Published in final edited form as:

Matrix Biol. 2005 December ; 24(8): 510–519. doi:10.1016/j.matbio.2005.08.006.

Hyaluronan secretion by synoviocytes is mechanosensitive

T.S. Momberger^a, J.R. Levick^b, and R.M. Mason^{c,*}

^aDivision of Biomedical Sciences, Faculty of Medicine, Imperial College London, London SW7 2AZ, United Kingdom

^b Physiology, Basic Medical Sciences, St. George's Hospital Medical School, London SW17 0RE, United Kingdom

^c Renal Section, Division of Medicine, Imperial College London, Hammersmith Hospital, London W12 0NN, United Kingdom

Abstract

Hyaluronan (HA) is an essential component of synovial interstitial matrix and synovial fluid, but the link between its production and joint use is unclear. HA secretion is enhanced by joint distension in vivo, but direct proof that synoviocytes exhibit mechanosensitive HA secretion is lacking. We tested this in vitro. Primary rabbit synoviocyte (PRS) cultures from microdissected synovial intima were subjected to 180 min of maintained 10% static stretch, or to 10 min of 10% static stretch followed by 170 min relaxation, in a Flexcell 2000 apparatus. Stretch stimulated HA secretion into the medium over 3 h by 57%. Notably, a short stretch (10 min) was as effective as sustained stretch. Actinomycin D and cycloheximide abolished stretch-stimulated HA secretion and also reduced basal HA secretion rate. RT-PCR showed that HAS2 was the major hyaluronan synthase expressed, but there was no increase in HAS2 mRNA (or other isoforms) in continuously stretched cells, and only a small increase (20%) at 180 min in cells stretched for the first 10–30 min. However HAS2 transcription increased 10-fold in response to TGF- β 1 and IL-1 β . Thus HA secretion by intimal synoviocytes is regulated by a mechanosensitive pathway which depends on transcription and de novo protein synthesis, possibly of HAS2, but also of other proteins involved in HA secretion.

Keywords

Synovium; Hyaluronan; Hyaluronan synthase; Stretch; Mechanosensitive

1. Introduction

The synovial intima layer, or 'synovium', is a vascularised sheet of specialized connective tissue enclosing the synovial cavity. The intima is about 10–20 μ m thick in the rabbit and comprises one to three layers of specialised cells, namely fibroblast-related synoviocytes or 'type B' cells (67%), and a smaller proportion of non-specific esterase-positive macrophages or 'type A' cells (Henderson and Edwards, 1987; Levick and McDonald, 1989a,b). The intima backs onto a thicker layer (~100 μ m) of less specialised, loose areolar connective tissue, the subsynovium. The surface synoviocytes have a characteristic branched morphology, abundant rough endoplasmic reticulum (Wilkinson et al., 1992), and synthesise and secrete hyaluronan (HA) and lubricin into the intraarticular synovial fluid, which is primarily an ultrafiltrate of fenestrated capillary plasma (Mason et al., 2002).

*Corresponding author. Tel.: +44 208 383 2718; fax: +44 208 383 2062. E-mail address: roger.mason@imperial.ac.uk (R.M. Mason).

Hyaluronan, a glycosaminoglycan composed of repeating units of D-glucuronic acid and D-N acetylglucosamine, linked $\beta(1-4)$ and $\beta(1-3)$, is synthesised at the plasma membrane (Prehm, 1983). Elongation takes place at the intracellular reducing end of the chain whilst the non-reducing end is simultaneously extruded through a trans-membrane pore into the extracellular space. This allows the polymer to grow extremely large. The enzyme responsible, hyaluronan synthase (HAS), has three mammalian isoforms (Itano et al., 1999). In the rabbit synovium, HAS2 is expressed predominantly, whilst HAS3 is expressed at only a very low level (Ohno et al., 2001).

HA is present in synovial fluid, on the cartilage surfaces, and in the synovial interstitial matrix, and has long been recognised as essential for rheological properties such as lubrication, mechano-protection and shock absorption (Balazs and Denlinger, 1985). Synovial fluid HA also has a major role in the attenuation of fluid loss from the joint cavity during periods of raised pressure, such as occur during a sustained joint flexion (Coleman et al., 1999; Lu et al., 2004).

The way in which HA secretion into mature joints is regulated, and especially the relation between joint use and HA secretion, has received little experimental investigation. However, we found evidence suggesting a brisk increase in rabbit synovial HA synthesis in response to synovial lining stress in vivo (Price et al., 1996). A second study extended this observation by showing that HA secretion into the rabbit knee joint cavity over 3-6 h is increased by ~25% following synovial lining distension by 2 ml intra-articular saline (Coleman et al., 1997). Electron microscope studies confirmed that the joint distension causes a marked stretching of the synovial lining cells (McDonald and Levick, 1988; Levick and McDonald, 1989b). These observations led to the hypothesis under investigation here, that mechanical deformation is a major regulator of hyaluronan secretion by synoviocytes. The present study tested this hypothesis directly by measuring HA secretion in cultured rabbit synoviocytes subjected to a standardised mechanical deformation in vitro. We also tested the roles of transcription and translation in the response, since potential mechanisms for increasing HA secretion include the upregulation of HAS expression and/or other proteins associated with HA synthesis/secretion. In addition, HAS isoform expression was assessed to investigate whether increased HAS transcription, as opposed to increased translation or increased activity of existing HAS, might underlie mechanically induced stimulation of HA secretion.

2. Results

2.1. Phenotype of cultured synoviocytes

The cultured synoviocytes, derived from explants of rabbit synovial intima, had a branching, extended morphology which resembled that of intimal cells in situ (McDonald and Levick, 1988; Levick and McDonald, 1989b). They developed a thick pericellular coat (Fig. 1A, C) that was readily digested by *Streptomyces* hyaluronidase (Fig. 1B, D), a well documented characteristic of HA-secreting cells (Fraser and Laurent, 1996). Moreover, they stained negatively for NSE (Fig. 1E), whereas preparations of non-adherent cells from synovial explants showed positive NSE staining (Fig. 1F). NSE is known not to be expressed by B-type synoviocytes, but it is expressed by the synovial lining macrophages (Henderson and Edwards, 1987).

Synoviocytes cultured on uncoated plastic culture dishes expressed the following integrin subunits, as detected by RT-PCR: $\alpha 1$, $\alpha 2$, $\alpha 5$, $\alpha 6$, αv , $\beta 1$ and $\beta 3$ (data not shown). The $\alpha 1$, $\beta 6$, αv and $\beta 1$ subunits were particularly strongly expressed. This is in keeping with the synoviocyte phenotype in situ, which expresses $\alpha 1\beta 1$, $\alpha 5\beta 1$, $\alpha v\beta 1$, (Johnson et al., 1993;

Rinaldi et al., 1997a; Pirilä and Heino, 1996), $\alpha v \beta 3$ (Rinaldi et al., 1997b; Tak et al., 1997) and $\alpha 6 \beta 1$ (Pirilä et al., 2001).

Collectively, the method used to obtain and culture cells from the synovial intima, their formation of hyaluronan coats, absence of non-specific esterase activity, and their pattern of integrin expression, are consistent with these cells being type B intimal synoviocytes.

2.2. Stretch-induced stimulation of HA secretion by rabbit synoviocytes

Theoretical calculations based on the radius of curvature of the rabbit femoral condyle (0.5cm) indicate a maximum cell stretch during flexion in vivo of the order +36%, but in reality it may be much less, because the synovial lining is furrowed rather than completely flat in extension (McDonald and Levick, 1988). Acute joint effusions stretch synovial cells in situ by up to 42% (McDonald and Levick, 1988; Levick and McDonald, 1989b). Stretches of nominal mean magnitude 5%, 10%, 20% and 30% were initially tested on cells which were cultured in collagen 1-coated wells. Type 1 collagen is a major extracellular protein for synoviocyte attachment via integrins in vivo and pilot studies showed that it supported cell proliferation of rabbit synoviocytes at the same rate as uncoated wells, or fibronectin-coated plates (FlexCell International Corp), and that cells cultured on uncoated plates strongly expressed collagen binding integrins ($\alpha 1$, $\alpha 2$ and $\beta 1$). Preliminary results showed that a 10% stretch of cells grown on type I collagen elicited the most consistent stimulation of HA secretion over 3 h (see below). 5% stretch stimulated HA secretion but to a less consistent degree. 20% stretch produced a smaller stimulation than 10% stretch, and 30% stretch caused no increase in HA secretion. Thus 10% stretch was selected for the studies reported below.

15 separate experiments were performed in which replicate cultures of synoviocytes in 6 well Flexcell plates were stretched by +10% for 180 min or, if controls, not stretched (Fig. 2A, right side). In 9 of these experiments, a 10% stretch was applied to further sets of plates (6 wells/plate) for 10 min followed by 170 min rest (Fig. 2A, left side). Basal HA secretion in the controls averaged 0.91 ± 0.11 ng HA/1000 cells/3 h ($n = 15$, 6 wells per experiment), but mean values ranged widely (6 fold) between experiments, from 0.33 ± 0.05 to 2.00 ± 0.09 ng HA/1000 cells/3 h (Fig. 2A). This was in keeping with in vivo measurements where basal HA secretion rate varies over a similarly wide range of 1.7-8.5 $\mu\text{g/h}$ (5 fold) in intact rabbit knee joints (Anggiansah et al., 2003).

Both of the stretch protocols stimulated HA secretion significantly, demonstrating unequivocally for the first time that the synoviocyte is mechanosensitive (Fig. 2A). A period of 180 min sustained stretch stimulated HA secretion rate by $57 \pm 7\%$ from 0.91 ± 0.11 ng HA/1000 cells/3 h to 1.41 ± 0.16 ng HA/1000 cells/3 h ($p < 0.0001$, $n = 15$, paired t test). Of particular interest was the finding that a 10 min stretch followed by 170 min rest stimulated HA secretion by an almost identical amount, namely by $57 \pm 18\%$, from 0.82 ± 0.17 ng HA/1000 cells/3 h to 1.32 ± 0.21 ng HA/1000 cells/3 h ($p = 0.001$, $n = 9$, paired t test). This indicates that increased HA secretion is triggered by an early event after the initial stretch and does not require a continuously maintained stimulus. The size of the response to stretch varied considerably between experiments, from 25% to 100%, in keeping with a similar wide variability of the HA secretory response of intact rabbit knees in vivo to both chemical (phorbol ester, Anggiansah et al., 2003) and mechanical stimuli (distension, Coleman et al., 1997). The magnitude of the response correlated significantly with the magnitude of the basal secretion rate, as shown in Fig. 2B (correlation coefficient $r^2 = 0.64$ for 10 min stretch and 0.90 for 180 min stretch; $p = 0.005$ and < 0.0001 , respectively). The cells appeared morphologically normal on microscopic inspection after each experiment and excluded trypan blue.

2.3. HAS isoform expression in non-stretched and stretched synoviocytes

RT-PCR of RNA from unstretched synoviocytes gave cDNA bands of the predicted size for HAS2 (409 bp), which was the main HAS isoform expressed, and for HAS3 (332 bp), which was expressed at only a very low level (data not shown). DNA sequencing validated the identity of these PCR products. RT-PCR also identified HAS2 as the major isoform expressed in the intact synovial intima of the rabbit knee (results not shown). HAS3 was not detected. These results confirm the findings of Ohno et al. (2001) who reported that rabbit synoviocytes express HAS2 strongly, with low expression of HAS3. We also amplified a cDNA band from synoviocyte RNA using published HAS1 primer sequences (Kawakami et al., 1998), but sequencing showed that it had homology with HAS2 and was therefore not attributable to HAS1. Kawakami et al. (1998) did not report sequencing their amplification product, making their findings uncertain. We designed new PCR primers manually for human HAS1 after determining a region of low homology between HAS1 and HAS2, but these also failed to amplify a HAS1 transcript in rabbit synoviocyte RNA.

Three different experiments were performed to examine the effect of 10%, 20% and 30% static stretch on HAS isoform expression. In view of the striking finding that 10 min stretch followed by 170 min rest stimulated HA secretion as effectively as 180 min sustained stretch, we also measured HAS transcript after different periods of stretch. In each experiment synoviocytes on 6-well plates were stretched for periods of 10, 30, 60 or 180 min and the RNA harvested 180 min after initiating the stretch. Non-stretched cultures were harvested as controls (Fig. 3). HAS2 was the main mRNA transcribed in the stretched synoviocytes. However there was no significant increase in HAS2 transcript overall; the HAS2/hactin transcript ratio averaged 0.68 ± 0.11 in the controls ($n = 3$ experiments) and 0.75 ± 0.06 for the combined group of all degrees and durations of stretch ($n = 12$ experiments; $p = 0.63$, unpaired t test). Since 10% stretch is the most effective stimulus for HA secretion, this subgroup was assessed separately but the change in transcript level was again slight (control 0.84, stretched 0.94 ± 0.03 , $n = 4$), as shown in Fig. 3. Although there was a consistent, minor (~20%) increase in transcript in cultures subjected to 10 min stretch ($p = 0.039$, $n = 3$, paired t test), this was evident not only with 10% stretch but also with 20% and 30% stretch, which are ineffective stimuli for HA secretion, so it is unlikely to have functional significance. The very low HAS3 mRNA levels in non-stretched cultures were not detectably enhanced by stretch (results not shown) and HAS1 was not detected.

In view of the overall lack of change in HAS2 mRNA levels in rabbit synoviocytes exposed to stretch, we tested whether gene expression could be upregulated in response to other stimuli. IL1 β and TGF β have been shown to stimulate HAS activity and HA secretion in a range of cell lines (Kawakami et al., 1998; Haubeck et al., 1995; Recklies et al., 2001; Suzuki et al., 2001; Tanimoto et al., 2004; Heldin et al., 1992). Thus rabbit synoviocytes were cultured in complete medium for 24 h after seeding, then maintained in serum-free medium for 12 h, after which they were treated for a further 24 h in serum free medium with 1-5 ng/ml of either IL1 β or TGF β , or with a combination of both cytokines. Each cytokine induced HAS2 expression in a dose responsive manner, as assessed by RT-PCR (result not shown). The HAS2: h-actin ratio increased about 2.9 fold in response to 5 ng/ml IL1 β and 1.8 fold in response to 5 ng/ml TGF β , whilst both agents together at these concentrations acted synergistically to induce an approximately 10 fold increase. Thus HAS2 is inducible in rabbit synoviocytes in response to appropriate stimuli.

2.4. Inhibition of translation blocks mechanosensitive HA secretion

To test whether stretch-induced HA secretion depends on de novo synthesis of protein, cells were either stretched or not stretched in the presence or absence of 10 μ g/ml cycloheximide (Fig. 4A). Mechanosensitive stimulation of synoviocyte HA secretion was again confirmed,

as was the equal effectiveness of short and sustained stretch. In the untreated cells, 10 min stretch and 180 min stretch increased HA secretion over 3 h by almost identical amounts, namely, $65.1 \pm 17.3\%$ and $65.3 \pm 11.8\%$, respectively (Fig. 4A). Cycloheximide treatment reduced basal HA secretion rate in non-stretched cultures by $52.2 \pm 9.4\%$ ($p < 0.01$, Bonferroni's test). Moreover, cycloheximide totally blocked the response to 10 or 180 min stretch, reducing the hyaluronan secretion rate by 66% and 65%, respectively ($p < 0.001$, Bonferroni's test). The rate of HA secretion by cells stretched for 10 or 180 min during treatment with cycloheximide was not significantly higher than that of unstretched cells treated with cycloheximide ($p > 0.05$, Bonferroni's test). The inhibitory effect of cycloheximide was not attributable to cytotoxicity, since the synoviocyte microscopic morphology remained normal and trypan blue was still excluded after treatment and stretch.

2.5. Inhibition of transcription blocks mechanosensitive HA secretion

Since the mechanosensitive response depended heavily on the synthesis of unidentified protein(s), yet HAS2 transcript levels did not change substantially as determined by semi-quantitative RT-PCR, we investigated whether the mechanosensitive response is dependent on *de novo* transcription. Synoviocytes were either stretched, or not stretched, in the presence or absence of $1 \mu\text{g/ml}$ Actinomycin D (Fig. 4B). Stretch again increased the HA secretion rate in non-treated cells, in this case by $37 \pm 19.5\%$ ($p < 0.05$, Bonferroni's test, $n = 6$). Treatment with Actinomycin D reduced the basal HA secretion rate of non-stretched cells by $38.9 \pm 13.7\%$ ($p < 0.05$, Bonferroni's test). Actinomycin D almost totally blocked the stretch-induced response, reducing the secretion rate of stretched synoviocytes by $45 \pm 11\%$ ($p < 0.001$, Bonferroni's test). The HA secretion rate of stretched cells treated with Actinomycin D was not significantly higher than that of unstretched cells treated with Actinomycin D ($p > 0.05$, Bonferroni's test). The reduction of HA secretion in Actinomycin D treated cells was not simply a cytotoxic effect, because the microscopic morphology of the cells remained normal and they continued to exclude trypan blue after treatment and stretch.

3. Discussion

Microdissection of the synovial intima is essential to provide a source of synovial lining cells which excludes the more abundant subsynovium, a loose areolar tissue containing fibroblasts. It is likely that the grossly dissected tissue samples used as culture sources in the past consist mainly of subsynovial cells. The normal phenotype of rabbit intimal synoviocytes was well preserved in our low passage cultures, as demonstrated by cytoplasmic process morphology, non-specific esterase negativity, appropriate integrin expression, stretch-induced HA secretion, and appropriate HAS isoform expression. HAS2 mRNA was the chief isoform expressed both *in situ* and *in vitro*, with very low levels of HAS3 mRNA, and no detectable HAS1 mRNA expression. These latter results confirm those of Ohno et al. (2001).

The variability of HA secretion rate between cultures was substantial (see Figs. 2, 4) but this is also a feature of physiological HA secretion into intact rabbit knee joints *in vivo* (Coleman et al., 1997; Anggiansah et al., 2003). Nevertheless, the mean HA basal secretion rate of hyaluronan by synoviocytes was ~ 18 times faster than by cultured keratinocytes ($0.4 \text{ ng HA}/1000 \text{ cells}/24 \text{ h}$) despite HA being a major component of epidermal matrix (Rilla et al., 2004). This underlines the fact that the synoviocyte phenotype is exceptionally specialised for rapid HA secretion. The high HA secretion rates are needed to maintain the high, lubricating concentration of HA in synovial fluid in the face of its continual seepage out of the joint cavity (Sabaratnam et al., 2004). Cell coat HA was not quantified here since cell layers were used for cell counting, but it may constitute about 20% of total extracellular HA. Pulse-chase experiments in chondrocytes, another cell with a HA coat, showed that

over 80% of newly synthesised hyaluronan passes into the medium within 3.5 h (Mason et al., 1982) and the major part of HA synthesized by fibroblast cultures (~80%) is released into the medium (Suzuki et al., 2001).

Our results show that cultured synoviocytes, like the intact synovial lining of a joint (Coleman et al., 1997), respond to mechanical deformation by an acute increase in HA secretion over 3 h. This provides a mechanism by which increased joint usage is accompanied by increased lubricant production, a link that is vital to effective joint function. A 10% stretch is probably well within the physiological range of stretches experienced by a synoviocyte in vivo, especially in a joint with an effusion. However 30% stretch did not stimulate HA secretion in vitro and too much stretch (100%) may shut down HA synthesis altogether, as shown in rat articular discs (Carvalho et al., 1995). The cited strain in the Flexcell is an average across the well, with the central cells experiencing relatively less stretch and the peripheral cells relatively more. This models synoviocytes in situ in joints, which almost certainly experience non-uniform strains on joint flexion. In principle an alternative mechanical stimulus to the 10-180 min strain could be the transient fluid shear stress occurring during the displacement of the Flexcell membrane; but since the movement phase is virtually instantaneous, strain seems the more likely physical stimulus.

Interestingly, a 10% stretch for 10 min increased HA secretion over 180 min as effectively as continuous stretch for the whole period (Fig. 2, 4). Thus the mechanical stimulus must trigger an early biochemical event with a long-lasting effect. It seems unlikely that increased expression of HAS isoforms is responsible for the increased HA secretion. No increase in HAS2 mRNA level was observed when the cells were subjected to 10% strain continuously for 3 h and the small change in HAS2 mRNA level detected at 180 min, after stretch for the first 10-30 min (Fig. 4), only just reached statistical significance. Interestingly, Recklies et al. (2001) reported that HAS2 mRNA levels in synovial fibroblasts, chondrocytes and osteosarcoma cells do not necessarily reflect the rate of HA secretion into the culture medium. Additionally, in contrast to Dowthwaite et al. (1999) and Mascarenhas et al. (2004) who found, respectively, that cyclical stretch of chick fibrocartilage cells or human lung fibroblasts induced HAS3 expression, we could not detect any stretch-induced increase in the very low mRNA levels of this HAS isoform in synoviocytes in vitro. Since our experimental set up provided insufficient RNA for analysis by Northern blotting, we analysed HAS mRNA by RT-PCR. This methodology may be incapable of detecting very small changes in mRNA pool size due to its semi-quantitative nature and lack of sensitivity, but it seems unlikely that marked changes in HA secretion could be effected by only a very small change in transcription of either HAS 2 or 3. Nevertheless it is clear that transcription and translation of a gene(s) is required for stretch-stimulated increases in HA secretion since the latter was completely blocked by Actinomycin D and cycloheximide. This may involve HAS 2 transcription, but also that of other protein factors obligatory for HA synthesis. A ~50% suppression of basal HA secretion over 3 h by Actinomycin D and cycloheximide indicated a fairly rapid turnover ($t_{1/2} \sim 180$ min) of a protein(s) involved in HA secretion in cultured synoviocytes, possibly HAS2. Similarly, protein factors involved in hyaluronan synthesis in cultured chondrocytes turn over rapidly (Bansal and Mason, 1986).

Dowthwaite et al. (2002) proposed that early increases in secreted HA (20 min) in stretched chick embryo fibroblast cultures were due, in part, to release from the pericellular coat. It seems unlikely that release of pre-formed HA from the pericellular coat of synoviocytes could account for increased medium HA following stretch, because the latter is blocked by inhibitors of intracellular events, namely, Actinomycin D, cycloheximide (Fig. 4), and protein kinase C inhibitors (Anggiansah et al., 2003). Moreover if, as proposed above, pericellular coat HA constitutes about 20% of total extracellular HA, it would be insufficient to account for the observed increase in secretion into the medium, from 0.9 ng/1000 cells/3 h

in the basal state to 1.4 ng/1000 cells/3 h in the stretched state. In vivo, the extracellular matrix HA concentration around rabbit synovial cells is 0.59 mg per ml, the total synovial intima volume is 0.021-0.036 ml, and the full-thickness interstitial volume fraction is 0.66, giving a total tissue HA mass of 8-14 μ g (Price et al., 1996). Since the joint lining secretes 26-35 μ g HA over a 6 h period (Coleman et al., 1997; Anggiansah et al., 2003), release of pre-formed pericellular HA cannot account for most of the HA secretion. Thus, we conclude that increased HA secretion by stretched synoviocytes is mediated by a mechanosensitive pathway which induces new HA synthesis. Stretch also increases HA synthesis and/or HAS expression in some other types of cell, for example cultured chondrocytic cells (Dowthwaite et al., 1999; Karjalainen et al., 2002) and lung fibroblasts (Mascarenhas et al., 2004). In the latter, the newly synthesised hyaluronan was of lower molecular weight than in non-stretched cells. Whilst we have no data on the molecular weight of hyaluronan synthesized by stretched and non-stretched rabbit synoviocytes, the mean chain length of hyaluronan synthesized by stretched and non-stretched synovium in vivo is unchanged (Coleman et al., 1997). Hyaluronan chain length may relate to the HAS isoform expressed in a particular cell type (Itano and Kimata, 2002).

Since release of HA from a pericellular store is unlikely to account for the average 57% increase in HA secretion in stretched synoviocytes, and in the absence of marked changes in HAS isoform expression, it seems likely that another mechanism must increase HA production in response to static stretch. There is evidence for increased hyaluronan synthase activity following its phosphorylation by protein kinase C (Klewes and Prehm, 1994). Moreover, the activation of protein kinase C by phorbol ester massively stimulates HA secretion by cultured mesothelial cells (Heldin et al., 1992) and by synovium in rabbit knee joints in vivo. The latter effect is totally abolished by the protein kinase C inhibitor bisindolylmaleimide (Anggiansah et al., 2003). Preliminary results indicate protein kinase C-ERK1/II kinase activation in stretch-induced HA stimulation in vitro (Momberger et al., 2003). Whether or not HAS itself is phosphorylated in stretch-stimulated synoviocytes remains to be demonstrated.

In conclusion, our results showed directly, for the first time, that synovial HA secretion is regulated by a mechanosensitive pathway in statically stretched rabbit intimal synoviocytes: demonstrated the predominance of the HAS2 isoform, but little if any increase in its transcription following stretch: demonstrated that a relatively brief stretch was as effective as sustained stretch in eliciting the response; and indicated a dependence of basal and stretch-induced HA secretion on the transcription and translation of unidentified protein(s). The new results indicate that mechanical deformation is likely to be a major regulator of the intra-articular hyaluronan content, and the results thus provide a basis for the physiologically crucial link between joint use and the secretion of a vital intra-articular polymer.

4. Experimental procedures

4.1. Isolation and culture of rabbit synoviocytes

The synovial intima layer was microdissected from the thicker underlying subsynovium of rabbit knee joints under a stereomicroscope, as described previously (Price et al., 1996). Separation of intima from subsynovium by this technique was confirmed histologically (Price et al., 1996). Small, diced pieces of the intimal synovium were transferred into T25 cm² tissue culture flasks (Corning, Amsterdam, NL) and cultured for 1 day in 1 ml Hams F12 medium (Khaighn's modification) (Invitrogen Life Technologies, Paisley, UK), supplemented with 30% fetal calf serum (FCS) (Invitrogen Life Technologies) and 2% penicillin/streptomycin/fungizone (PSF) (Invitrogen Life Technologies). Medium supplemented with 20% FCS and 1% PSF (complete medium) was added daily over the next 5 days to a final medium volume of 6 ml. Cells with a highly branched phenotype,

characteristic of surface synoviocytes in situ (McDonald and Levick, 1988), grew out from the explant after 4 days. The medium (complete) was changed every 2 days after day 6. When the cells were confluent, the tissue fragments were removed and the cells trypsinised (0.25% trypsin/0.03% EDTA, Invitrogen Life Technologies) before reseeding into fresh flasks (3×10^5 cells per T25 flask, or 1×10^6 cells per T75 flask).

Passage 6-10 cultures were used for experiments. For any given experiment, cells from the same parent rabbit synoviocyte culture were seeded on to 6 well plates since basal HA secretion rates could differ several-fold between different parent cultures (e.g. Fig. 4). Variability of basal HA secretion rate is a characteristic of the synoviocytes per se, rather than of changes induced by culture, because basal HA secretion rate into intact rabbit knee joints likewise shows a wide, 4-fold range (Anggiansah et al., 2003). The recombinant human cytokines used in some experiments were from R&D Systems Europe Ltd, Abingdon, UK.

4.2. Non-specific esterase (NSE) activity

Freshly outgrown synoviocytes and passage 10 synoviocytes were tested for non-specific esterase (NSE) activity, an enzymatic marker for macrophages (Chayen et al., 1973). Intimal type B synoviocytes are NSE negative whilst macrophage-like type A cells are NSE positive (Henderson and Edwards, 1987). Cells were grown on cover slips in complete medium for 24 h, washed 3 times with PBS and incubated in a standard reaction solution for 15-30 min at 37 °C (Chayen et al., 1973). After washing in 30% ethanol-0.1% acetic acid, followed by distilled water, the cells were mounted in Farrant's medium (TCS Microbiology, Botolph Claydon, UK). Non-adherent cells isolated from synovial explants were used as a positive control for NSE activity. A proportion of macrophages become non-adherent during cell culture (Spaniel-Borowski and Ricken, 1997). To obtain non-adhering cells, microdissected synovial tissue was explanted into 1ml RPMI 1649 medium (Invitrogen Life Technologies) containing 30% FCS and 1% PSF. 1ml of fresh medium plus supplements was added daily over the next 5 days, after which the medium was transferred to a Falcon tube and centrifuged ($3000 \times g$, 5 min) to recover non-adherent cells. The cell pellet was washed with fresh medium and incubated in reaction solution, on a coverslip, as above.

4.2.1. Pericellular HA coat formation

Erythrocyte-excluding HA-pericellular coats are characteristic of HA-secreting cells (Fraser and Laurent, 1996). Freshly outgrown synoviocytes and passage 10 synoviocytes were seeded into 6 well plates ($3-5 \times 10^4$ cells/well), and maintained in complete medium until about 50% confluent, when the medium was changed to one containing 0.5% FCS for 24 h. Then, glutaraldehyde-fixed horse erythrocytes (Sigma Aldrich, Poole, UK) were washed extensively with phosphate-buffered saline (PBS), re-suspended at a density of 5×10^8 cells/ml in PBS containing 1 mg/ml bovine serum albumin, and 1×10^7 cells added per 1ml of synoviocyte culture medium. After 20 min, the cultures were inspected at 20 magnification. Three of the six wells were then washed 5 times with PBS to remove erythrocytes and treated with medium containing 5 units/ml Streptomyces hyaluronidase (Calbiochem, Nottingham, UK) and 0.5% FCS for 1 h at 37 °C. The other three wells were incubated in the absence of hyaluronidase, as a control. After incubation, red cells were again added to the wells and the cultures inspected for HA coats.

4.2.2. Integrin expression

Rabbit synoviocytes were cultured with complete medium on uncoated tissue culture dishes. RT-PCR (see below) was then used to assess their integrin expression. Published PCR primers were used to probe for $\alpha 1$ and $\alpha 6$ (Ozaki et al., 1998), $\alpha 2$ (Loeser et al., 2000), $\alpha 3$

(Anderson et al., 1999), $\alpha 5$, $\beta 1$ (Argraves et al., 1987), αv and $\beta 3$ (Tang et al., 1995) integrin subunits.

4.2.3. Hyaluronan assay

A commercial HA binding kit (Corgenix Inc., Westminster, Colorado, USA) was used to measure HA in freshly aspirated medium, or in medium which had been stored at -20°C , following the manufacturers instructions. The assay was calibrated using HA standards of concentration 50-800 ng/ml. Optical densities were measured at 450 nm using a multiwell plate reader (Fluostar Galaxy, BMG Labtech GmbH, Offenburg, Germany).

4.2.4. Mechanical stretching of cultured synoviocytes

Rabbit synoviocytes were seeded at a density of $3-5 \times 10^4$ cells per well onto type I collagen coated 6-well Flexcell plates (Flexcell International Corporation, Dunn Labortechnik GmbH, Asbach, Germany) and cultured in complete medium for 48 h, when they were 50-60% confluent. Then, further cell division was arrested by replacing the medium with serum-free medium, modelling as closely as possible the synovium in vivo where synoviocytes occur as single cells, or small clusters, separated by extracellular matrix. The serum free medium was renewed 24 h later and the plates placed in a Flexcell 2000 apparatus (Flexcell International Corporation) housed in the incubator. The apparatus establishes a vacuum in a chamber beneath the wells which have flexible silicone membrane bases. Stretch of the membrane is a non-linear function of the vacuum pressure applied. A static stretch of magnitude 10-30% was applied and sustained for the stated time interval. The change in strain was immediate on application of the vacuum. The stated strain (e.g. 10%) is a nominal, average value and was determined from the measured applied pressure in kPa and a calibration factor supplied by the manufacturer. In reality the strains of peripheral and central cells will be distributed around the 10% average due to the curved geometry of the system.

Stretch experiments were carried out over 3 h because stimulation of HA secretion has previously been demonstrated in vivo over this interval (Coleman et al., 1997, 1999). Cells were stretched for either 10 min, followed by 170 min without stretch, or throughout the whole 180 min. Cells were then assessed visually for detachment, morphology, damage, and in three experiments, viability (trypan blue test, Sigma Aldrich). The culture medium was collected for HA analysis. Control plates were kept unstretched throughout. Cell number was determined using crystal violet staining by reference to a standard curve of absorbance versus cell number (Gillies et al., 1986).

4.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of integrins and HAS isoforms by rabbit synoviocytes was assayed semi-quantitatively by RT-PCR. Oligonucleotide primers based on human cDNA sequences for HAS1 (Kawakami et al., 1998), HAS2 and HAS3 (Nishida et al., 1999) were used for PCR since the sequences of rabbit HAS genes (Ohno et al., 2001) were unknown when the study started. Poly (A)⁺RNA was isolated from trypsinised, sub-confluent rabbit synoviocytes using TRI[®] reagent (Sigma Aldrich). 3 μg of each RNA was reverse-transcribed with an oligo(dT) primer using the Superscript II first strand cDNA synthesis kit (GibcoBRL, Gaithersburg, MD, USA). PCR amplification was carried out in a Hybaid Omnigene thermocycler (Waltham, MA, USA). MgCl_2 concentration and cycle number were selected to obtain specific PCR products for HAS2 (409 bp, 1.5 μM MgCl_2 , 30 cycles) and HAS3 (331 bp, 3 μM MgCl_2 , 40 cycles) under non-saturating conditions. HAS1 was not detected (see Results). h-actin (840 bp, 3 μM MgCl_2 , 25 cycles) was amplified with each reaction to control for equivalent loading on subsequent electrophoresis on 3% agarose gels. For

quantification two separate RT reactions were performed for each RNA sample and each RT-product was subjected to two PCR reactions. Each PCR reaction was analysed on three separate agarose gels. Bands were visualised with ethidium bromide, photographed and the photographs scanned three times to measure band intensities, using densitometry software (NIH Image 6). Measurements were normalized to the corresponding h actin band. Each product was cleaned and sequenced at the Advanced Biotechnology Centre (Imperial College London, UK). Sequences were subjected to a Blast analysis to confirm the product sequence (Altschul et al., 1997). Insufficient RNA was available from the Flexcell experiments for Northern blotting.

Two to three kilogram New Zealand rabbits were killed by an overdose of sodium pentobarbitone (Euthatal, May and Baker Ltd, Dagenham, UK), the synovial lining harvested (Price et al., 1996), and the tissue snap frozen for 1 min in liquid nitrogen. It was stored at -80°C prior to homogenisation, RNA extraction and RT-PCR, as above.

4.4. Role of gene transcription and de novo protein synthesis in hyaluronan secretion by stretched synoviocytes

Rabbit synoviocytes plated on type 1 collagen-coated 6-well plates (5×10^4 cells/well) were grown overnight in F12 K medium supplemented with 15% FCS, after which they were maintained in serum-free medium for 24 h for to prevent cell division. Ten minutes before the experiment the cells were treated with serum-free medium containing either cycloheximide ($10 \mu\text{g/ml}$) to inhibit translation, or with Actinomycin D ($1 \mu\text{g/ml}$) to inhibit gene transcription. At time zero the medium was replaced with fresh medium of the same composition and the wells subjected to 10 min or 180 min 10% stretch, as described above. Medium was collected after 3 h for HA analysis and the cells were counted using the crystal violet assay.

4.4.1. Statistical procedures

Multiple comparisons were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc multiple comparison test. Two-way ANOVA was used where two variables changed, e.g. stretch and time. The one-sample *t* test and Student's *t*-test were used where appropriate. Means are followed by their standard errors (SEM). Statistical analysis was implemented using GraphPad Prism (San Diego, CA, USA). *p* values of 0.05 or less were regarded as statistically significant.

Acknowledgments

The research was funded by a Wellcome Trust Prize Studentship 059339 (TSM) and by Wellcome Trust grant 056983/Z/99.

References

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 1997; 25:3389–3402. [PubMed: 9254694]
- Anderson R, Fassler R, Georges-Labouesse E, Hynes RO, Bader BL, Kreidberg JA, Schaible K, Häsmann J, Wylie C. Mouse primordial germ cells lacking beta1 integrins enter the germ line but fail to migrate normally to the gonads. *Development.* 1999; 126:1655–1656. [PubMed: 10079228]
- Anggiansah CL, Scott D, Coleman PJ, Baldrick E, Mason RM, Levick JR. Regulation of hyaluronan secretion into rabbit synovial joints in vivo by protein kinase C. *J. Physiol.* 2003; 550:631–640. [PubMed: 12766248]

- Argaves WS, Suzuki S, Arai H, Thompson K, Pierschbacher MD, Ruoslahti E. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 1987; 105:1183–1190. [PubMed: 2958481]
- Balazs EA, Denlinger JL. Sodium hyaluronate and joint function. *J. Equine Vet. Sci.* 1985; 5:217–228.
- Bansal MK, Mason RM. Evidence for rapid metabolic turnover of hyaluronate synthetase in Swarm rat chondrosarcoma chondrocytes. *Biochem. J.* 1986; 236:515–519. [PubMed: 3092811]
- Carvalho RS, Yen EH, Suga DM. Glycosaminoglycan synthesis in the rat articular disc in response to mechanical stress. *Am. J. Orthod. Dentofac. Orthop.* 1995; 107:410–411.
- Chayen, J.; Bitensky, L.; Butcher, RG. *Practical Histochemistry*. Wiley; London: 1973. p. 131-139.
- Coleman PJ, Scott D, Ray J, Mason RM, Levick JR. Hyaluronan secretion into the synovial cavity of rabbit knees and comparison with albumin turnover. *J. Physiol.* 1997; 503:645–656. [PubMed: 9379418]
- Coleman PJ, Scott D, Mason RM, Levick JR. Characterization of the effect of high molecular weight hyaluronan on trans-synovial flow in rabbit knees. *J. Physiol.* 1999; 514:265–282. [PubMed: 9831732]
- Dowthwaite GP, Ward AC, Flannely J, Suswillo RF, Flannery CR, Archer CW, Pitsillides AA. The effect of mechanical strain on hyaluronan metabolism in embryonic fibrocartilage cells. *Matrix Biol.* 1999; 18:523–532. [PubMed: 10607914]
- Dowthwaite, GP.; Thomas, S.; Flannery, CR.; Pitsillides, AA.; Archer, CW. Hyaluronan metabolism in response to mechanical strain is modulated by matrix depletion. In: Kennedy, JF., et al., editors. *Hyaluronan*. Vol. 2. Woodhead Publishing; Cambridge: 2002. p. 375-385.
- Fraser, JRE.; Laurent, TC. *Hyaluronan, Extracellular Matrix*. Comper, WD., editor. Vol. 2. Harwood Academic Publishers; Amsterdam: 1996. p. 141-199.
- Gillies RJ, Didier N, Denton M. Determination of cell number in monolayer cultures. *Anal. Biochem.* 1986; 159:109–113. [PubMed: 3812988]
- Haubeck HD, Kock R, Fischer DC, Van de Leur E, Hoffmeister K, Greiling H. Transforming growth factor beta 1, a major stimulator of hyaluronan synthesis in human synovial lining cells. *Arthritis Rheum.* 1995; 38:669–677. [PubMed: 7748222]
- Heldin P, Asplund T, Ytterberg D, Thelin S, Laurent TC. Characterization of the molecular mechanism involved in the activation of hyaluronan synthetase by platelet-derived growth factor in human mesothelial cells. *Biochem. J.* 1992; 283:165–170. [PubMed: 1567364]
- Henderson, B.; Edwards, JCW. *The Synovial Lining*. Chapman and Hall; London: 1987.
- Itano N, Kimata K. Mammalian hyaluronan synthases. *IUBMB Life.* 2002; 54:195–199. [PubMed: 12512858]
- Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, Shinomura T, Hamaguchi M, Yoshida Y, Ohnuki Y, Miyauchi S, Spicer AP, McDonald JA, Kimata K. Three isoforms of mammalian HAS have distinct enzymatic properties. *J. Biol. Chem.* 1999; 274:25085–25092. [PubMed: 10455188]
- Johnson BA, Haines GK, Harlow A, Koch AE. Adhesion molecule expression in human synovial tissue. *Arthritis Rheum.* 1993; 36:137–146. [PubMed: 7679271]
- Karjalainen, H.; Sironen, R.; Elo, M.; Helminen, HJ.; Lammi, MJ. Cyclical stretching increases the expression of aggrecan and hyaluronan synthase-2 (HAS-2) in chondrocytic cells. *Fed. Eur. Connect. Tis. Soc. XVIIIth Meeting, Abstract C3*; 2002.
- Kawakami M, Suzuki K, Matsuki Y, Ishizuka T, Hidaka T, Konishi T, Matsumoto M, Kataharada K, Nakamura H. Hyaluronan production in human rheumatoid fibroblastic synovial lining cells is increased by interleukin 1 beta but inhibited by transforming growth factor β 1. *Ann. Rheum. Dis.* 1998; 57:602–605. [PubMed: 9893571]
- Klewes L, Prehm P. Intracellular signal transduction for serum activation of hyaluronan synthase in eukaryotic cell lines. *J. Cell. Physiol.* 1994; 160:539–544. [PubMed: 8077292]
- Levick JR, McDonald JN. Synovial capillary distribution in relation to altered pressure and permeability in knees of anaesthetized rabbits. *J. Physiol.* 1989a; 419:477–492. [PubMed: 2621638]
- Levick JR, McDonald JN. Ultrastructure of transport pathways in stressed synovium of the knee in anaesthetized rabbits. *J. Physiol.* 1989b; 419:493–508. [PubMed: 2621639]

- Loeser RF, Sadiev S, Tan L, Goldring MB. Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins in mediating chondrocyte adhesion to types II and VI collagen. *Osteoarthr. Cartil.* 2000; 8:96–105. [PubMed: 10772239]
- Lu Y, Levick JR, Wang W. Concentration polarization of hyaluronan on the surface of the synovial lining of infused joints. *J. Physiol.* 2004; 561:559–573. [PubMed: 15579541]
- Mascarenhas MM, Day RM, Ochoa CD, Won-Il C, Yu L, Ouyang B, Garg HG, Hales CA, Quinn DA. Low molecular weight hyaluronan from stretched lung enhances interleukin-8 expression. *Am. J. Respir. Cell Mol. Biol.* 2004; 30:51–60. [PubMed: 12738686]
- Mason RM, Kimura JH, Hascall VC. Biosynthesis of hyaluronic acid in cultures of chondrocytes from the Swarm rat chondrosarcoma. *J. Biol. Chem.* 1982; 257:2236–2245. [PubMed: 7061420]
- Mason, RM.; Coleman, PJ.; Scott, D.; Levick, JR. Role of hyaluronan in regulating joint fluid flow. In: Hascall, VC.; Kuettner, KE., editors. *The Many Faces of Osteoarthritis*. BirkHauser-Verlag; Basel: 2002. p. 179-187.
- McDonald JN, Levick JR. Morphology of surface synoviocytes in situ at normal and raised joint pressure, studied by scanning electron microscopy. *Ann. Rheum. Dis.* 1988; 47:232–240. [PubMed: 3355260]
- Momberger T, Levick JR, Mason RM. Mechanosensitive modulation of hyaluronan (HA) secretion involves protein kinase C, Ca^{2+} and MAPK. *J. Vasc. Res.* 2003; 40:295.
- Nishida Y, Knudson CB, Nietfield JJ, Margulis A, Knudson W. Antisense inhibition of hyaluronan synthase-2 in human articular chondrocytes inhibits proteoglycan retention and matrix assembly. *J. Biol. Chem.* 1999; 274:21893–21899. [PubMed: 10419509]
- Ohno S, Tanimoto K, Fujimoto K, Ijuin C, Honda K, Tanaka N, Doi T, Nakahara M, Tanne K. Molecular cloning of rabbit hyaluronan synthases and their expression patterns in synovial membrane and articular cartilage. *Biochim. Biophys. Acta.* 2001; 1520:71–78. [PubMed: 11470161]
- Ozaki I, Yamamoto K, Mizuta T, Kajihara S, Fukushima N, Setoguchi Y, Morito F, Sakai T. Differential expression of laminin receptors in human hepatocellular carcinoma. *Gut.* 1998; 43:837–842. [PubMed: 9824613]
- Pirilä L, Heino J. Altered integrin expression in rheumatoid synovial lining type-B cells—in vitro cytokine regulation of $\alpha 1\beta 1$, $\alpha 6\beta 1$ and $\alpha \text{v}\beta 5$ integrins. *J. Rheumatol.* 1996; 23:1691–1698. [PubMed: 8895142]
- Pirilä L, Aho H, Roivainen A, Konttinen YT, Pelliniemi LJ, Heino J. Identification of $\alpha 6\beta 1$ integrin positive cells in synovial lining layer as type B synoviocytes. *J. Rheumatol.* 2001; 28:478–483. [PubMed: 11296945]
- Prehm P. Synthesis of hyaluronate in differentiated teratocarcinoma cells. Mechanism of chain growth. *Biochem. J.* 1983; 211:191–198.
- Price FM, Levick JR, Mason RM. Glycosaminoglycan concentration in synovium and other tissues of rabbit knee in relation to hydraulic resistance. *J. Physiol.* 1996; 495:803–820. [PubMed: 8887784]
- Recklies AD, White C, Melching L, Roughley PJ. Differential regulation and expression of hyaluronan in human articular cartilage, synovial cells and osteosarcoma cells. *Biochem. J.* 2001; 354:17–24. [PubMed: 11171074]
- Rilla K, Pasonen-Seppanen S, Rieppo J, Tammi M, Tammi R. The hyaluronan synthesis inhibitor 4-methylumbelliferone prevents keratinocyte activation and epidermal hyperproliferation induced by epidermal growth factor. *J. Invest. Dermatol.* 2004; 123:708–714. [PubMed: 15373776]
- Rinaldi N, Schwarz-Eywill M, Weiss D, Leppepmann-Jansen P, Lukoschek M, Keilholz U, Barth TF. Increased expression of integrins on fibroblast-like synoviocytes from rheumatoid arthritis in vitro correlates with enhanced binding to extracellular matrix proteins. *Ann. Rheum. Dis.* 1997a; 56:45–51. [PubMed: 9059141]
- Rinaldi N, Weiss D, Brado B, Schwarz-Eywill M, Lukoschek M, Pezzutto A, Keilholz U, Barth TF. Differential expression and functional behaviour of the αv and $\beta 3$ integrin subunits in cytokine-stimulated fibroblast-like cells derived from synovial tissue of rheumatoid arthritis and osteoarthritis in vitro. *Ann. Rheum. Dis.* 1997b; 56:729–736. [PubMed: 9496152]

- Sabaratnam S, Mason RM, Levick JR. Flow-dependence of hyaluronan reflection by joint-to-lymph barrier in rabbit knees; evidence for concentration polarisation. *J. Physiol.* 2004; 557:909–922. [PubMed: 15073278]
- Spanel-Borowski K, Ricken AM. Evidence for the maintenance of macrophage-like cells in long term bovine granulosa cell cultures. *Cell Tissue Res.* 1997; 288:529–538. [PubMed: 9134865]
- Suzuki M, Asplund T, Yamashita H, Heldin CH, Heldin P. Stimulation of hyaluronan biosynthesis by platelet-derived growth factor-BB and transforming growth factor-beta 1 involves activation of protein kinase C. *Biochem. J.* 2001; 307:817–821. [PubMed: 7741713]
- Tak PP, Thurkow EW, Daha MR, Kluin PM, Smeets TJM, Meinders AE, Breeveld FC. Expression of adhesion molecules in early rheumatoid synovial tissue. *Clin. Immunol. Immunopathol.* 1997; 77:236–242. [PubMed: 7586733]
- Tang D, Diglio CA, Bazaz R, Honn KV. Transcriptional activation of endothelial cell integrin alpha v by protein kinase C activator 12 (S)-HETE. *J. Cell Sci.* 1995; 108:2629–2644. [PubMed: 7593304]
- Tanimoto K, Suzuki A, Ohno S, Honda K, Tanaka N, Doi T, Yoneno K, Ohno-Nakahara M, Nakatani Y, Ueki M, Tanne K. Effects of TGF-beta on hyaluronan anabolism in fibroblasts derived from the synovial membrane of the rabbit temporomandibular joint. *J. Dent. Res.* 2004; 83:40–44. [PubMed: 14691111]
- Wilkinson LS, Pitsillides AA, Worrall JG, Edwards JCW. Light microscopic characteristics of the fibroblast-like synovial intimal cell (synoviocyte). *Arthritis Rheum.* 1992; 35:1179–1184. [PubMed: 1329776]

Fig. 1. Characterisation of synoviocyte cultures. Frames (A)-(D) Pericellular, hyaluronidase-sensitive coats around rabbit synoviocytes were revealed by exclusion of glutaraldehyde-fixed horse erythrocytes. (A),(C). The HA coat is seen as a broad, red cell excluding zone (thin red arrow marks its periphery) outside the cell membrane (thick blue arrows). (B),(D) Treatment with *Streptomyces* hyaluronidase removed the coat. Original magnification $\times 20$. Scale bars; (A) 28 μm , (B) 16 μm , (C) 21 μm (D) 28 μm . Frames (E), (F) Non-specific esterase activity. (E) Passage 10 synoviocytes were grown on cover slips for 24 h after and then tested histochemically for NSE activity. Cells were esterase-negative. (F) Non-adherent putative type A synovial cells were tested for NSE activity as a control. Blue reaction product shows positive staining for non-specific esterase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

The effect of 10% stretch on HA secretion into culture medium by rabbit synoviocytes over 3 h. (A) Absolute HA secretion rates (ng HA/1000 cells/3 h) are shown on the left for non-stretched control plates and paired sets of plates subjected to 10% stretch for 10 min at the start of the 180 min secretion period (9 separate experiments), and, on the right, for control plates and paired sets of plates subjected to 10% stretch throughout the whole of 180 min period (15 separate experiments). Each point is the mean value for the 6 wells on the plate. The stimulation of HA secretion by stretch is highly significant ($p = 0.001$ and 0.0001 , respectively, paired t test). (B) Stretch-stimulated secretion rate plotted as a function of basal secretion rate. The correlations are highly significant ($p = 0.005$ and <0.0001 for 10 and 180 min stretch, respectively). Dashed line is regression line through 10 min stretch results; solid line is regression line fitted to 180 min stretch results. Neither the slopes of the two regression lines nor their intercepts are significantly different ($p = 0.32$, 0.28 , respectively, analysis of co-variance).

Fig. 3.

The expression of HAS2 by control and stretched synoviocytes. Panel (A) shows the representative PCR gels from three experiments in which synoviocytes were either not stretched (control), or stretched at 10% (experiment 1), 20% (experiment 2) or 30% strain (experiment 3). In each experiment the cells were stretched for either 10, 30, 60, or 180 min and harvested at 180 min, after which RNA was extracted, pooled from the six wells of each plate for each experimental condition, and analysed for HAS2 and h-actin mRNA by RT-PCR. Panel (B) A mean densitometric value was calculated for the 12 PCR analyses for each RNA sample. The graph shows the change in HAS2 mRNA level at 180 min in response to different magnitudes and durations of strain. HAS2 mRNA was expressed as a fraction of h-actin, the housekeeping gene. Overall there was no significant increase in HAS2 expression.

Fig. 4.

Effect of inhibitors of transcription and translation on basal and stretch-induced HA secretion. Panel (A) Rabbit synoviocytes were seeded into six 6-well Flexcell plates, cultured, and then treated with cycloheximide (10 μ g/ml), as described in the Experimental procedures section. Plates were treated as follows: (1) control; (2) unstretched but treated with cycloheximide for 180 min; (3) 10% stretch for 180 min; (4) 10% stretch for 180 min in the presence of cycloheximide; (5) 10% stretch for the first 10 min of the 180 min secretion period; (6) 10% stretch for the first 10 min of the 180 min period and treated with cycloheximide throughout. The medium from each well was then collected and analysed for hyaluronan content. The cell layer was analysed for cell number. Two independent experiments were performed, giving a total of 12 HA secretion values per 1000 cells under each test condition. (Means $\pm$ SEM; * p < 0.05; ** p < 0.01; *** p < 0.001, one-way ANOVA and Bonferroni's post hoc test). Panel (B) Rabbit synoviocytes were seeded into four 6-well Flexcell plates, cultured and then treated with Actinomycin D (1 μ g/ml), as described in the Experimental procedures section. Plates were treated as follows: (1) control; (2) unstretched but treated with Actinomycin D for 180 min; (3) 10% stretch for 180 min; (4) 10% stretch for 180 min and treated with Actinomycin D. Media and cell layers from each well were analysed individually as above. Statistical analysis, as above. The difference in basal rate

between panels (A) and (B) is typical of the variation seen between cultures (Fig. 2) and also in intact joints in vivo (Anggiansah et al., 2003; Coleman et al., 1997).