool size of 0.35 nmol and a half-life of 12 min[3], the cultures synthesize ~ 0.9 nmol UDP-glucuronate per h per 10^6 cells.

The UDP-glucuronate pool size increased dramatically in cultures treated with DON. This occurred without further expansion of the UDP-hexose pool in glutamine deprived cultures, and was independent of large differences in ^{35}S-proteoglycan synthesis, as seen in glutamine-supplemented and glutamine-deprived cultures treated with 10 μg mL^{-1} DON. Thus whilst DON induces the same expansion of the pool in both sets of conditions, the flux through the pool must vary according to the demand for chondroitin sulphate synthesis. It seems likely that changes in the level of proteoglycan synthesis in vivo would be accommodated by a change in the rate of flux through the appropriate UDP-sugar pools rather than by a mechanism involving constant flux and a change in pool size.

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