Expression of human hyaluronan synthases in response to external stimuli

Annica JACOBSON*, Jonas BRINCK*, Michael J. BRISKIN†, Andrew P. SPICER‡ and Paraskevi HELDIN*[1]

*Department of Medical Biochemistry and Microbiology, Unit of Biochemistry, Uppsala University, Biomedical Centre, Box 575, S-751 23, Uppsala, Sweden, †LeukoSite, 215 First St., Cambridge, MA 02142, U.S.A., and ‡Rowe Program in Genetics, Department of Biological Chemistry, University of California, School of Medicine, Tupper Hall, Davis, CA 95616, U.S.A.

In the present study we have investigated the expression of mRNAs for hyaluronan synthase isoforms (HAS1, HAS2 and HAS3) in different cells in response to various stimuli. Human mesothelial cells, which synthesize large amounts of hyaluronan, express mRNAs encoding all three HAS isoforms, whereas their transformed counterparts, mesothelioma cells, which produce only minute amounts of hyaluronan, express only HAS3 mRNA. Human lung fibroblasts and the glioma cell line U-118 MG express only the *HAS2* and *HAS3* genes. The expression of the transcripts was higher in subconfluent than in confluent cultures and was well correlated with the production of hyaluronan by the cells. Stimulation of mesothelial cells with platelet-derived growth factor-BB induced an up-regulation of mRNA for HAS2 to a maximum after 6 h of stimulation; *HAS1* and *HAS3* genes were only induced slightly. Transforming growth factor-β1 reduced HAS2 mRNA slightly, and hydrocortisone reduced it strongly, within 6 h of stimulation in mesothelial cell cultures but did not significantly affect the expression of mRNAs for HAS1 and HAS3. Induction of HAS1 and HAS2 protein levels in response to the stimuli above correlated with HAS transcript levels. Thus the expression of the three HAS isoforms is more prominent in growing cells than in resting cells and is differentially regulated by various stimuli suggesting distinct functional roles of the three proteins.

Key words: biosynthesis, growth factors, regulation, signal transduction.

INTRODUCTION

Hyaluronan, a major constituent of the extracellular matrix, is a linear high-molecular-mass glycosaminoglycan composed of alternating units of D-glucuronic acid and *N*-acetylglucosamine. Hyaluronan interacts with hyaluronan-binding proteins, collectively referred to as hyaladherins, and is involved in a variety of biological processes, such as maintenance of tissue architecture, cell proliferation, migration, differentiation, angiogenesis, wound healing and tumourigenesis [1–5]. Increased hyaluronan levels in tissues and body fluids can be detected in several inflammatory conditions such as lung fibrosis, rheumatoid arthritis, myocardial infarction and transplant rejection, as well as in some invasive tumours [1]. Such accumulation of hyaluronan often leads to organ dysfunction.

Genes encoding hyaluronan synthase (HAS) have been characterized from bacteria [6,7] and vertebrates [8–13]. More recently a virally encoded HAS has been identified within the *Paramecium bursaria* chlorella virus (PBCV-1) genome [14]. The streptococcal hyaluronan synthase, HasA, and the three mammalian HAS isoforms are predicted plasma-membrane proteins with molecular masses of 42 and 64 kDa, respectively. Each protein is predicted to span the plasma membrane several times, with the sequences specifying catalytic activity residing on the inner face of the membrane [15]. The vertebrate HAS proteins share 55–71 % sequence identity whereas homologous isoforms of human and mouse share about 96–99 % sequence identity [16,17].

The mechanisms that regulate hyaluronan synthesis in normal tissues, as well as in pathological processes such as tumour metastasis and inflammatory foci are not well understood. Recently, the molecular mechanisms that regulate hyaluronan biosynthesis have been partially unravelled. We, and other groups, found that growth factors such as platelet-derived growth factor-BB (PDGF-BB), transforming growth factor-β1 (TGF-β1), insulin growth factor, fibroblast growth factor and follicle-stimulating hormone stimulate hyaluronan synthesis in various cells of mesenchymal origin [18–22]. The growth factors mediate their stimulatory effects on hyaluronan synthesis partly through the activation of protein kinase C and protein kinase A, and in part through *de novo* protein synthesis [20,23]. However, the stimulatory effects on hyaluronan synthesis in foreskin fibroblast cultures by PMA, an activator of protein kinase C, were not dependent on protein synthesis [20]. In the present study we have attempted to elucidate the expression of HAS isoform mRNAs and proteins in various cell types and to investigate the effect of exposure to various growth factors. We report that HAS1, HAS2 and HAS3 are expressed differentially in several cell types. Expression of hyaluronan synthases is higher in subconfluent cultures compared with growth-arrested cultures. Furthermore, we report that *HAS1*, *HAS2* and *HAS3* genes are differentially inducible by growth factors.

MATERIALS AND METHODS

Materials

Primary cultures of normal human mesothelial (NHM) cells were obtained from biopsies of patients undergoing surgery for coronary disease, as described previously [24]. The project was approved by the Ethical Committee, Uppsala University Hospital, Uppsala, Sweden. The human mesothelioma cell line,

Abbreviations used: HAS, hyaluronan synthase; PDGF-BB, platelet-derived growth factor-BB; TGF-β1, transforming growth factor-β1; NHM, normal human mesothelial; NHLF, normal human lung fibroblast; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

[1] To whom correspondence should be addressed (e-mail Paraskevi.Heldin@medkem.uu.se).

Mero-14, was provided kindly by Dr M. Versnel (Erasmus University, Rotterdam, The Netherlands). The glioma cell line U-118 MG was a kind gift from Dr B.Westermark (Uppsala University, Uppsala, Sweden). Foreskin fibroblasts (AG 1519) and normal human lung fibroblasts (NHLFs; 2801-1) were purchased from the Human Mutant Repository (Camden, NJ, U.S.A.) and Clontech (Palo Alto, CA, U.S.A.), respectively. Recombinant PDGF-BB and TGF-β1 were provided kindly by Dr C.-H. Heldin and Dr P. ten Dijke (Ludwig Institute for Cancer Research, Uppsala, Sweden), and epidermal growth factor was purchased from Sigma, Stockholm, Sweden. Foetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium and the other cell-culture reagents were purchased from SVA (Statens Veterinärmedicinska Anstalt, Uppsala, Sweden).

Cell culture and determination of hyaluronan chain length and content

NHM cells were cultured in Ham's F-10 medium supplemented with 15% FCS, 4 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml epidermal growth factor and 0.4 μg/ml hydrocortisone. The cells were examined for mesothelial characteristics by light microscopy and stained positive with monoclonal antibodies to cytokeratin 19, as described previously [19]. Mero-14, U-118 MG, AG 1519 and NHLF cells were cultured in DMEM containing 10% FCS, 4 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. NHLF culture medium was supplemented with insulin (5 ng/ml).

Hyaluronan synthesis was estimated using a hyaluronan detection kit (HA-kit; Pharmacia, Uppsala, Sweden). This kit is based on the specificity of a hyaluronan-binding protein isolated from bovine cartilage for hyaluronan [25]. Hyaluronan content in conditioned media and cell-associated hyaluronan from subconfluent and confluent cultures was determined after 24 h of stimulation with 10% FCS. The conditioned media were centrifuged for 10 min at 10000 ***g*** to remove cell debris prior to hyaluronan determination and crude membranes were prepared as described [26], with slight modifications. Briefly, cells were washed with PBS and lysed on ice in a hypotonic buffer containing 10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris/HCl, pH 7.4, 1 mM dithiothreitol and a cocktail of protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 10000 KIU/ml trasylol and 1 mM pefabloc). Lysates were freeze-thawed twice, homogenized and centrifuged at 400 ***g*** for 5 min to pellet nuclei. The supernatants were then re-centrifuged (25000 ***g***, 45 min), the membrane pellets resuspended in PBS containing protease inhibitors and hyaluronan content was determined.

The size of hyaluronan chains synthesized *de novo* by unstimulated NHM cells and by cells stimulated with 15% FCS, 50 ng/ml PDGF-BB, 5 ng/ml TGF-β1 and 10 nM PMA was determined essentially as described in [27]. The external stimulators were used at concentrations giving maximum effects [18]. Briefly, 1×10^5 cells were starved for 24 h and then stimulated for another 24 h in the presence of 10 μCi of [^{3}H]glucosamine. Conditioned media were harvested and dialysed against 0.1 M sodium acetate buffer, pH 5.0, containing 5 mM EDTA. Each dialysate was then divided into two aliquots. One aliquot was treated overnight with 10 units/ml *Streptomyces* hyaluronidase at 37 °C whereas the other was treated in a similar way but in the absence of the enzyme. After enzyme inactivation the samples were subjected to gel chromatography on a gel Sephacryl-HR column (106 cm × 1 cm) and the radioactivity in the fractions was measured.

Preparation of mRNA and Northern analysis

Confluent [$(6–10) \times 10^6$ cells/dish] or subconfluent [$(3–5) \times 10^6$ cells/dish] cultures, depending on cell type, were cultured in 175 cm^2 Falcon bottles for 24 h in complete media before mRNA preparation. Media were aspirated and fresh media, containing 0.25% FCS (mesothelial cells) or 0.1% FCS (Mero-14, U-118MG, AG 1519 and NHLF cells) were added and the cells were cultured for another 24 h. At this time the media were aspirated, fresh media added and the cultures were incubated in the absence or presence of 50 ng/ml PDGF-BB, 5 ng/ml TGF-β1, 10 nM PMA or various concentrations of hydrocortisone for various periods of time. mRNA was prepared using a commercially available kit essentially according to manufacturer's instructions (Mini Message Maker; R & D Systems, Oxford, U.K.), except that cells in culture dishes were lysed directly with lysis buffer and harvested from the bottles with a rubber policeman.

To quantify the mRNA expression of the three HAS isoforms in untreated and treated cells, mRNAs (3 μg) were separated on 1% agarose gels containing formaldehyde and vacuum-transferred overnight to Hybond N$^+$ nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Following cross-linking with UV light, the membranes were incubated with 1 × Denhardt's reagent and Rapid Hybe buffer (Amersham Pharmacia Biotech) for 1 h at 65 °C and hybridized with ^{32}P-labelled probes for 2 h at 65 °C under conditions recommended by the manufacturer. The labelled probes were prepared by random priming (Mega Prime, Amersham Pharmacia Biotech) and 1.5×10^6 c.p.m./ml was added to each hybridization mix. Each membrane was hybridized sequentially with two or three ^{32}P-labelled cDNA probes representative for HAS1 (1110 bp nucleotide sequence; *Sac*II digest of HAS1 cDNA in pcDNA3 vector [10]), HAS2 (360 bp nucleotide sequence; *Cla*I/*Xho*I digest of mouse Has2 cDNA in pCIneo [12]) or HAS3 (500 bp nucleotide sequence; *Eco*RI/*Pst*I digest of mouse Has3 cDNA in pCIneo [13]) and exposed to X-ray film overnight at −70 °C using intensifying screens. Following exposure the membrane was stripped by boiling for 1 min in 0.1% SDS, exposed to X-ray film overnight to ensure the complete removal of the probe and then rehybridized with ^{32}P-labelled cDNA probe for another HAS isoform. In order to control for variation in sample loading the membranes were also probed with a ^{32}P-labelled cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In addition, mRNA expression levels were quantified by PhosphoImaging of Northern blots using a Bio-Rad PhosphoImager and associated software (Bio-Rad).

Polyclonal peptide antisera against HAS1 and HAS2

A rabbit polyclonal peptide antiserum against the C-terminal part of HAS1 (GVRRLCRRRTGGYRVQV; termed GVR) has been described previously [28]. We also developed an antiserum against the C-terminal portion of mouse Has2 (TIYKESKKPFSESKQT; termed TIY). Both antisera were affinity purified as described previously [28].

Immunoblotting

Expression of HAS1 and HAS2 proteins was determined by immunoblotting after 24 h of culture under control (untreated) conditions, or growth factor, PMA or hydrocortisone treatments. Membranes were prepared as described in [26] and protein content was determined, using Bio-Rad Protein Assay.

Samples of crude membrane preparations were mixed with SDS/PAGE reducing sample buffer, boiled for 3 min at 95 °C, separated on a 10 % polyacrylamide gel and electrotransferred to nitrocellulose membranes. The membranes were blocked overnight in 5 % non-fat dry milk powder in TBS-T (20 mM Tris/HCl, 137 mM NaCl and 0.1 % Tween 20, pH 7.6), followed by incubation with 10 μg/ml affinity purified GVR or TIY antibodies for 3 h. Following one wash in TBS-T, membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies, which were diluted 5000-fold in TBS-T (donkey anti-rabbit IgG, Amersham Pharmacia Biotech). To check the specificity of GVR or TIY antibodies, the antibodies were preincubated for 30 min with a 100-fold molar excess of GVR or TIY peptides before incubation with membranes. Immune complexes were detected by enhanced chemiluminescence according to recommendations of the manufacturer (Amersham Pharmacia Biotech).

RESULTS

Differential expression of HAS isoform mRNAs

We used Northern blotting to investigate the expression of HAS isoform mRNAs by subconfluent and confluent cultures of U-118 MG, Mero-14, NHM, AG 1519 and NHLF cells (Figure 1). A HAS1 mRNA transcript of approx. 2.4 kb was expressed by subconfluent cultures of mesothelial cells and, to a lower degree, by confluent cultures. HAS2 transcripts migrating as 5.0 and 3.4 kb components (their actual sizes are 4.4 and 3.0 kb according to their sequences), were expressed abundantly by subconfluent cultures of U-118 MG and mesothelial cells, and to a lesser extent by NHLFs. The expression of HAS2 was diminished in confluent cultures of these cell types. HAS2 transcripts were undetectable in subconfluent and confluent cultures of Mero-14 and AG 1519. HAS3 transcripts of approx. 5.2 and 3.2 kb components (their actual sizes are 4.5 and 3.2 kb according to their sequences) were expressed by all cell types tested and at highest levels in NHM and Mero-14 cells (Figure 1).

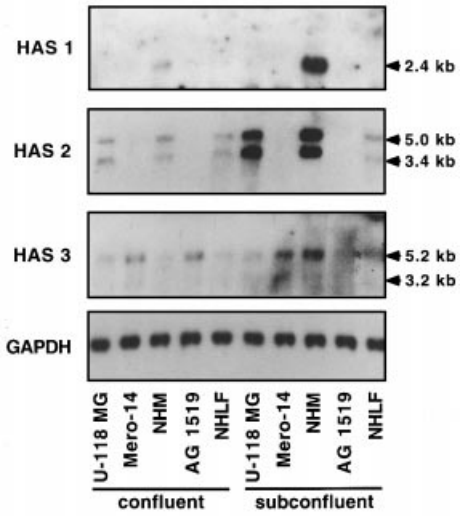

Figure 1 Differential expression of mRNA for HAS1, HAS2 and HAS3

mRNA (3 μg/well) obtained from subconfluent or confluent cultures of U-118 MG, Mero-14, NHM cells, AG 1519 and NHLFs was subjected to 1% agarose-gel electrophoresis and transferred to nitrocellulose membranes. The filters were hybridized sequentially with ^{32}P-labelled cDNA probes for HAS1, HAS2 and HAS3 and exposed to autoradiographic film. Variation in sample loading was determined by hybridization with a GAPDH probe. The data shown are representative of two separate experiments.

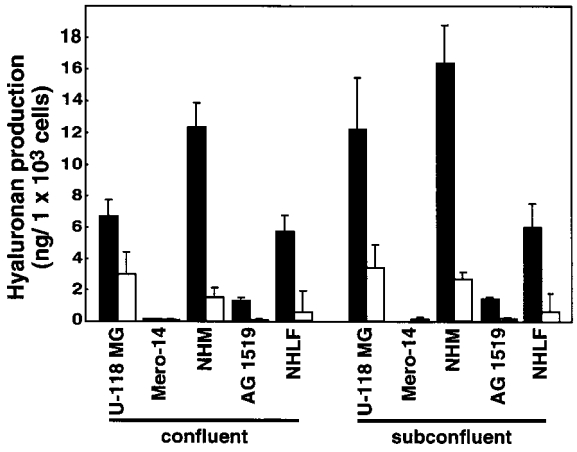

Figure 2 Hyaluronan production

Hyaluronan content in 24-h-conditioned media from subconfluent and confluent cell cultures was measured using a commercial kit. The amount in the corresponding cell layers was also determined; cells were washed once and harvested by membrane preparation. The data represent means ± S.E.M. of three separate experiments. Black bars, hyaluronan released in media; white bars, cell-surface-associated hyaluronan.

Our previous studies on the molecular mechanisms behind the stimulation of HAS isoform activities in mesothelial cell cultures revealed that the enzymic activity was accompanied by a time-dependent increase in hyaluronan amount in the medium; a major increase of synthesized hyaluronan was found in the conditioned media after 24 h of stimulation [19]. Therefore, we determined the amounts of hyaluronan in 24-h-conditioned media from subconfluent and confluent cultures of U-118 MG, NHM cells, AG 1519, and NHLFs; the hyaluronan content correlated well with the expression of mRNAs for the three HAS isoforms. However, conditioned media from Mero-14 cells produced only minute amounts of hyaluronan (Figure 2), despite the fact that HAS3 transcripts were expressed (Figure 1). The amount of cell-associated newly synthesized hyaluronan varied considerably among the cell types tested. About 25 % of the hyaluronan synthesized by the malignant U-118 MG cells was membrane-associated compared with about 10 % of that synthesized by primary cultures of NHM cells and NHLFs (Figure 2). No hyaluronan was detected in membrane preparations isolated from Mero-14 and AG 1519 cell cultures.

Thus the expression patterns of the HAS isoforms varied between cell types of mesenchymal origin obtained from different organs. Moreover, the expression was generally higher in subconfluent compared with confluent cultures.

Effects of PDGF-BB, TGF-β1 and PMA on *HAS* gene expression

PDGF-BB has been shown to be a powerful stimulator of hyaluronan synthesis in fibroblast and NHM cell cultures [18–20]. Therefore, we investigated the effect of PDGF-BB on the expression of each HAS isoform by NHM cells using Northern blotting. Treatment of NHM cells with PDGF-BB for various time periods led to an early induction of *HAS1* gene, as early as 1 h after treatment, which then remained high for at least 24 h. Stimulation with PDGF-BB also induced a 2-fold up-regulation of HAS2 mRNA at 1 h after treatment with a maximum increase

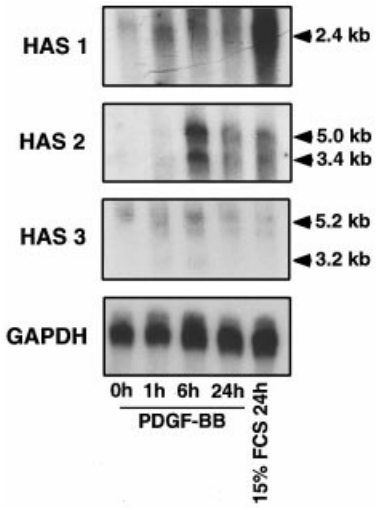

Figure 3 Time course of the induction of mRNA for each HAS isoform by PDGF-BB in NHM cells

Subconfluent cultures of NHM cells were starved for 24 h in Ham's F-10 medium with 0.25% FCS and then treated for the indicated times with 50 ng/ml PDGF-BB, as well as for 24 h with 15% FCS. mRNA was prepared and subjected to Northern analysis. The filters were hybridized sequentially with ^{32}P-labelled HAS1, HAS2 and HAS3 probes followed by a ^{32}P-labelled probe for GAPDH. The data shown are representative of three separate experiments.

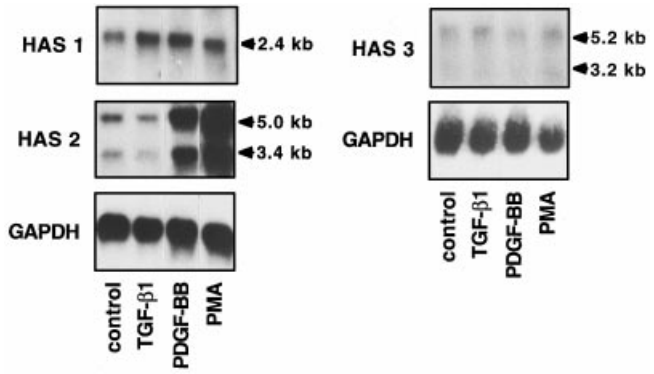

Figure 4 Effects of TGF-β1, PDGF-BB and PMA on HAS isoform gene expression in NHM cells

Subconfluent cultures of mesothelial cells were starved for 24 h in Ham's F-10 medium containing 0.25% FCS and then treated with 5 ng/ml TGF-β1, 50 ng/ml PDGF-BB or 10 nM PMA for 6 h. mRNA was extracted and subjected to Northern analysis. The data shown are representative of three separate experiments.

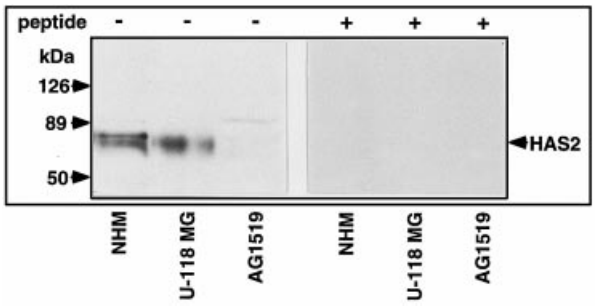

Figure 5 Expression of HAS2 protein in several cell lines

Crude membrane preparations obtained from NHM, U-118 MG and AG 1519 cells were subjected to SDS/PAGE (20 μg of protein/well) and transferred to a nitrocellulose filter. The filter was incubated with affinity purified TIY peptide antibodies (10 μg/ml) against mouse Has2, in the absence or presence of 100-fold molar excess of blocking peptide. The data shown are representative of two separate experiments.

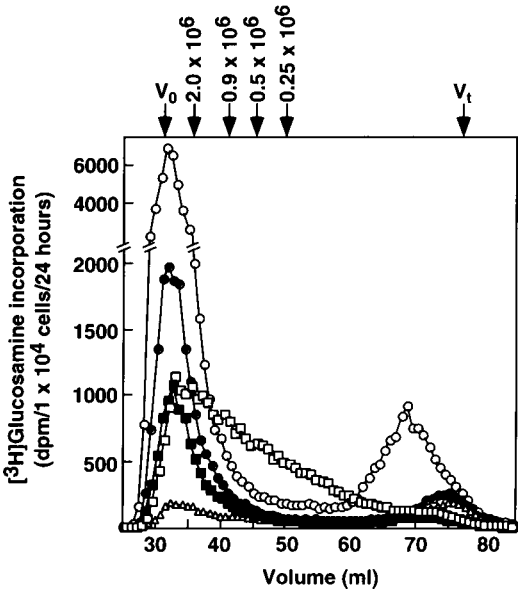

Figure 6 Sephacryl-HR gel chromatography of glycosaminoglycans synthesized by NHM cells

Starved cells (1×10^5) were stimulated for 24 h with 15% FCS (○), 0.25% FCS (Δ), 50 ng/ml PDGF-BB (●), 5 ng/ml TGF-β1 (■) or 10 mM PMA (□) in the presence of [^{3}H]glucosamine. Samples were analysed on a Sephacryl-HR column (see the Materials and methods section). Only material not treated with *Streptomyces* hyaluronidase is shown. Arrows show elution positions of hyaluronan molecular-mass markers.

of about 8-fold at 6 h followed by a slight decrease at 24 h after treatment. HAS3 transcript was also slightly induced in response to growth factor with a maximum at 6 h (Figure 3). Culturing of the cells for 24 h in media containing FCS led to 6- and 2-fold increases of HAS1 and HAS2 transcripts, respectively, over the level in non-stimulated cells.

Similarly to PDGF-BB stimulation, treatment of NHM cells with PMA for 6 h resulted in an increase in HAS2 expression. Slight induction of HAS1 and HAS3 expression was observed. However, treatment of NHM cells with TGF-β1 slightly attenuated the HAS2 expression but increased HAS1 mRNA expression about 2-fold. Under the same conditions, HAS3 transcript levels were not affected (Figure 4). Furthermore, culturing of the cells in the presence of the protein-synthesis inhibitor cycloheximide increased the expression of all three HAS transcripts (results not shown). Thus it is likely that TGF-β1 influences hyaluronan synthesis in mesothelial cells primarily through *HAS1* gene induction, whereas PDGF-BB and PMA exert their effects through *HAS2* gene induction.

Characterization of polyclonal antibodies against mouse Has2

The specificity of the affinity purified antibodies raised against the Has2 synthetic peptide was determined by immunoblotting (Figure 5). Cell lysates from mesothelial and glioma cells, which express large amounts of HAS2 mRNA, as well as lysates from

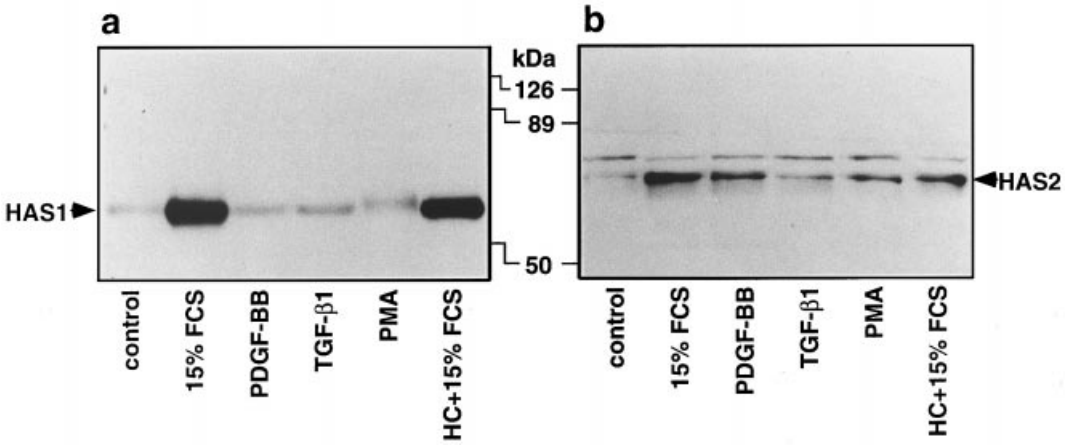

Figure 7 Expression of HAS1 and HAS2 proteins in NHM cells

Membranes isolated from untreated NHM cells (Ham's F-10 medium supplemented with 0.25% FCS; control) or NHM cells treated with 50 ng/ml PDGF-BB, 5 ng/ml TGF-β1, 10 nM PMA, 15% FCS or 15% FCS + 125 μg/ml hydrocortisone (HC) were subjected to SDS/PAGE followed by immunoblotting (**a**, 10 μg of protein/well; **b**, 20 μg of protein/well). The blots were incubated with specific peptide antibodies against HAS1 or against mouse Has2 (see the Materials and methods section). The data shown are representative of two separate experiments.

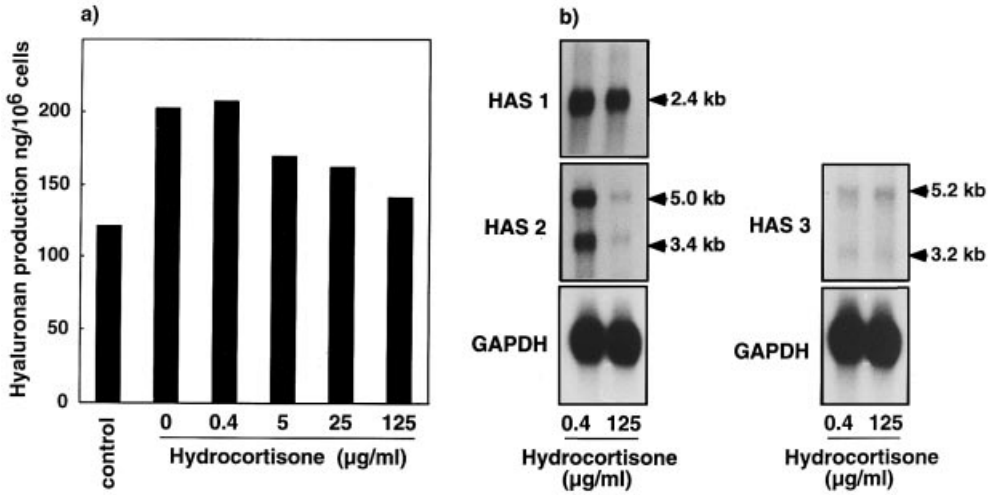

Figure 8 Effects of hydrocortisone on *HAS* gene expression

(**a**) Subconfluent cultures of NHM cells were cultured for 6 h in Ham's F-10 medium supplemented with 0.25% FCS (control) or in medium containing 15% FCS and various concentrations of hydrocortisone. The amount of hyaluronan in conditioned media was measured using a commercial kit. (**b**) mRNA was extracted from the cells, cultured in the presence of 0.4 or 125 μg/ml hydrocortisone, and equal amounts (3 μg/well) were subjected to Northern analysis. One blot was hybridized sequentially with probes for HAS1 and HAS2, and the other was hybridized with a HAS3 probe. Variation in sample loading was determined by hybridization with a GAPDH probe. The data shown are representative of two separate experiments.

foreskin fibroblasts, which do not express HAS2 mRNA (Figure 1), were subjected to immunoblotting in the absence or presence of blocking peptides. Proteins migrating at approx. 71 and 74 kDa were detected in lysates derived from mesothelial and glioma cells but not from foreskin fibroblasts. Immunoreactive bands were not detected when the antibody was preincubated with a 100-fold molar excess of TIY peptide.

Effects of PDGF-BB, TGF-β1 and PMA on hyaluronan chain length as well as on HAS1 and HAS2 protein expression

The stimulation of NHM cells with FCS, PDGF-BB or TGF-β1 led to synthesis of hyaluronan chains with high molecular mass, larger than 2×10^6 kDa. The amount of shed hyaluronan at 24 h after stimulation was increased 20-, 7- and 4-fold, respectively, compared with unstimulated cells cultured in 0.25% FCS. PMA led to 10-fold increase in the production of hyaluronan; however, interestingly the hyaluronan produced was of polydisparse size (Figure 6). The radioactivity eluted was sensitive to *Streptomyces* hyaluronidase treatment, indicating that it represented hyaluronan, except for the radioactive peaks eluted close to the total column volume (V_t), which thus probably represented other glycosaminoglycans [27]. Thus the amounts and sizes of the shedding hyaluronan chains differ in response to various external stimuli.

The effects of PDGF-BB, TGF-β1 and PMA on HAS proteins were also investigated by immunoblotting using affinity purified polyclonal antibodies, which were raised against synthetic pep-

tides corresponding to the deduced primary sequences of human HAS1 [10,28] and mouse Has2 [12]. Antibodies against HAS1 and Has2 detected proteins of approx. 59 kDa as well as of 71 and 74 kDa, respectively, in membrane preparations from NHM cells cultured for 24 h in medium containing 15% FCS (Figure 7). Treatment of the cells grown in the presence of 0.25% FCS with PDGF-BB, TGF-β1 or PMA led to a slight increase of HAS1 protein, whereas treatment with 15% FCS led to a marked increase. Treatment of NHM cells with FCS, PDGF-BB and PMA increased the 71 kDa HAS2 protein, whereas the effect of TGF-β1 treatment was minimal (Figure 7), consistent with the effect observed on mRNA expression levels for each HAS isoform (Figure 4). Overall, these results suggest that HAS1 and HAS2 protein levels are differently affected by various stimuli, and that increased HAS protein levels are a reflection of increased HAS transcript levels.

Hydrocortisone suppresses *HAS2* gene expression

Previous studies, by ourselves and others, have revealed that high concentrations of hydrocortisone (125 μg/ml) suppress hyaluronan synthesis by mesothelial cells and fibroblasts [29,30]. We investigated whether a decrease in expression of one or more of the three HAS isoforms could account for the decrease of hyaluronan synthesis by these cell types. When cells were cultured in complete medium containing physiological concentrations of hydrocortisone (0.4 μg/ml), hyaluronan levels were approx. 2-fold over those of quiescent cells. However, culture with an increased concentration of hydrocortisone (125 μg/ml) led to a decrease of hyaluronan synthesis to levels similar to those of quiescent cells (Figure 8a). As shown in Figure 8(b), the HAS2 mRNA expression was decreased by approx. 80%, as quantified by PhosphoImager, when NHM cells were cultured in medium supplemented with 15% FCS and high hydrocortisone concentrations. Under the same conditions, the expression of the *HAS1* gene was slightly decreased whereas HAS3 mRNA levels were not affected. The inhibitory effects of hydrocortisone on hyaluronan production in NHM cells were also detected at the level of HAS protein expression; an attenuation of HAS1 and HAS2 proteins was seen after 24 h of treatment (Figure 7). Thus it is likely that hydrocortisone at high pharmacological concentrations regulates mesothelial hyaluronan metabolism primarily through down-regulation of *HAS2* gene expression.

DISCUSSION

In this study we have shown that the expression of mRNAs for HAS1, HAS2 and HAS3 is dependent on cell type and is modulated by cell density and by various growth factors. The length of hyaluronan chains synthesized by NHM cells in response to external stimuli varied (Figure 6); FCS, TGF-β1 and PDGF-BB led to synthesis of hyaluronan chains of high molecular mass, whereas PMA induced synthesis of polydisparse hyaluronan. Polydisparse hyaluronan is found during the inflammatory processes such as lung fibrosis and rheumatoid arthritis. Furthermore, hyaluronan of low molecular mass is characteristic of certain invasive forms of cancer. It would be of interest to investigate whether cell types other than NHM cells respond in a similar way to these external stimuli since it is known that hyaluronan of different sizes have different effects on cells [31]. The expression of HAS transcripts was well correlated to the production of hyaluronan in the cell cultures tested, except for the mesothelioma cell line tested, Mero-14, which did not synthesize hyaluronan despite expressing HAS3 transcripts (Figures 1 and 2). In patients with malignant mesotheliomas an accumulation of hyaluronan is seen in pleural fluids and ascites of the patients [32]. Previous studies *in vitro* in our laboratory revealed that mesothelioma cells do not produce hyaluronan by themselves; rather the cells release factors that stimulate hyaluronan synthesis by mesothelial cells and fibroblasts [24,30]. It is likely that transformation of normal mesothelial cells to their transformed counterparts, mesothelioma cells, is acompanied by a down-regulation of *HAS* gene expression. It will be of great importance to elucidate the regulatory mechanisms underlying the decrease of hyaluronan synthesis in mesothelioma cells, and to investigate the possibility that loss of ability to synthesize hyaluronan is important for malignant transformation of mesothelial cells.

Among the three HAS isoforms, HAS2 transcript levels were up-regulated to the greatest extent in response to FCS, PDGF-BB and PMA in NHM cell cultures. HAS1 transcript levels were also markedly induced in response to FCS, but only to a lower extent after PDGF-BB and PMA treatment (Figures 3 and 4). The mRNA levels correlated to the levels of HAS proteins and to production of hyaluronan. Investigation of the respective promoter sequences and identification of potential enhancer sequences for the three *HAS* genes will be necessary to understand the differences in their responses to stimulation by various factors. In this respect, the promoter region of the mouse *Has1* gene has been characterized recently [33].

In cultured mesothelial cells, *HAS2* gene expresssion was slightly suppressed in response to TGF-β1 and strongly suppressed in response to hydrocortisone. We have shown previously that TGF-β1 induces an increase in hyaluronan synthesis with slower kinetics than PDGF-BB [19]. It is possible that TGF-β1 initially induces *HAS1* gene expression and suppresses HAS2 expression, and also induces other factors that ultimately stimulate hyaluronan synthesis in mesothelial cells. These findings suggest that TGF-β1 regulate HAS1 and HAS2 differently. The same conclusion was also reached by Sugiyama and his colleagues [34], who studied the expression of *HAS* genes in human skin fibroblasts. However, the authors observed an up-regulation of both *HAS1* and *HAS2* genes in response to TGF-β1, which differs from our observations. One possible explanation could be that different cell types were studied; it is likely that the regulation of *HAS* genes is cell-type-specific. Our observation that hydrocortisone inhibits the expression of the *HAS2* gene in NHM cells (Figure 8b) may have physiological implications. Intra-articular administration of hydrocortisone has been reported to result in decreased hyaluronan content in the joints and to promote the reduction of synovial effusions [35]. The decreased hyaluronan content in joints might not be exclusively dependent on the reduction of *HAS2* gene only, since treatment of NHLFs with hydrocortisone resulted in a considerable decrease in the expression of both *HAS2* and *HAS3* genes (results not shown).

Finally, we observed that treatment of NHM cultures with PDGF-BB and the RNA-synthesis inhibitor actinomycin D for 6 h inhibited entirely the induction of mRNAs for HAS1 and HAS2, but did not affect the relative levels of HAS1 and HAS2 proteins during that time period (results not shown). Thus it is likely that HAS1 and HAS2 proteins exhibit low turnover. Further studies are needed to understand the regulation of *HAS* gene expression and the post-transcriptional and post-translational regulation of their gene products in response to various stimuli. In turn, this will permit a greater understanding of factors influencing the composition and function of the extracellular matrix.

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