Differential regulation and expression of hyaluronan synthases in human articular chondrocytes, synovial cells and osteosarcoma cells

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Recently three isoforms of hyaluronan synthase (HAS), the enzyme responsible for hyaluronate/hyaluronan (HA) biosynthesis, have been cloned, allowing us to study their expression pattern. Our objective was to determine which of the HAS isoenzymes were expressed in human articular chondrocytes, synovial fibroblasts and osteosarcoma cells, whether their expression could be modulated by growth factors (insulin-like growth factor-1, basic fibroblast growth factor and transforming growth factor (TGF- β 1) and cytokines [interleukin 1 β 1 (IL-1 β)], and whether changes in the rate of HA synthesis by the cells correlated with changes in mRNA levels for one or more of the HAS isoforms. All three HAS isoforms were found to be expressed in the cultured cells analysed in this study, although the relative proportions varied for each cell type. HAS2 mRNA was usually predominant in chondrocytes, whereas synovial cells contained increased amounts of HAS1. HAS3 was always the least abundant message. The rapidly growing osteosarcoma cells contained almost exclusively HAS2 message. HAS usage in uncultured cartilage and synovial tissues was similar to that in the cultured cells, with HAS2 message being the predominant species in cartilage and HAS1 usually being the

predominant species in synovium. HA synthesis was stimulated by the growth factors, but the extent of the response was cell-type specific. Synovial cells responded particularly well to IL-1 β , and showed a unique synergistic response when IL-1 β was used in combination with TGF- β 1. This response was much reduced in articular chondrocytes and absent in the osteosarcoma cells. Analysis of changes in HAS message levels indicated that there was often no correlation with the changes in HA secretion following exposure to growth factors. Although HAS-1 mRNA was increased in synovial cells after exposure to TGF- β 1/IL-1 β , the magnitude of the change was far less than the effect on HA synthesis. Our data thus suggest that HAS gene usage is tissue specific, and the regulation by growth factors is unique for each HAS gene and is further modulated by cell-specific factors. In addition, regulation of HA biosynthesis appears to be multifaceted, with control of HAS gene expression and mRNA levels being only one aspect of this process.

Key words: cartilage, cytokines, extracellular matrix, glycosaminoglycan.

INTRODUCTION

Hyaluronan (HA) is a biosynthetic product of articular chondrocytes and synovial cells as well as most other vertebrate cells. In articular cartilage HA functions as a backbone for the aggregation of aggrecan, localizing these molecules in the cartilage matrix and endowing the cartilage with its unique property to absorb shock. In synovial fluid HA is important for its rheological properties, providing the level of viscosity necessary for the frictionless movement of diarthrodial joints. Synthesis of HA, a polymer of alternating units of *N*-acetylglucosamine and glucuronic acid, connected by β 1-4 and β 1-3 linkages, respectively, is thought to occur at the plasma membrane [1,2], with newly synthesized HA being extruded through a pore into the extracellular milieu. *In vitro*, newly synthesised HA has been shown to form a pericellular coat [3], the organisation of which is mediated by the HA receptor CD44 [4,5]. Whether this pericellular organisation of HA plays a role *in vivo* is not clear at present.

Although numerous studies have been performed with respect to the importance of HA in a large number of biological and pathological processes, the enzymes responsible for its synthesis have been elusive until recently. The cloning of the streptococcal HA synthase (HAS) [6] led to the identification of three mammalian enzymes referred to as HAS1, HAS2 and HAS3 [7–12].

All HASs are capable of directing the synthesis of HA, although some differences have been noted with respect to the size of the polymers being produced [13]. Comparison of the deduced amino acid sequences shows a high degree of homology between the three HAS isoenzymes, particularly HAS2 and HAS3. They all contain seven putative membrane-spanning domains, two of which are located at the N-terminal end of the molecules while the other five are located at the C-terminal end. The loop between these two regions is thought to contain the catalytic site and binding sites for the two UDP-bound substrate units. The arrangement of the enzyme in the plasma membrane is thought to result in the cytoplasmic localization of both the N- and C-termini [14]. This model, however, awaits experimental verification.

The demonstration of the existence of three isoenzymes for HA synthesis raises the question whether they are present in similar proportions in all cells and whether their expression is regulated in a similar fashion. Since HA synthesis is such an important aspect of the physiology and pathology of diarthrodial joints, we investigated the expression of HASs in human chondrocytes and synovial cells and compared their expression with that exhibited by an osteosarcoma cell line as an example of a rapidly proliferating cell. In addition, we determined whether various growth factors and cytokines could modulate expression of the

Abbreviations used: bFGF, basic fibroblast growth factor; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hyaluronate/hyaluronan; HAS, hyaluronan synthase; IGF-1, insulin-like growth factor-1; IL-1 β , interleukin-1 β ; MMuLV, Moloney murine leukaemia virus; RT, reverse transcriptase; SSC, saline sodium citrate (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate); TGF- β 1, transforming growth factor- β 1.

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various HAS genes in a similar manner in the different cell types and whether effects on HAS gene expression are mirrored by changes in the rate of hyaluronan synthesis.

MATERIALS AND METHODS

Materials

Human recombinant interleukin-1 β (IL-1 β) was purchased from Boehringer-Mannheim (Montreal, QC, Canada), porcine platelet transforming growth factor (TGF- β 1), recombinant human insulin-like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF) from R&D Systems (Minneapolis, MN, U.S.A.), and fetal calf serum (FCS) from Biomed (Montreal, QC, Canada). Omniscript reverse transcriptase (RT) was purchased from Qiagen (Santa Clarita, CA, U.S.A.), *Thermus aquaticus* (Taq) polymerase from GIBCO (Toronto, ON), RNasin from Boehringer Mannheim, U.S.A., and [α ³²P]-dCTP from Amersham (Montreal, QC, Canada). Aggrecan used in the HA binding assay was purified by standard procedures from bovine nasal cartilage [15]. The 5D4 monoclonal antibody against keratan sulphate was purchased from ICN (Montreal, QC), and the alkaline phosphatase conjugate of goat anti-mouse immunoglobulin was obtained from Dimension Laboratories (Mississauga, ON, Canada). Immulon2 96-well plates were supplied by VWR/Canlab (Montreal, QC, Canada). All other reagents were of the purest grade available.

Cells and tissues

Human articular chondrocytes were prepared from cartilage specimens harvested at autopsies from femoral chondyles and the tibial plateau, as described previously [16]. For this study, cartilage derived from 3-month-old, 2-year-old and 48-year-old donors was used. Cells were maintained in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and antibiotics (penicillin, streptomycin) in a humidified atmosphere of 95% air, 5% CO₂ at 37 °C. For direct extraction of total RNA from cartilage, tissue was obtained from individuals aged 2 months, 3 years and 79 years.

Synovium was obtained from the knee of arthritic patients undergoing arthroplasty. Synovial cell cultures were prepared by collagenase digestion of the tissue as described by Golds et al. [17]. All cell cultures were maintained in DMEM supplemented with 10% FCS as above. The cells used in the present study were between passage levels 8 and 11.

The human osteosarcoma cell line, MG-63, was a generous gift from Dr. T. Glant (Rush Medical Center, Chicago, IL, U.S.A.). The cells were maintained in DMEM supplemented with 10% FCS and antibiotics.

Cell culture and stimulation with growth factors

To determine HA release and analyse mRNA levels for HAS, cells were seeded into T150 culture flasks at 25 000 cells/cm². The cells were allowed to adhere for 48 h in the presence of DMEM containing 10% FCS. The medium was then replaced by serum-free medium for 24 h, and growth factors (IGF-1 at 50 ng/ml, bFGF at 5 ng/ml, TGF- β 1 at 5 ng/ml, IL-1 β at 2 ng/ml, or a combination of TGF- β 1 and IL-1 β at 5 and 2 ng/ml respectively) were added 24 h later in 10 ml of serum-free medium. Medium containing 5% FCS was used as a control. The medium was not changed over the ensuing 4-day culture period. At day 4 the medium was collected for analysis of HA levels, and the cells were trypsinized and counted then used for the preparation of total cellular RNA. All media and RNA

samples were stored frozen at -20 °C or -80 °C, respectively, until analysis.

Analysis of HA levels in culture media

HA levels in culture media were determined using the competitive binding assay described by Goldberg [18]. 96-well plates were coated with rooster comb hyaluronic acid (0.1 mg/ml in 0.2 M sodium carbonate buffer, pH 9.2), blocked with 5% BSA in PBS, and stored sealed at 4 °C until use. The incubation buffer for aggrecan-HA binding consisted of PBS/Tris (40 mM), pH 7.3, supplemented with 1% BSA, 0.1% Tween 20 and 0.05% NaN₃. A D1 preparation of aggrecan, purified from bovine nasal cartilage, was used as an HA-binding agent. The HA used as a standard in the assay was purified from a commercial preparation extracted from human umbilical cord. For assay, medium samples (diluted to 100 μ l in PBS, 1% BSA if necessary) were incubated for 4 h at room temperature with 100 μ l of incubation buffer containing 2 μ g/ml aggrecan. The samples were then transferred to HA-coated plates and incubated overnight at 4 °C. Bound aggrecan was determined using a monoclonal antibody to keratan sulphate, followed by goat anti-mouse immunoglobulin serum conjugated to alkaline phosphatase. Standard curve parameters for calculation of sample HA concentrations were calculated by fitting the binding data to a sigmoidal dose-response curve, using the program Graphpad Prism. The assay was found to be linear in a concentration range of 3 to 50 ng of HA/assay sample (equivalent to 30 to 500 ng/ml in undiluted culture medium). Intra-assay variation was found to be less than 5%, and inter-assay variation was between 8 and 15%. The identity of the HA in the media samples was confirmed by its sensitivity to digestion with *Streptomyces* hyaluronidase and its resistance to degradation by proteinase K.

Isolation of total cellular RNA

Total cellular RNA was isolated from cultured cells, and from fresh synovial tissue and cartilage specimens, using the guanidine thiocyanate extraction method described by Chomczynski and Sacchi [19]. These RNA preparations were used both for Northern blotting and analysis by quantitative RT-PCR.

Northern blotting

Total cellular RNA preparations (10 μ g/lane) were separated by electrophoresis in 1% agarose/1.11% formaldehyde gels and capillary-blotted onto positively charged nylon membranes in 20 $\times$ SSC. Gel-purified probes representing base pairs 740 to 1196 for HAS1 (GenBank® accession no. U59269), 806 to 1312 for HAS2 (GenBank® accession no. U54804) and 878 to 1194 for HAS3 (GenBank® accession no. AF232772) were labelled with [α ³²P]-dCTP on one strand using specific primers and reagents from the Multiprime DNA labelling system (Amersham). Hybridization was performed using the RapidHyb buffer system (Amersham), and bound probes were detected by radioautography using X-ray film and exposure times of 4 days.

Analysis of mRNA levels

Message levels for the three HAS genes were quantitated by a competitive RT-PCR protocol, based on that described previously for the analysis of proteoglycan messages [20]. Reverse transcription was carried out independently for each message using the specific downstream primer to be later used in PCR amplification and Omniscript reverse transcriptase. To allow quantification, multiple reactions were carried out for each message in which total cellular RNA (250 ng) was mixed with

Table 1 Oligonucleotide primers for competitive RT-PCR analysis

Numbering under 'Location' refers to cDNA sequences contained in the following references: HAS1 [9]; HAS2 [7]; HAS3 [12]; G3PDH [21].

Gene	Primers (5' → 3')	Strand	Location	Message product (bp)	Standard product (bp)	Annealing temperature (°C)
HAS 1	CGGCCTGTTCCCTTCTTCGTG	+	1259–1280	348	425	65
	TCGTGTGCTACGCTGCGGACCA	—	1606–1585			
HAS 2	CACAGCTGCTTATATTGTTG	+	613–632	358	423	51
	AGTGGCTGATTTGTCTCTGC	—	970–951			
HAS 3	CAGCCTCTCCAGCAGTTCC	+	878–897	317	423	55
	TAACCGTGGCAATGAGGAAG	—	1194–1175			
G3PDH	GGTATCGTGGAAGGACTCAT	+	538–557	341	426	57
	ACCACCTGGTGCTCAGTGTA	—	878–859			

differing copy numbers (10^3 to 10^8) of a synthetic RNA standard possessing the same primer binding sites as the authentic message. Reactions were carried out in a 20 μ l volume using 4 units of reverse transcriptase, and 5 μ l of each reaction mixture was then used for subsequent PCR amplification in a 50 μ l reaction volume, as described previously [20]. The nucleotide sequences of primers, the size of products obtained from message and standards, and the annealing temperatures used are listed in Table 1. The identities of the PCR products for each HAS gene were confirmed by cDNA sequence analysis. Furthermore, when cDNA plasmids for each HAS gene were used as template for PCR, a product was only obtained when the corresponding primer/template combination was used. In addition to the three HAS genes, message levels for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were also analysed [21]. All RNA standards were prepared and purified by the same techniques as used previously [20].

Quantification of PCR products

The PCR products (10 μ l) were analysed by electrophoresis in a 10 % agarose gel containing ethidium bromide, and the resulting bands were visualized by UV transillumination. A digital image of the gel was recorded using a Gel Print 2000i Video System, and analysis of the image was carried out using Scion Image (the NIH Image software ported to Windows) to generate intensity levels for both the product of the authentic message and that of the standard. After correcting the intensity levels for the variation in length between the two products, copy number of the authentic message was derived from the point of equivalence on plots of log intensity ratio against log copy number of the standard.

Validation of PCR quantification

The reliability of the competitive PCR protocol for message quantitation was first optimized at the level of reverse transcription. Omniscript reverse transcriptase used in conjunction with the specific downstream primer was found to result in maximal product yield, compared to alternative protocols involving other reverse transcriptases (e.g. Moloney murine leukaemia virus (MMuLV) reverse transcriptase) or other primers (e.g. oligo-dT or random hexamers). Secondly, the accuracy of the PCR amplification protocol was assessed by the quantification of known amounts of plasmids containing the genes of interest.

Full length cDNAs spanning the coding region of both the human HAS1 and HAS2 genes were prepared by RT-PCR using message preparations from synovial cells and chondrocytes, respectively. Reverse transcription was performed using 250 ng total cellular RNA with oligo-dT priming (1 μ M) for 2 h.

Amplification of HAS1 cDNA was performed using the primers 5'-GCCTTCGCCCTGCTCATCCT and 5'-CCCCTCCACTCCTCAAATCC at an annealing temperature of 62 °C to yield a product of 1890 bp (bp 114–2003; [9]). Amplification of HAS2 cDNA was performed using the primers 5'-ATTTAAGACTCCCCTATCCT and 5'-CCTTTGGCATTTCATTTGGT at an annealing temperature of 52 °C to yield a product of 2290 bp (bp 360–2649; [7]). In both cases the Expand Long Template System (Roche Molecular Biochemicals) was used, according to the manufacturer's instructions. The resulting PCR products were then cloned into the PCR-TOPO vector using the TOPO-TA cloning kit (Invitrogen), according to the manufacturer's instructions. The authenticity of the cDNA inserts was verified by nucleotide sequence analysis. A plasmid containing a human HAS3 cDNA insert was generously given by Dr. Andrew Spicer (Department of Biological Chemistry, University of California, Davis, CA, U.S.A.).

RESULTS

Human articular chondrocytes, synovial cells and osteosarcoma cells were maintained in monolayer culture for 4 days in the presence of growth factors and cytokines which have been implicated in the homeostasis of the tissues of the diarthrodial joint. Expression of the three HAS genes, as reflected by their respective mRNA levels, and the cumulative production of HA was evaluated after 2 days of stimulation with growth factors and at the end of the culture period. As the values of HA production determined at day 2 were generally about half of those determined at day 4, suggesting that changes in the rate of biosynthesis had stabilized by 2 days and remained at that level, only the latter are shown here.

HA production was monitored in the medium only, as preliminary experiments showed that greater than 90 % of the HA was in this compartment. Cultures were repeated with chondrocytes and synovial cells obtained from different sources to verify that variation in HA production and HAS message ascribed to different growth factors was independent of cell source. Consistent trends were observed, and results are reported below for representative experiments for each cell type.

HA secretion by cultured cells

The secretion of HA from connective tissue cells is affected by the culture environment of the cells. However, the direction as well as the magnitude of the response is dependent on the cell type, as described below and illustrated in Figures 1 to 3.

HA secretion by articular chondrocytes increases from 0.2 to 2.0 μ g/ml medium for cultures maintained in the absence or presence of serum (Figure 1A). Addition of growth factors gives

Figure 1 HA secretion by cultured chondrocytes

Chondrocytes were maintained in monolayer culture in the presence of growth factors for 4 days. At this point cell numbers and the HA content of the culture supernatants were determined. HA content is expressed as $\mu\text{g/ml}$ medium (A) and $\mu\text{g}/10^6$ cells at the end of the culture period (B). Cultures were carried out in DMEM (1) or in DMEM supplemented with IGF-1 (2), bFGF (3), TGF- β 1 (4), IL-1 β (5), TGF- β 1 + IL-1 β (6), or FCS (7).

Figure 3 HA secretion by cultured osteosarcoma cells

Osteosarcoma cells were maintained in monolayer culture for 4 days and cell density and HA content of the culture medium were analysed. HA content is expressed as $\mu\text{g/ml}$ medium (A) and $\mu\text{g}/10^6$ cells at the end of the culture period (B). Cultures were carried out in DMEM (1) or in DMEM supplemented with IGF-1 (2), bFGF (3), TGF- β 1 (4), IL-1 β (5), TGF- β 1 + IL-1 β (6), or FCS (7).

Figure 2 HA secretion by cultured synovial cells

Synovial cells were maintained in monolayer culture for 4 days, and cell density and HA content of the culture medium were analysed. HA content is expressed as $\mu\text{g/ml}$ medium (A) and $\mu\text{g}/10^6$ cells at the end of the culture period (B). Cultures were carried out in DMEM (1) or in DMEM supplemented with IGF-1 (2), bFGF (3), TGF- β 1 (4), IL-1 β (5), TGF- β 1 + IL-1 β (6), or FCS (7).

rise to intermediate values, with their ability to stimulate HA secretion varying in the order TGF- β 1 < IGF-1 < bFGF < IL-1 β . The most effective stimulation of HA release was observed in the combined presence of TGF- β 1 and IL-1 β , where values approached those obtained in the presence of FCS. As the growth factors used may also stimulate cell division, final cell densities were determined and HA secretion re-evaluated on a per cell basis (Fig. 1B). The overall response pattern of chondrocytes was similar, with HA release ranging from 3 to 28 $\mu\text{g}/10^6$ cells over the 4 day culture period for control and TGF- β 1/IL-1 β -stimulated cultures. The only notable change was the decrease in the value associated with FCS stimulation, due to the more pronounced influence of this supplement on cell proliferation.

For synovial cells the observed trends in HA secretion were independent of whether results were normalized to initial (Figure 2A) or final (Figure 2B) cell density. All individual growth factors stimulated HA release relative to the control. The response pattern to the growth factors was different from that observed with chondrocytes, with the ability to stimulate varying in the order IGF-1 < bFGF < TGF- β 1 < IL-1 β . FCS and TGF- β 1 induced similar levels of HA secretions. In general, HA production was higher by synovial cells than chondrocytes with values ranging from 1 $\mu\text{g/ml}$ (10 $\mu\text{g}/10^6$ cells) for control to 8 $\mu\text{g/ml}$ (80 $\mu\text{g}/10^6$ cells) for IL-1 β stimulation. However, the greatest level of HA stimulation by far was observed for cultures exposed to both TGF- β 1 and IL-1 β , which attained levels of up to 70 $\mu\text{g/ml}$ (700 $\mu\text{g}/10^6$ cells). This dramatic stimulation in HA production was unique to synovial cells, indicating that TGF- β 1 and IL-1 β act in a synergistic manner on these cells.

The osteosarcoma cell line MG-63 exhibited the least response to growth factors or FCS stimulation (Figure 3A), with only IGF-

Table 2 HAS message analysis in cultured chondrocytes

Culture conditions	Copies (number/250 ng of RNA)				Ratio HAS1/HAS2/HAS3
	HAS1	HAS2	HAS3	G3PDH	
Control	1.8×10^6	2.4×10^6	1.6×10^5	1.1×10^8	0.75:1:0.07
IGF-1	1.4×10^6	2.6×10^6	1.2×10^5	1.2×10^8	0.54:1:0.05
bFGF	3.4×10^5	7.0×10^6	5.6×10^3	1.0×10^8	0.05:1: < 0.01
TGF β	3.7×10^5	3.8×10^5	5.5×10^3	1.5×10^8	0.97:1:0.01
IL-1 β	3.8×10^5	1.4×10^6	1.7×10^4	1.0×10^8	0.27:1:0.01
TGF β + IL-1 β	6.9×10^5	2.4×10^5	5.8×10^3	0.8×10^8	2.9:1:0.02
FCS	3.2×10^5	3.5×10^5	1.6×10^5	1.4×10^8	0.91:1:0.45

Table 3 HAS message analysis in cultured synovial cells

Culture conditions	Copies (number/250 ng of RNA)				Ratio HAS1/HAS2/HAS3
	HAS1	HAS2	HAS3	G3PDH	
Control	5.8×10^6	1.6×10^6	1.1×10^5	1.4×10^8	3.6:1:0.07
IGF-1	2.2×10^6	1.8×10^6	1.6×10^5	1.9×10^8	1.2:1:0.09
bFGF	5.0×10^6	1.4×10^6	6.8×10^4	1.8×10^8	3.6:1:0.05
TGF β	5.0×10^6	5.8×10^5	3.2×10^3	1.2×10^8	8.6:1: < 0.01
IL-1 β	3.1×10^6	2.4×10^6	1.2×10^5	1.2×10^8	1.3:1:0.05
TGF β + IL-1 β	8.6×10^6	3.6×10^5	1.2×10^4	1.7×10^8	23:1:0.03
FCS	1.9×10^6	4.4×10^5	1.8×10^4	1.9×10^8	4.3:1:0.04

1 and FCS affecting HA production. This increase was completely accounted for by their effects on cell proliferation (Figure 3B), with values ranging between 2–4 $\mu\text{g}/10^6$ cells over the 4 day culture period. This rate of synthesis is lower than that exhibited by chondrocytes or synovial cells. Also, unlike synovial cells and, to a much lesser degree chondrocytes, these cells exhibited no synergistic response in the presence of both TGF- β 1 and IL-1 β . There was a similar lack of response with respect to HA accumulation in cultures of human fetal lung or skin fibroblasts (results not shown). Thus amongst the cell lines investigated, synovial cells produce much higher levels of HA, and the rate of synthesis is much more responsive to the presence of growth factors in the culture milieu.

HAS gene utilization by cultured cells

To address the issue of which HAS gene is responsible for HA production, and whether this varies with cell type or growth factor stimulation, RNA was isolated from the cells after 4 days of exposure to the various culture conditions and analysed using a quantitative competitive RT-PCR protocol. This technique allows expression levels of the three HAS genes to be compared.

Of the three isoforms, HAS2 was found to be the most abundant message in articular chondrocytes (Table 2). Perhaps surprisingly, culture in the presence of FCS and most growth factor conditions resulted in decreased levels of the HAS2 message. The exception was bFGF which stimulated HAS2 expression 3-fold, while HAS1 mRNA levels were reduced to about 20% of the control values. HAS1 message levels were usually lower than those of HAS2, except after culture in the presence of TGF- β 1 plus IL-1 β . All growth factors and FCS resulted in decreased expression of HAS1, but in the case of TGF- β 1 plus IL-1 β the inhibitory effect was much more pronounced on HAS2 expression, allowing HAS1 levels to become more predominant. HAS3 message levels were always least abundant and were particularly low in the presence of bFGF,

TGF- β 1, IL-1 β and TGF- β 1/ IL-1 β . In these latter cases, it is likely that message levels of HAS3 may represent the detection of illegitimate transcription.

Synovial cells showed an opposite trend to the chondrocytes, with HAS1 message levels always being more abundant than that for HAS2, irrespective of growth factor or FCS treatment (Table 3). Exposure of the cells to any of the individual growth factors or FCS resulted in a decrease in HAS1 message levels, and only the combination of TGF- β 1 and IL-1 β caused an increase in HAS1 message levels. In contrast, this growth factor combination decreased HAS2 message levels, resulting in HAS1 message being 20 fold greater than HAS2 message under conditions where HA production by the synovial cells is maximal. HAS2 message levels were also decreased by FCS, bFGF and TGF- β 1, but were stimulated by IGF-1 and IL-1 β . This stimulatory effect was however insufficient to permit HAS2 message levels to exceed those of HAS1. As with the chondrocytes, HAS3 message levels were always least abundant, and never exceeded 10% of the HAS2 message level under any of the culture conditions.

HAS2 message levels always predominated in osteosarcoma cells, and relative to controls the levels were reduced by all growth factors and FCS (Table 4). In contrast to the synovial cells and chondrocytes, HAS3 message levels were always more abundant than HAS1 message levels in the osteosarcoma cells. Indeed, HAS1 levels were always less than 1% of those of HAS2 and may represent the consequence of illegitimate transcription. HAS3 message levels were usually less than 7% of HAS2 levels and also decreased in abundance with most growth factors. However, HAS3 message levels were increased by both IL-1 β and FCS, and in the former case they may attain 30% of the level of the HAS2 message.

To obtain an independent verification of the trends observed by the quantitative RT-PCR technique, HAS message levels were also studied by Northern blotting. This approach does not allow ready comparison of the expression levels of different HAS types, but changes in the expression of individual HAS types in response

Table 4 HAS message analysis in cultured osteosarcoma cells

Culture conditions	Copies (number/250 ng of RNA)				Ratio HAS1/HAS2/HAS3
	HAS1	HAS2	HAS3	G3PDH	
Control	4.2×10^3	4.5×10^6	3.3×10^5	1.3×10^8	$< 0.01:1:0.07$
IGF-1	6.1×10^3	2.8×10^6	1.3×10^4	2.0×10^8	$< 0.01:1: < 0.01$
bFGF	1.5×10^4	3.9×10^6	2.7×10^5	1.3×10^8	$< 0.01:1:0.07$
TGF β	4.2×10^3	1.0×10^6	1.7×10^4	9.6×10^7	$< 0.01:1:0.02$
IL-1 β	1.8×10^4	1.9×10^6	5.8×10^5	9.4×10^7	$< 0.01:1:0.31$
TGF β + IL-1	9.2×10^3	1.3×10^6	2.6×10^4	9.6×10^7	$< 0.01:1:0.02$
FCS	2.0×10^4	2.5×10^6	4.5×10^5	2.4×10^8	$0.01:1:0.18$

Figure 4 Northern blot analysis of RNA from cultured chondrocytes

Total cellular RNA was isolated from the chondrocyte cultures used to generate the data shown in Figure 1 and analysed by Northern blotting for the expression of mRNA for HAS1 (A), HAS2 (B) and G3PDH (C). The cells were cultured in unsupplemented DMEM (1) or in DMEM supplemented with IGF-1 (2), bFGF (3), TGF- β 1 (4), IL-1 β (5), TGF- β 1 + IL-1 β (6), or FCS (7).

Figure 5 Northern blot analysis of RNA from cultured synovial cells

Total cellular RNA was isolated from the synovial fibroblast cultures used to generate the data shown in figure 2 and analysed by Northern blotting for the expression of mRNA for HAS1 (A), HAS2 (B) and G3PDH (C). The cells were cultured in unsupplemented DMEM (1) or in DMEM supplemented with IGF-1 (2), bFGF (3), TGF- β 1 (4), IL-1 β (5), TGF- β 1 + IL-1 β (6), or FCS (7).

to growth factors can be evaluated. HAS3 was undetectable by Northern blotting in all RNA preparations, and in the case of the osteosarcoma cells, HAS1 message was also undetectable. Transcripts for both HAS1 and HAS2 were readily detectable in total

Figure 6 HAS gene expression in human articular cartilage and synovial tissues.

Total RNA was extracted from fresh tissue samples and HAS message levels were determined by quantitative PCR. The cartilage samples were from specimens aged 2 months (1), 3 years (2) and 79 years (3). Synovial specimens were obtained from patients with rheumatoid arthritis (1,2) or osteoarthritis (3,4). All mRNA levels were normalized to the copy numbers determined for G3PDH. ●, HAS1; ○, HAS2; ◇, HAS3. Ratios of HAS1 to HAS2 are given underneath the Figure for the specimens shown, in numerical order.

cellular RNA preparations from chondrocytes (Figure 4) and synovial cells (Figure 5).

Generally, the trends in mRNA distribution determined by PCR analysis were confirmed. Changes in abundance of both HAS1 and HAS2 message were apparent in chondrocytes exposed to the different growth factors, and the trends differed for the two genes (Figure 4). The most apparent effects were the reduction in both HAS1 and HAS2 message levels by TGF- β 1. While this effect was reversed by IL-1 β for HAS1, this was not the case for HAS2.

In synovial cells (Figure 5), the blotting data confirmed the increase in HAS1 message levels by TGF- β 1 plus IL-1 β , and the decrease in the expression of both HAS messages by FCS. Similar trends between blotting (results not shown) and PCR were also apparent for the osteosarcoma cells, with TGF- β 1 causing the most pronounced decrease in HAS2 message abundance.

HAS message expression by tissues

In order to address whether the relative abundance of the different HAS messages observed in cultured cells reflects the situation occurring *in vivo*, RNA was extracted directly from fresh specimens of articular cartilage and synovium and analysed by the quantitative RT-PCR procedure. As shown in Figure 6, the relative abundance of the HAS subtypes in these tissues was significantly different and followed a pattern similar to that observed in the isolated and cultured cells. In articular cartilage, HAS2 message levels were always most abundant and HAS3 message levels always least abundant. In the three specimens examined HAS1 message levels were 10–50 % of those of HAS2, whereas HAS3 message levels were less than 10 % of those of HAS2. HAS1 message levels in synovial tissue were similar to or greater than those for HAS2, whereas HAS3 message levels were always least abundant. Thus, as observed in the cultured cells, native chondrocytes express predominantly HAS2 message, whereas native synovial cells tend to express greater amounts of HAS1 message. In addition, the total copy numbers for all HAS messages relative to G3PDH is much higher in synovial tissues than in articular cartilage at any of the ages investigated, suggesting a much higher level of expression of the HAS genes and consequently a much increased level of HA biosynthesis in this tissue.

DISCUSSION

Several features are apparent from the present data with respect to HA synthesis by cells of the skeletal system: (1) the extent of utilization of the three HAS genes is cell-specific; (2) HAS message levels are often of low abundance within the cells; (3) growth factors and cytokines can stimulate HA production but have differing effects on different cell types; and (4) an increase in HA production need not necessarily correspond with an increase in HAS gene expression. In contrast to other reports we find evidence for the expression of all three HAS isoforms in all the cell types studied, although the relative ratios of the isoforms vary considerably. Thus while HAS2 is the major expressed isoform in chondrocytes and cartilage, there are appreciable levels of HAS1 in contrast to reports by Nishida et al. [22] and Hiscock et al. [23] who could not find any evidence for the expression of HAS1 in human articular chondrocytes and cartilage. As the identity of the PCR fragments was confirmed by sequencing and the PCR data mirror those obtained by Northern blotting, we conclude that the connective tissue cells analysed in this study do indeed express HAS1. The failure to detect HAS1 mRNA is most likely due to differences in methodology, particularly the choice of reverse transcriptase and primers. In our studies we found that MMuLV reverse transcriptase performed very poorly with several sets of downstream primers as template, particularly for HAS1. When this reverse transcriptase was used with oligo-dT as downstream primer, the relative efficiencies of reverse transcription were vastly different for the three HAS isoforms. For HAS3 this may be due to the large size of the 3'-untranslated region, the sequence for which has recently been deposited (GenBank® accession no. AF232772). The use of the Omniscript reverse transcriptase proved superior and copied all three mRNA species with similar efficiency. These observations may explain the differences between our data and those referred to above.

The quantitative RT-PCR protocol allowed message levels within the cells to be accurately established. Message levels for HAS1 and HAS2 never exceed 10^7 copies/250 ng total cellular RNA, whereas those for HAS3 may be below 10^4 copies/250 ng RNA. If one accepts that a typical cell may contain about

25 pg RNA [24], then 250 ng RNA corresponds to about 10^4 cells and many of the values obtained for HAS3 represent less than 1 message/cell. Such low levels most likely reflect the products of illegitimate transcription. In contrast, HAS2 message levels range from about 20–700 copies/cell, depending on the stimulus being used in culture. HAS1 message levels fall within a similar range for the chondrocytes and synovial cells, but are below 1 copy/cell in the osteosarcoma cells. The HAS message levels can be compared to those obtained for the house-keeping gene, *G3PDH*, which is usually above 10^8 copies/250 ng RNA giving message levels in the order of 10000 copies/cell. The relatively low cellular abundance of HAS messages under many conditions, may help explain the paucity of sequence tags for HAS genes present in the expressed-sequence-tag database (dbEST), even though HA synthesis is a feature of most cell types.

It would appear from the current work that the low level of HAS gene expression is not just a feature of cultured chondrocytes, but also of chondrocytes *in vivo* where HAS message levels are also about 1–4 % of those for *G3PDH* message. In the case of HAS2 and HAS3, similar low levels of message expression have been previously reported in cultured human chondrocytes [22], with 2×10^7 and 5×10^5 copies/ μ g RNA, respectively. The present report, however, is the first to describe HAS1 expression by chondrocytes, and determine how message expression can be affected by growth factors. In the case of the synovial cells HAS message expression appears to be higher *in vivo* than in the cultured cells. At least in the arthritic synovium studied in the present work HAS message levels were between 10–50 % those for *G3PDH*. Such high levels of HAS gene expression may not be too surprising in view of the large effusion of HA-containing synovial fluid that characterizes such joints.

The ability of growth factors to regulate HA production is not too surprising, nor is the fact that the growth factors exhibit different effects on different cell types, as their needs for HA differ. In the case of chondrocytes, however, it is perhaps surprising that HA synthesis is stimulated by IL-1 β , a cytokine that down-regulates aggrecan synthesis, yet is down-regulated by TGF- β 1, a growth factor that stimulates aggrecan synthesis. If one believes that the major role of HA in cartilage is to support proteoglycan aggregate formation by aggrecan then one might have expected a more co-ordinated synthesis of the two molecules. The stimulation of synovial cell HA production by TGF- β 1 or IL-1 β has been previously reported [25,26], in agreement with our observations. However, the synergistic effect of these two growth factors on HA synthesis has not been observed previously. The magnitude of this response is remarkable and represents one of the few instances where these two cytokines actually work in a synergistic fashion with respect to the synthesis of an extracellular matrix component. While TGF- β 1 is not generally considered a pro-inflammatory cytokine with respect to cartilage physiology and pathology, its effect on immune-cell mediated processes may be pro-inflammatory [27] and the increased production of HA by the synovium is, indeed, thought of as part of the inflammatory response in synovitis. Recruitment of immune cells is an important aspect of inflammation, and elevated levels of HA may aid in this process, particularly for the CD44-mediated homing of lymphocytes [28–30]. It is interesting to note that the synergism with HA production was also observed in cultured chondrocytes, although with a much reduced magnitude.

It is apparent from the present studies that increases in HA secretion need not be accompanied by increases in HAS message levels, suggesting that the regulation of HA synthesis is often a post-transcriptional event. In such cases HA synthesis may be regulated by changes in the rate at which the message is translated, changes in the availability of the precursors for HA synthesis,

or changes in the activity of the synthases themselves, and evidence in support of all these mechanisms can be found [31]. One must also consider that the measurement of HA secretion reflects cumulative production throughout the culture period, whereas HAS message analysis reflects levels at the termination of the culture period, and one cannot guarantee that either value was constant for the entire culture period. Analysis of HA levels in the cultures used in this study after 2 days of stimulation suggests that the rate of HA release from the cells is fairly constant over the entire culture period. However, whether the turnover of the enzymes themselves is constant remains to be determined.

Finally, it is apparent that none of the three cell types under study express significant amounts of the HAS3 gene, and that they make different use of the HAS1 and HAS2 genes. Synovial cells preferentially utilize the HAS1 message, whereas chondrocytes and osteosarcoma cells preferentially utilize the HAS2 message. This preferential use of HAS1 gene expression is particularly apparent when HA production by the synovial cells is maximally stimulated by TGF- β 1 plus IL-1 β . Thus increased expression of the HAS1 gene may be part of the inflammatory response of connective tissues. The reason for the difference in HAS gene usage is not readily apparent, but it is tempting to speculate that the different HAS isoforms may have different enzymic properties, such as their rate of synthesis, the size of the HA they produce, and their ability to retain the HA in a pericellular coat rather than direct its secretion to a remote site. Transfection of COS cells with constructs encompassing the various HAS genes provides evidence that such a scenario may exist [13,32]. Such experiments indicate that HAS1 transfection results in smaller pericellular HA coats than transfection with HAS2 or HAS3, and that the size of the HA secreted by HAS2 transfectants is larger than that produced following transfection with HAS1 or HAS3. *In vitro* studies also demonstrated that the three synthases exhibit different kinetic properties. As chondrocytes, synovial cells and osteosarcoma cells produce HA for different purposes, it is possible that different synthases are required to meet these needs. In particular, one might predict that HAS1 favours the production of HA for tissue secretion, whereas HAS2 favours its production for retention in the extracellular matrix.

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