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Extracellular ATP and UTP stimulate cartilage proteoglycan and collagen accumulation in bovine articular chondrocyte pellet cultures

L.J. Croucher [a], A. Crawford [b], P.V. Hatton [c], R.G.G. Russell [a], D.J. Buttle [a,*]

[a] *Division of Human Metabolism and Clinical Biochemistry, University of Sheffield Medical School, Beech Hill Road, Sheffield, S10 2RX, UK*

[b] *DiscoveryBioscience, AstraZeneca, Bakewell Road, Loughborough, LE11 5RH, UK*

[c] *Centre for Biomaterials and Tissue Engineering, School of Clinical Dentistry, University of Sheffield, Claremont Crescent, Sheffield, S10 2TA, UK*

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Abstract

Bovine articular chondrocytes were maintained in high density pellet cultures with and without serum and nucleotide triphosphates for different periods of time. Despite half-lives in culture of about 3 h, adenosine triphosphate and uridine triphosphate in the presence of serum increased sulphated glycosaminoglycan and collagen deposition above control levels. In the presence of serum a single dose of uridine triphosphate on the first day of culture was sufficient to induce significant increases in subsequent proteoglycan and collagen deposition. We conclude that both adenine triphosphate and uridine triphosphate are anabolic for articular chondrocytes, and that this effect on the chondrocyte is long-term.

Keywords: Extracellular nucleotide triphosphate; Chondrocyte; Proteoglycan; Collagen

1. Introduction

Proteoglycans and type II collagen form the major components of the extracellular matrix (ECM) of cartilage [1]. These and the other molecules of the ECM are synthesised and degraded by the resident cells of cartilage, the chondrocytes. In healthy adult articular cartilage the rate of turnover of the ECM is relatively low. However, in degenerative joint diseases such as osteoarthritis (OA), cartilage ECM is gradually lost as the rate of breakdown exceeds that of synthesis and incorporation into the matrix [2–5]. This eventually results in cartilage depletion and loss of joint function.

Proinflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor-α stimulate proteolytic activity in cartilage [6–8], and both cytokines and proteinases have been targeted in attempts to reduce joint inflammation and cartilage loss [9–12]. Less well studied are agents that promote synthesis of ECM components by chondrocytes. Insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor have been shown to promote matrix synthesis and cartilage repair [13–15]. Extracellular adenosine triphosphate (ATP), acting via various purinoceptor subtypes, is known to have a range of effects on many different cells, including chondrocytes [16,17]. ATP has been detected in arthritic synovial fluid [18]

* Corresponding author. Fax: +44 (114) 2713781; E-mail: d.j.buttle@sheffield.ac.uk

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and may also be released from the chondrocyte itself. Studies measuring cell responses such as cytosolic Ca^{2+} flux and prostaglandin E_2 (PGE_2) release have demonstrated that extracellular ATP mediates these effects via purinoceptors of the P_2 class present on the surface of the chondrocyte [19]. Investigation of gene expression and use of purinoceptor agonists has shown that receptors of the P_2Y_2 subtype, at which ATP and UTP are equipotent, are present on articular chondrocytes [20].

In an earlier report we demonstrated that ATP had opposing effects on cartilage proteoglycan turnover depending on the source of cartilage. In explants of bovine nasal cartilage extracellular ATP increased both aggrecanase activity and proteoglycan loss. In contrast, ATP increased proteoglycan synthesis in bovine articular cartilage explants. In both experimental systems ATP appeared to initiate a shift in the phenotype of the chondrocytes, in that transient exposure to the nucleotide was sufficient to cause subsequent changes in the levels of proteoglycan breakdown or synthesis [21].

Measuring increments of cartilage matrix assimilation over the high amounts of material already present in cartilage explants presents a problem in accurate quantitation. Using a radiolabel to measure incorporation may analyse only a relatively small pool of rapidly turning over molecules. We have therefore utilised pellet cultures of articular chondrocytes, where the initial amount of extracellular matrix is essentially zero, to examine in more detail the effects of nucleotide triphosphates on total proteoglycan and collagen synthesis and deposition.

2. Materials and methods

2.1. Materials

Dulbecco's modification of Eagle's medium (DMEM) was supplemented in all experiments with glutamine (2 mM), streptomycin (100 μg/ml), penicillin (100 IU/ml), amphotericin B (0.25 μg/ml), all supplied by Gibco BRL Life Technologies (Paisley, UK), and gentamicin (25 μg/ml) and L-ascorbic acid (50 μg/ml), from Sigma-Aldrich (Poole, Dorset, UK). Newborn calf serum (NCS) was purchased from Gibco BRL Life Technologies and heat-inactivated at 56°C for 30 min. Dulbecco's phosphate buffered saline (PBS) and trypsin (EC 3.4.21.4) were also from Gibco BRL. Bacterial collagenase (clostridiopeptidase A, EC 3.4.24.3), ATP, adenosine diphosphate (ADP), uridine triphosphate (UTP), EDTA, 37% (w/v) paraformaldehyde and the lactate assay kit were all from Sigma-Aldrich. [^{35}S]Methionine was supplied by Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). Hydrochloric acid, trichloroacetic acid, molecular sieve 3A, uranyl acetate, lead citrate, OCT embedding medium, xylene and DPX mountant were purchased from B.D.H. Laboratory Supplies (Poole, Dorset, UK). Spurr's resin was supplied by Agar Scientific (Bishops Stortford, UK). 3-Aminopropyl triethoxysilane was obtained from I.C.N. Biomedicals (Thame, UK). All other reagents were of analytical grade.

2.2. Chondrocyte isolation and pellet culture

Full-thickness articular cartilage was dissected from the metacarpophalangeal joints of freshly killed cattle. Cartilage slices were washed in PBS and digested for 30 min at 37°C with 2.5 mg/ml trypsin, followed by an overnight incubation at 37°C in 3 mg/ml bacterial collagenase in DMEM. The isolated chondrocytes were washed once in PBS.

For pellet culture, the cells were resuspended at a density of 5×10^5 cells/ml in DMEM supplemented with 10% (v/v) NCS. 1 ml aliquots of the cell suspension were dispensed into 15 ml Falcon centrifuge tubes (Becton Dickinson, Oxford, UK), and centrifuged at $100\times g$ for 3 min. The chondrocyte pellets were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 7 days, unless stated otherwise. DMEM, without added serum, was replaced on days 3 and 5. ATP, ADP or UTP was added to chondrocyte suspensions in DMEM from 40× stock solutions in PBS, to give a final concentration of 500 μM. Unless stated otherwise, nucleotides were added on day 0 only.

2.3. Determination of $t_{0.5}$ for nucleotide triphosphates in pellet culture

Chondrocyte pellets were cultured as above with

ATP or UTP. Medium was removed from pellets incubated for 0.5, 1, 3, 5 and 24 h, filtered through 0.2 μm Acrodisc PF filters (Gelman Sciences, Northampton, UK), then snap-frozen and stored in liquid N_2. The freshly thawed medium was run through a 1 ml UNO Q1 anion-exchange column linked to a BioLogic chromatography system (Bio-Rad Laboratories, Hemel Hempstead, UK) using the following gradient: 120–360 mM NH_4HCO_3 over 17 min, followed by a return to 120 mM NH_4HCO_3 over 1 min. The flow rate was maintained at 1.5 ml/min. The column outflow was monitored at 254 nm and absorbance peaks representing each nucleotide were identified by comparison to those generated by running fresh standard preparations of 500 μM ATP or UTP. Culture time versus peak height was plotted and the $t_{0.5}$ was determined empirically by reading off the time taken for the peak heights to reduce to 50% of those of the 500 μM standards.

2.4. Quantitation of proteoglycan

Chondrocyte pellets were digested with papain and assayed for sulphated glycosaminoglycan (sGAG) using the dimethylmethylene blue metachromatic assay, as described previously [22].

2.5. Total collagen

Unprocessed chondrocyte pellets or pellets digested with papain for assay of sGAG (see above) were hydrolysed overnight with 6 N hydrochloric acid at 110°C and assayed for hydroxyproline content by the microassay method described previously [23].

2.6. Determination of type II collagen

CB11B, an epitope specific to type II collagen, was assayed by inhibition ELISA of proteinase-K-digested pellets as described [5].

2.7. DNA quantitation in chondrocyte pellets

Total DNA in proteinase-K-digested pellets was determined spectrophotometrically as previously described [24].

2.8. Protein synthesis

Protein synthesis was determined by [^{35}S]methionine incorporation as described previously [25]. Briefly, medium was removed from pellets on day 7 of culture and replaced with 1 ml of fresh medium containing 3 μCi/ml [^{35}S]methionine. After a further 2 h incubation, this medium was removed and the pellets were washed 3 times in 3% (w/v) trichloroacetic acid, followed by two rinses in water. The pellets were digested with papain as previously described [22] and incorporated ^{35}S was quantified as disintegrations per minute (dpm) in a scintillation counter. As a control, some pellets were killed by two freeze-thaw cycles prior to pulsing with [^{35}S]methionine.

2.9. Lactate determination

Medium (5–7 days) was assayed for lactate using the lactate oxidase/peroxidase method, provided in kit form.

2.10. Histochemistry

Pellets from 21 day cultures were frozen at −20°C and embedded in OCT medium. 7 μm thick sections were cut and transferred to slides precoated with 3-aminopropyltriethoxysilane as previously described [26]. Sections were fixed for 15 min in 4% (w/v) paraformaldehyde in PBS, washed 3 times with PBS and stained with toluidine blue for GAG [27] or van Geison stain for collagen, as described [28]. Stained sections were dehydrated through graded alcohols and xylene and mounted in DPX.

2.11. Transmission electron microscopy

Pellet cultures were prepared for energy dispersive X-ray microanalysis (EDS) in the transmission electron microscope (TEM). Pellets were first quench frozen in *n*-pentane at −140°C over liquid N_2. This freezing step was taken to prevent the movement or loss of ions from the specimen. Frozen specimens were transferred under a blanket of N_2 gas to polypropylene vials containing dry acetone and molecular sieve 3A. Freeze substitution was carried out at

−80°C for 7 days. The pellets were then returned to ambient temperature and washed with fresh, dry acetone. Specimens were infiltrated in Spurr's resin for 3 days and polymerised in fresh resin overnight at 60°C. Semi-thin sections (0.5–1.0 μm) were cut on an ultramicrotome using a diamond knife. Electron dense deposits in the cultures were viewed in a TEM (Philips CM10, Eindhoven, The Netherlands) and elemental analysis performed using an EDAX PV9800 EDS (EDAX UK, Haverhill, UK). Freezing artefacts and microstructure were assessed separately using ultrathin sections stained with uranyl acetate and lead citrate.

2.12. Statistical analyses

Within experiments, six pellet cultures per condition were tested. Experiments were performed at least twice using tissue from different animals. Statistical analyses were performed using the Mann-Whitney *U*-test for non-parametric data.

3. Results

3.1. The half-life of nucleotide triphosphates in pellet culture

Determined empirically as described in Section 2, the $t_{0.5}$ values for ATP and UTP in pellet culture were found to be 3.2 and 2.4 h, respectively. The half-life of ATP in bovine articular cartilage explants had previously been found to be 3 h [21].

3.2. The anabolic effects of extracellular nucleotides

Pelleted bovine articular chondrocytes from one joint were cultured in DMEM with a single initial dose on day 0 of 500 μM ATP, ADP or UTP. On day 7, the pellets were harvested and analysed for sGAG and hydroxyproline content. A representative experiment is shown in Fig. 1. Treatment with ATP or UTP increased the amount of sGAG laid down by the chondrocytes compared to controls, whereas ADP had no significant effect. UTP also significantly increased collagen (measured as hydroxyproline). A summary of such experiments carried out for 7 and 21 days is shown in Table 1. Extension of the culture period from 7 to 21 days led to few qualitative changes in anabolic response, with the exception that ADP as well as ATP and UTP produced increased quantities of sGAG and collagen in the pellets (Table 1). A specific assay for type II collagen [5] demonstrated that over 90% of the total collagen assayed as hydroxyproline was type II collagen (not shown).

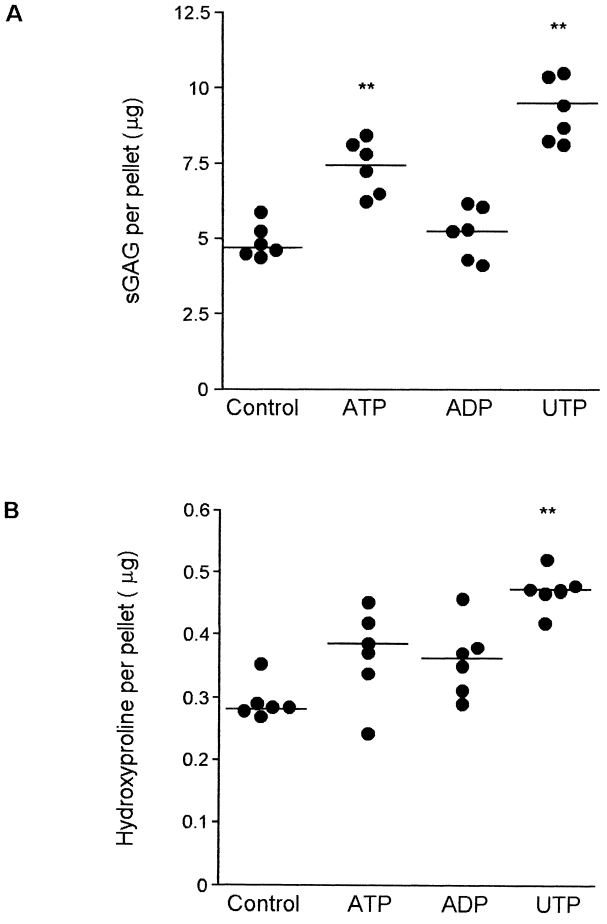

Fig. 1. The effects of extracellular nucleotide phosphates on matrix synthesis and incorporation in pellet culture. ATP, ADP or UTP (500 mM) was added to pellets (except controls) on day 0 only. 10% NCS was also present from day 0 to day 3. After 7 days, pellets were digested and assayed for sGAG (A) and hydroxyproline content (B); $^{**}P < 0.005$ compared to control.

3.3. Effects of repeated exposure to extracellular nucleotides on chondrocyte anabolic behaviour

In light of the effects of transient exposure to ATP and UTP on proteoglycan and collagen deposition

(Fig. 1 and Table 1), we investigated whether sGAG and collagen deposition varied according to the duration of exposure to nucleotides. In some pellets, 500 μM UTP was added to the medium on day 0 only, while in others UTP was replenished with fresh medium on day 3, or on both days 3 and 5. A representative experiment is shown in Fig. 2. While sGAG synthesis increased with repeated exposure of the chondrocytes to UTP, a single dose of UTP on day 0 was nonetheless sufficient to cause a significant increase in subsequent sGAG deposition into the matrix compared to controls. The amount of collagen deposited following a single exposure to UTP was not increased by further exposure to the nucleotide. A summary of similar experiments is presented in Table 2. These confirmed the significant anabolic effect of a single dose of UTP on sGAG and collagen accumulation in the matrix, with no further collagen deposition resulting from repeated exposure to the nucleotide. Our finding that exposure to serum need only be transient is also novel. For the experiments shown in Fig. 2 and Table 2 10% (v/v) NCS was only present up to day 3 of the 7 day cultures. Indeed, exposure to serum for as little as the first day of culture is sufficient for the extracellular nucleotides to subsequently exert their anabolic effects (results not shown).

These results suggest that chondrocytes undergo a long-term change to an anabolic phenotype in response to some extracellular nucleotide triphosphates and to serum. Repeated treatment with the nucleotides is not essential for maintenance of this phenotype, at least within the 7 or 21 day time course of these experiments.

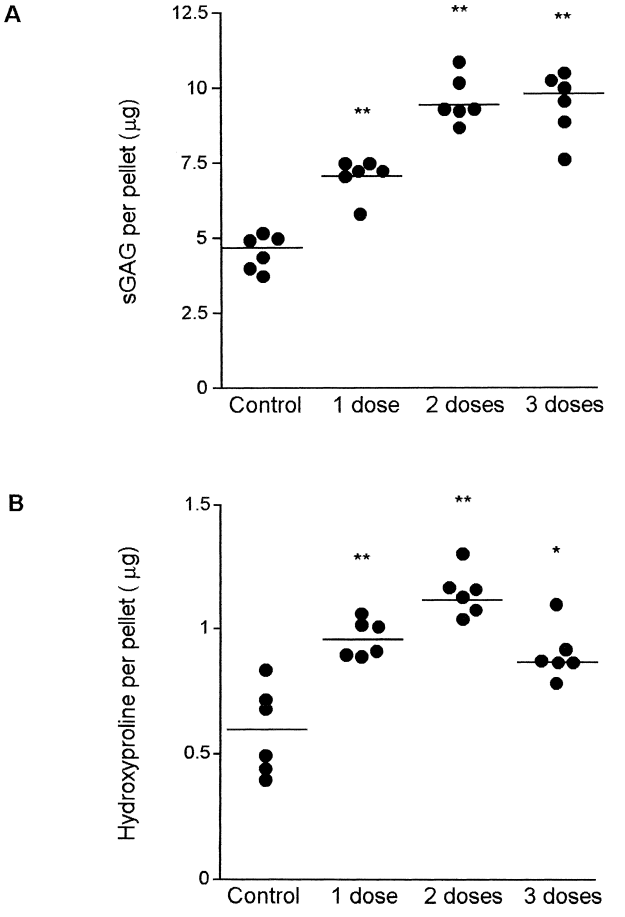

Fig. 2. The effects of repeated exposure to UTP on pellet matrix deposition. 500 μM UTP was added to all pellets except controls on day 0. Some pellets received only one dose of UTP, while in others UTP was replenished on day 3 (two doses) or on days 3 and 5 (three doses). 10% NCS was also added to all cultures on day 0 only. (A) sGAG; (B) hydroxyproline content. $*P < 0.05$ compared to control; $**P < 0.005$ compared to control.

Table 1
The effects of ATP, ADP and UTP on matrix synthesis and incorporation in chondrocyte pellets cultured over 7 and 21 days

		Nucleotide		
		ATP	ADP	UTP
7 days[1]	sGAG	1.58 ± 0.12***	1.03 ± 0.03	1.83 ± 0.10***
	Hydroxyproline	1.53 ± 0.10***	1.18 ± 0.11	2.10 ± 0.12***
21 days[2]	sGAG	1.27 ± 0.06***	1.10 ± 0.03**	1.45 ± 0.07***
	Hydroxyproline	4.31 ± 1.23***	1.22 ± 0.05**	4.06 ± 0.98***

Values represent means and standard errors of nucleotide-treated pellet cultures relative to controls (normalised to 1) for each experiment. Combined data are from experiments using chondrocytes from [1]seven and [2]five individual animals, $n = 6$ per animal. Statistical analysis is by the two-tailed Mann-Whitney U-test for non-parametric data; $**P < 0.005$; $***P < 0.0005$ compared to controls.

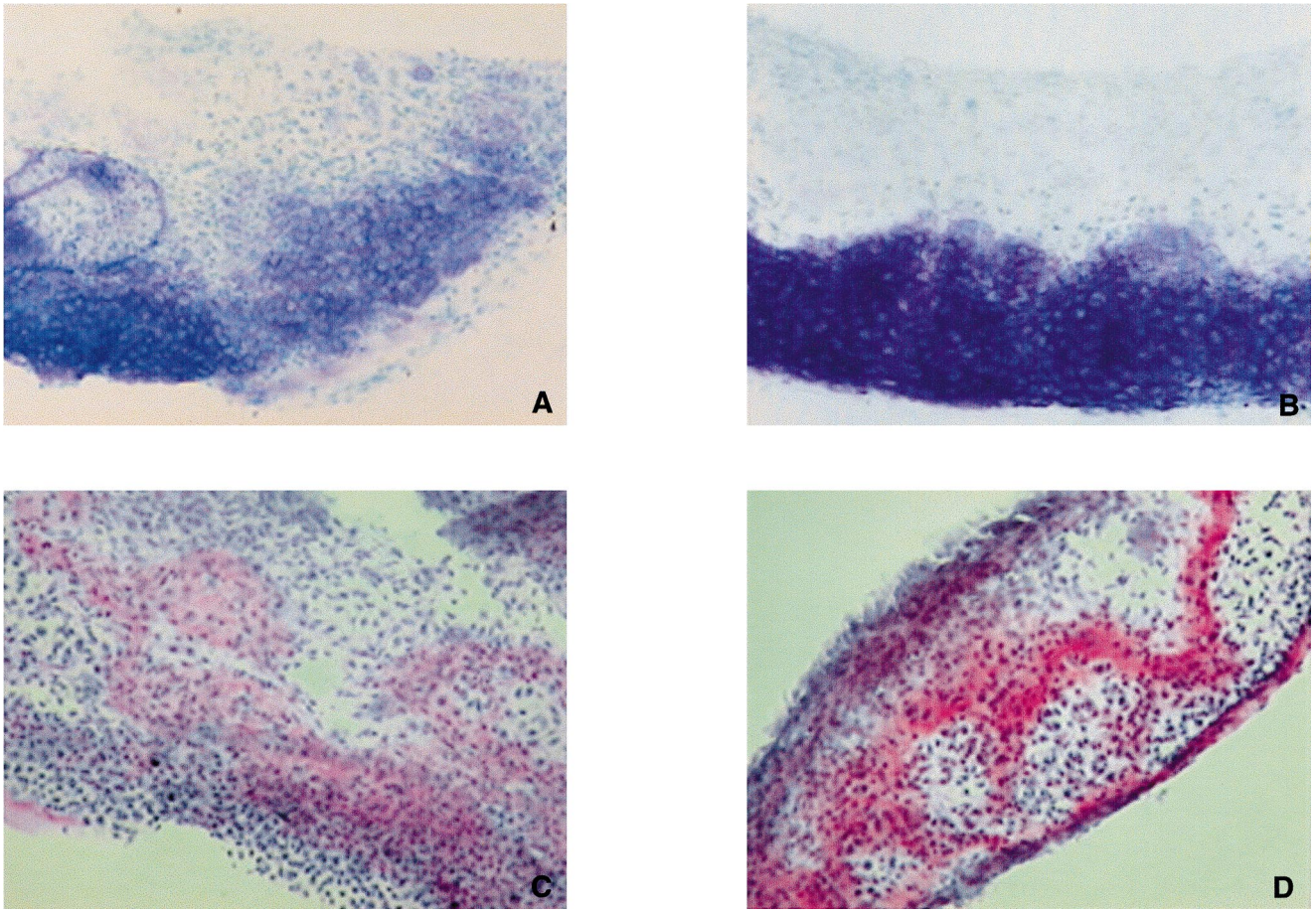

Fig. 3. Histochemical staining of pellet cultures. Pellets treated with ATP (B,D) and without ATP (A,C) were cultured for 21 days, sectioned and stained with toluidine blue for GAG (A,B) or van Geison for collagen (C,D).

3.4. Effect of extracellular nucleotides on cell number

In the above experiments, nucleotide triphosphates may have exerted anabolic effects via modulation of ECM synthesis, deposition and breakdown rates by chondrocytes, or by increasing chondrocyte number.

In order to determine the effects of extracellular nucleotides on chondrocyte number, chondrocytes were cultured in the presence or absence of ATP, ADP or UTP, and assayed for DNA content. A sample of the starting chondrocyte preparation (5×10^5 cells) used to establish the culture was also assayed. No significant increase in DNA content with nucleotide triphosphate treatment was seen fol-

Table 2
The effects of variable time of exposure to 500 μM UTP on pellet matrix deposition

	Exposure to 500 μM UTP		
	one dose	two doses	three doses
sGAG	1.42 ± 0.08***	1.84 ± 0.18***	1.96 ± 0.16***
Hydroxyproline	1.68 ± 0.20***	1.41 ± 0.08***	1.25 ± 0.06**

Combined data for experiments with chondrocytes from four different animals, $n=6$ for each. UTP was added on day 0 (one dose), day 0 and day 3 (two doses) and days 0, 3 and 5 (three doses) over the 7 day culture period. Values represent means and standard errors of UTP-treated pellets relative to controls (normalised to 1) for each experiment. Statistical analysis was by two-tailed Mann-Whitney *U*-test for non-parametric data; $^{**}P<0.005$; $^{***}P<0.0005$ compared to controls.

lowing 7 days in culture (not shown). While chondrocytes grown in pellets showed a 2–3-fold increase in DNA content during 21 days in culture compared to the initial number of chondrocytes, no significant differences were seen in the DNA content of pelleted chondrocytes between the three different treatments and the control (median values of 6.62 (range 5.95–10.61), 5.95 (5.41–7.52), 7.06 (5.17–8.56), 7.59 (6.37–10.28) μg DNA per pellet for control, ATP-, ADP- and UTP-treated 21 day pellet cultures, respectively). The increased sGAG and collagen synthesis seen in the presence of ATP or UTP can therefore be attributed to a direct effect of these nucleotides on the activity of the chondrocytes, rather than to an effect on cell number.

3.5. Histochemical analyses of chondrocyte pellets

ECM present in pellets was visualised directly by histochemical staining. After 21 days of culture, toluidine blue and van Geison staining showed focal areas of GAG and collagen deposition in all pellets (Fig. 3). The staining was asymmetrical, and we suspect that the side adjacent to the area of greatest staining was at the top of the pellet, nearest to the source of nutrients and with the shortest pathway for removal of metabolites. When cultures were left longer than 21 days cells in the interior of the pellets demonstrated signs of necrosis (not shown). There was no discernible difference in the distribution pattern of GAG and collagen between ATP-treated and control pellets. In agreement with the biochemical data, histochemical analyses of pellets after 7 and 14 days of culture demonstrated that the chondrocytes were actively depositing matrix during the entire 21 day culture period (not shown).

3.6. Chondrocyte viability

The effects of nucleotide triphosphates on the general metabolic state of chondrocytes in pellet culture were assessed by measurement of protein synthesis. Lactate production was also used as a general measure of cell metabolism, as chondrocytes respire anaerobically [29]. Nucleotide-treated pellets were pulsed on day 7 of culture with [^{35}S]methionine, digested and counted. All pellets, including controls, showed ^{35}S incorporation, indicative of protein synthesis, with median values of 7288 (range 6585–7547), 6148 (5353–6983), 7519 (7016–8576) and 6459 (5868–6993) dpm per pellet for control, ATP-, ADP- and UTP-treated pellets, respectively. Killed (frozen-and-thawed) pellets were included in the analysis to control for non-specific ^{35}S incorporation, and gave much lower counts (354 dpm, range 305–469) than those seen in the live pellets.

Medium (days 5–7) was assayed for lactate. No significant difference in lactate production was seen between control pellets and those treated with extracellular nucleotides, with median values of 210 (range 260–330), 235 (250–320), 225 (280–340) and 215 (270–320) μg lactate/ml for medium from control, ATP-, ADP- and UTP-treated pellets, respectively. This indicates that nucleotide treatment in this culture system is not cytotoxic to chondrocytes.

3.7. Mineral deposition

During the course of this study it was observed that the inclusion of 50 mM EDTA in the digestion mixture was required to obtain complete digestion of pellets by papain. We therefore investigated the possibility that mineral deposition was occurring during culture. Electron-dense deposits in sectioned pellets were analysed by EDS. The presence of deposits of calcium and phosphorus was confirmed in cultures treated with UTP and ADP, with no detectable quantities of other elements (not shown). Further work will be required to determine the exact nature of the mineral deposits, and whether their formation is dependent on the presence of nucleotide phosphates in the medium, or is an artefact of the culture system.

4. Discussion

We have previously demonstrated that ATP inhibits proteoglycan breakdown from articular cartilage explants whether or not the breakdown is driven by catabolic mediators such as proinflammatory cytokines and retinoic acid. By utilising radioactive tracer studies in the articular cartilage explant cultures we also demonstrated an apparent increase in cartilage proteoglycan synthesis in the presence of ATP [21]. In the present study we have utilised a pellet culture

system that is established as a model for cartilage matrix synthesis [13], and have demonstrated for the first time an increased accumulation of cartilage proteoglycan and collagen in the presence of ATP and UTP. Biochemical analyses, toluidine blue and van Geison staining of pellet culture sections confirmed that the chondrocytes continued to perform their role as matrix producers even after isolation from their original surroundings.

In previous studies the responsiveness of articular chondrocytes to extracellular ATP, in terms of elevated cytosolic calcium and PGE_2 production, have led investigators to conclude that chondrocytes express P_2 purinoceptors [19,20]. Although we have not yet analysed purinoceptor expression, the observation that in pellet culture articular chondrocytes respond to ATP and UTP in terms of increased sGAG and type II collagen deposition is in line with signalling pathways initiated by P_2 receptors. Receptors of the P_2Y_2 subtype (formerly known as P_2U) are activated by both ATP and UTP [30] and are refractory to the presence of ADP. A member of the P_2Y family, designated P_2Y_4, has recently been cloned from human placenta and human genomic DNA and found to respond most favourably to UTP and UDP, ATP being a partial agonist [31,32]. It is not yet known whether this receptor is expressed by mammalian chondrocytes.

We cannot yet discount the possibility that adenosine, formed from the breakdown of ATP in culture, is partly responsible for some of the effects we have described here, particularly as it is known that a P_1 receptor is expressed by chondrocytes [20]. However, this receptor does not appear to be involved in PGE_2 release [20], and in bovine nasal explant cultures neither adenosine nor AMP affected sGAG release whereas a slowly hydrolysed analogue of ATP had the same effect as ATP itself [21,33]. These data suggest that most of the effects of purines on cartilage are mediated through P_2 receptors.

In the presence of serum a single early exposure to UTP was sufficient to induce levels of sGAG and collagen synthesis significantly higher than those seen in cultures grown in the absence of the nucleotide. This finding is all the more remarkable in view of the short half-life of these nucleotides of around 2–3 h. The fact that serum is an essential requirement for the expression of anabolic effects of the extracellular nucleotides and also the observation that serum, as well as ATP or UTP, need only be present at the start of the culture for the subsequent manifestation of anabolism points to the existence of cross-talk between growth factor receptor and purinoceptor signalling pathways. A previous investigation has demonstrated that articular chondrocytes denuded of their surrounding matrix require serum or individual growth factors such as IGF-1 to increase new matrix synthesis above basal levels [13].

The in vivo significance of our findings has yet to be established, given that these data are based on the behaviour of chondrocytes removed from the influence of their original matrix. Damaged chondrocytes or those in a heightened state of metabolic activity, such as in OA and rheumatoid arthritis (RA), may release ATP into the surrounding extracellular matrix and synovial fluid. It is known that extracellular ATP is rapidly hydrolysed by synovial fluid, although at a slower rate by rheumatoid than by osteoarthritic and normal fluid [34]. Inhibition of the action of ectonucleotidases in the joint may therefore be one potential mechanism to promote the anabolic effects of the nucleotide triphosphates, which are potentially present in higher amounts in arthritis than in healthy joints, and at the same time inhibit the formation of pyrophosphate which is known to stimulate chondrocalcinosis [18,35]. The observation of the presence of mineral deposits containing calcium and phosphorus in our pellet cultures may be pertinent to the mechanisms of mineral deposition in joint pathology. It is known that rabbit growth plate chondrocytes grown in pellet cultures deposit a calcified matrix in the absence of exogenous ATP or pyrophosphate, and that this calcification is inhibited by low concentrations of transforming growth factor-β [36]. It is not known how mineral deposition and structure are modulated by the presence of ATP and UTP.

Our results suggest that therapeutic agents aimed at activating, in a number of potential ways, the receptors involved in the anabolic responses observed in this study may be beneficial in the treatment of degenerative diseases such as OA and RA. This proposal is supported by evidence from previous work demonstrating that ATP inhibits basal and IL-1-stimulated proteoglycan breakdown in articular cartilage explants [21]. Further work is required to de-

fine whether the increased accumulation of matrix is mostly due to an inhibition of breakdown or an increase in synthesis and successful deposition into the matrix, or a combination of both these effects.

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