Growth factor regulation of hyaluronan synthesis and degradation in human dermal fibroblasts: importance of hyaluronan for the mitogenic response of PDGF-BB

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The glycosaminoglycan hyaluronan is important in many tissue-repair processes. We have investigated the synthesis of hyaluronan in a panel of cell lines of fibroblastic and epithelial origin in response to PDGF (platelet-derived growth factor)-BB and other growth factors. Human dermal fibroblasts exhibited the highest hyaluronan-synthesizing activity in response to PDGF-BB. Analysis of *HAS* (hyaluronan synthase) and *HYAL* (hyaluronidase) mRNA expression showed that PDGF-BB treatment induced a 3-fold increase in the already high level of *HAS2* mRNA, and increases in *HAS1* and *HYAL1* mRNA, whereas the levels of *HAS*3 and *HYAL*2 mRNA were not affected. Furthermore, PDGF-BB also increased the amount and activity of HAS2 protein, but not of HYAL1 and HYAL2 proteins. Using inhibitors for MEK1/2 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase 1/2] (U0126) and for PI3K (phosphoinositide 3-kinase) (LY294002), as well as the SN50 inhibitor, which prevents translocation of the active NF-κB (nuclear factor κB) to the nucleus, we observed a complete inhibition of both *HAS2* transcriptional activity and hyaluronan synthesis, whereas inhibitors of other signalling pathways were without any significant effect. TGF-β1 (transforming growth factor-β1) did not increase the activity of hyaluronan synthesis in dermal fibroblasts, but increased the activity of HYALs. Importantly, inhibition of hyaluronan binding to its receptor CD44 by the monoclonal antibody Hermes-1, inhibited PDGF-BB-stimulated [^{3}H]thymidine incorporation of dermal fibroblasts. We conclude that the ERK MAPK and PI3K signalling pathways are necessary for the regulation of hyaluronan synthesis by PDGF-BB, and that prevention of its binding to CD44 inhibits PDGF-BB-induced cell growth.

Key words: dermal fibroblast, growth factor, hyaluronan synthase (HAS), hyaluronidase, platelet-derived growth factor (PDGF), signal transduction.

INTRODUCTION

Hyaluronan is a glycosaminoglycan consisting of repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine. It is found in particular in soft connective tissues and is the major component in synovial joint fluid, vitreous body and skin, both in dermis and epidermis, contributing both to its structural and functional integrity [1,2]. Hyaluronan is synthesized by three hyaluronan-synthesizing enzymes [HAS (hyaluronan synthase) 1, 2 and 3] located at the plasma membrane, and the newly synthesized molecule is extruded into the extracellular space while the synthesis is in progress [3–5]. Among the three HAS isoforms, the HAS2 isoform is required for embryonic development, since mice deficient in the *HAS2* gene die at E (embryonic day) 9.5; in contrast, mice deficient in *HAS1* and *HAS3* genes are viable [6].

Hyaluronan accumulation is a prominent feature of rapidly remodelling tissues. For example, during embryonic development, hyaluronan production facilitates the migration of differentiating cells and stimulates their transformation to a mesenchymal phenotype [6]. Furthermore, hyaluronan is involved in the skin wound-healing process from the early inflammatory process to re-epithelization and remodelling [7], and is involved in scarless fetal healing probably by reducing collagen deposition [7,8]. In addition, hyaluronan overproduction promotes tumorigenesis in several tumour types, whereas suppression of hyaluronan production decreases tumorigenicity [9,10]. Accumulation of hyaluronan synthesis can arise from both increased synthesis and decreased degradation. Hyaluronan is degraded by the action of HYALs (hyaluronidases), including HYAL1, HYAL2 and PH-20 that have demonstrated *bona fide* HYAL activity [11]. Hyaluronan degradation occurs locally in several tissues, for example in dermis where HYAL activities have been demonstrated [12,13]; however, the mechanisms of hyaluronan metabolism in the connective tissues under physiological and inflammatory conditions are still largely unknown.

Hyaluronan interacts with several cell-surface receptors, such as CD44, RHAMM (receptor for hyaluronic acid-mediated motility) and LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1), and extracellular components, such as versican and aggrecan [14,15]; through its complex interactions, hyaluronan affects a variety of physiological cellular events, such as cell proliferation, differentiation and migration. Because of its hygroscopic and viscoelastic properties, hyaluronan also affects cellular behaviour via remodelling of the macro- and micro-environments around cells [16–19].

Abbreviations used: BCA, bicinchoninic acid; bFGF, basic fibroblast growth factor; CREB, cAMP-response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HABP, hyaluronan-binding protein; b-HABP, biotinylated HABP; HAS, hyaluronan synthase; HEK-293, human embryonic kidney; HRP, horseradish peroxidase; HYAL, hyaluronidase; IκB, inhibitor of nuclear factor κB; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MEK, MAPK/ERK kinase; NF-κB, nuclear factor κB; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; STAT, signal transducer and activatory of transcription; TBS-T, Tris-buffered saline with Tween 20; TGF-β1, transforming growth factor-β1.

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Growth factors play central roles during both normal and pathological conditions, including embryogenesis, wound healing and tumour invasion, i.e. biological processes characterized by increased amounts of hyaluronan. PDGF (platelet-derived growth factor)-BB and TGF (transforming growth factor)-β1, which are produced by both normal and transformed cells, are powerful stimulators of hyaluronan production by certain mesenchymal cells [16,20–22]. PDGF-BB mediates its cellular effects through activation of α- and β-tyrosine kinase receptors. Ligand binding causes receptor dimerization, leading to autophosphorylation of specific tyrosine residues in the intracellular parts of the receptors. This creates docking sites for SH2 (Src homology 2) domain-containing signalling molecules, whereby several signalling pathways are activated [23]. PDGF receptors can act synergistically with $\alpha v\beta 3$ integrin to promote tumour cell tissue infiltration [24]. However, recent studies in our laboratory on primary cultures of human dermal fibroblasts revealed that high concentrations of exogenous hyaluronan negatively affect PDGF β-receptor activation and cell migration in a CD44-dependent manner [25]. In normal fibroblast cultures, the intracellular signalling pathways that regulate the turnover of hyaluronan in response to external stimuli have not yet been clarified. Here, we demonstrate that the effect of PDGF-BB on hyaluronan synthesis in human dermal fibroblast cultures is due to induction of HAS2. In contrast, TGF-β1 has no effect on hyaluronan synthesis, but enhances hyaluronan degradation by stimulation of HYAL activity. Moreover, we show that the Ras-ERK (extracellular-signal-regulated kinase) MAPK (mitogen-activated protein kinase) and PI3K (phosphoinositide 3-kinase) pathways are important for PDGF-BB-stimulated hyaluronan production in these cells, and that hyaluronan binding to its receptor CD44 is important for PDGF-BB-induced cell growth.

MATERIALS AND METHODS

Cell cultures

Biopsies were taken, after approval, from patients undergoing breast reduction surgery at the Department of Plastic Surgery of the University Hospital, Uppsala, Sweden, essentially as described previously [25]. Briefly, the biopsies were transported to the laboratory in chilled PBS without Ca^{2+} and Mg^{2+}, supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) (Veterinary Institute, Uppsala, Sweden), washed with 70 % ethanol, followed by washing with PBS, and minced to 1–2 mm^3 pieces with a razor blade. After an overnight incubation in 25 mg of dispase/10 ml of DMEM (Dulbecco's modified Eagle's medium) (grade II, 0.5 unit/ml) (Boehringer Mannheim) at 4 °C, dermis and epidermis were separated, using fine forceps. Fibroblasts from the dermal sheet explants were then cultured in complete medium [DMEM containing 10 % FBS (fetal bovine serum) and antibiotics], and used between passages 6 and 10 for all experiments. Normal human epidermal keratinocytes were released from the epidermal sheets by incubation with 4 mg/ml trypsin and 4 mg/ml glucose in 0.02 % EDTA at 37 °C for 1 h. After gentle pipetting, 10 % FBS was added to stop the enzymatic activity, whereafter keratinocytes were washed and collected by centrifugation. Keratinocytes (60–70 % confluence, passage 3) were grown in EpiLife™ keratinocyte medium (Sigma, #E0151), containing bovine pituitary extract-free keratinocyte medium supplement and 10 % FBS.

DMEM supplemented with 10 % FBS was also used in the culture of HEK-293 (human embryonic kidney) cells, Cos-1 cells, NIH-3T3 cells, human foreskin fibroblasts (AG1518, AG1523; Human Mutant Cell Repository, Camden, NJ, U.S.A.), MEFs (mouse embryonic fibroblasts), the mouse epithelial cell line NMuMG (kindly provided by Anita Moren, Ludwig Institute for Cancer Research, Uppsala, Sweden; medium also contained 10 μg/ml insulin), and fibroblasts derived from keloid-like dermal scars (generously provided by Bengt Gerdin, Akademiska Hospital, Uppsala, Sweden).

Polyclonal antibodies against human HAS2 and HYAL2, and peptide synthesis

To raise polyclonal antibodies against HAS2 and HYAL2, we chose the peptide CGRRKKGQQYDMVLD that corresponds to amino acid residues 537–549 at the C-terminal domain of HAS2 and is conserved in both human and mouse HAS2 [26,27], and the peptide PGHAPGEPQLRPVG that corresponds to amino acid residues 398–411 at the C-terminal domain of human HYAL2 [28]. The peptides were synthesized using the Fmoc (fluoren-9-ylmethoxycarbonyl) strategy which resulted in a C-terminal amidated domain to facilitate subsequent coupling to KLH (keyhole-limpet haemocyanin) for rabbit immunization. Furthermore, a cysteine residue was added to PGH peptide at the C-terminal domain, in order to facilitate a subsequent coupling to a SulfoLink resin (Pierce), and used to affinity-purify the antibodies. The peptides were purified by reverse-phase HPLC, and rabbits were immunized (service by Eurogenetec). The antisera were used directly or after purification on a HiTrap Protein G HP column (Amersham Biosciences), according to the instructions of the manufacturer.

Determination of hyaluronan synthesis

Cells (3×10^4 cells/well in 12-well plates, or as indicated) were incubated in complete medium for 24 h, whereafter the medium was replaced with DMEM containing 0.1 % FBS (starvation medium). Then, the quiescent cells received fresh starvation medium and were stimulated for an additional 24 h with PDGF-BB, other growth factors or growth factor combinations, or 10 % FBS. In parallel, non-stimulated and PDGF-BB-stimulated human dermal fibroblasts were incubated with the following inhibitors of downstream signalling molecules: the MEK1/2 (MAPK/ERK kinase 1/2) inhibitor U0126 (10 μM), PI3K inhibitor LY294002 (20 μM), Src inhibitor SU6656 (1.5 μM), p38 inhibitor SB203580 (10 μM) and NF-κB (nuclear factor κB) SN50 inhibitor peptide (16 μM), as well as the NF-κB SN50M inactive control peptide (16 μM) (all Calbiochem).

The hyaluronan content in 24 h-conditioned media was quantified, essentially as described previously [9]. This assay is based on the highly specific and irreversible capture of hyaluronan molecules in the samples with immobilized HABP (hyaluronan-binding protein) domain of aggrecan, from bovine cartilage [29]; proteins that bind hyaluronan with such high affinity are not present in the fibroblast cultures [30]. The HABP–hyaluronan complexes were then detected using b-HABP (biotinylated HABP) and streptavidin-biotinylated HRP (horseradish peroxidase) followed by a chromogenic reaction. The assay was carried out on MaxiSorb 96-well Nunc-Immuno Plates precoated overnight with 1 μg/ml HABP in 50 mM carbonate buffer, pH 9.5. After three washes in PBS containing 0.5 % (v/v) Tween 20, and blocking in PBS supplemented with 1 % BSA, the hyaluronan standards (0–100 ng/ml; molecular mass $0.5–1.5 \times 10^6$ Da) (Genzyme Hylumed) and samples, at appropriate dilutions in blocking solution, were added, and the plates were incubated for 1 h at 37 °C. Following washing, 100 μl of b-HABP (1 μg/ml) was added, and samples were incubated for 1 h. Then, after removing excess b-HABP, the b-HABP bound to the hyaluronan trapped by the HABP-coated

plates was detected by incubation for 1 h with streptavidin-biotinylated HRP (Amersham Biosciences), followed by the addition of 100 ml of TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution (Sigma) for 15 min. Then, 50 μl of 2 M H_2SO_4 was added, and the absorbance at 450 nm was measured. The hyaluronan content in the conditioned media was calculated by comparing with a standard curve made from known concentrations of hyaluronan. It is unlikely that HABPs secreted into the conditioned medium affected the result, since treatment of the conditioned medium with proteases before the assay had no effect (B. Bernert and P. Heldin, unpublished work).

Detection of HYAL activity in human dermal fibroblast cultures

The activity of HYALs in fibroblast conditioned media and cell-layers harvested after 6 h from unstimulated and PDGF-BB- or TGF-β1-stimulated cells was estimated quantitatively using a microtitre-based assay essentially as described by Frost and Stern [31]. In this assay, biotinylated hyaluronan (substrate) is covalently coupled to Covalink-NH 96-well microtitre plates. After removal of the coupling solution and three washings with PBS supplemented with 2 M NaCl and 50 mM $MgSO_4$, the plates were equilibrated with assay buffer (0.1 M formate, pH 3.7, 0.15 NaCl and 1 % Triton X-100).

Conditioned medium from each sample supplemented with 1 μg/ml aprotinin was cleared by centrifugation at 1000 ***g*** for 5 min, and dialysed against 0.01 M formate, pH 3.7, and 0.015 M NaCl containing aprotinin overnight at 4 °C. Cell layers extracted for 30 min with 1 ml of ice-cold 0.01 M formate, pH 3.7, 0.015 M NaCl and 0.1 % Triton X-100 containing DNase I (0.5 mg/ml), aprotinin (1 μg/ml) and Pefabloc (0.5 mg/ml) were sheared through a 20 gauge needle and centrifuged at 10 000 ***g*** for 15 min. Then, the samples were concentrated 10-fold and the protein content was measured using a BCA (bicinchoninic acid) kit (Pierce). Samples of conditioned media and of cell layers as well as *Streptomyces* hyaluronidase standards ($1.0–1 \times 10^{-6}$ units/ml) (Sigma–Aldrich) were diluted with the assay buffer at appropriate dilutions, and added in the Covalink plates with immobilized biotinylated hyaluronan. After 1 h of incubation, under shaking at 37 °C, the reaction was stopped with 6 M guanidinium chloride followed by three washes with PBS supplemented with 2 M NaCl, 50 mM $MgSO_4$ and 0.05 % (v/v) Tween 20. Then, the residual coupled biotinylated hyaluronan was detected in a streptavidin-biotinylated HRP reaction and the complexes were visualized as described above.

Immunoblotting

Human dermal fibroblasts (4×10^5 cells in a 10-cm-diameter dish) were cultured in DMEM containing 10 % FBS for 24 h. After incubation in starvation medium for 24 h, quiescent cells or cells stimulated with various concentrations of PDGF-BB (5–50 ng/ml) or TGF-β (2.5 and 5 ng/ml), were incubated in the absence or presence of 10 μM U0126 or 20 μM LY294002. At different time intervals after treatment, the medium was removed and cell layers were washed twice with ice-cold PBS followed by lysis in 50 μl of ice-cold RIPA buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 % Nonidet P40, 0.1 % SDS, 1 % deoxycholate and 1 mM dithiothreitol) containing phosphatase and protease inhibitors (50 mM NaF, 1 mM Na_3VO_4, 0.5 mg/ml Pefabloc, 10 μM leupeptin, 2 mM pepstatin and 1 μg/ml aprotinin) and mixing at 4 °C for 30 min. Cell lysates were scraped into microfuge tubes on ice, sheared by a 20 gauge needle, incubated for an additional 30 min on ice and centrifuged at 14 000 ***g*** for 15 min at 4 °C, in order to remove insoluble debris. The protein content in the supernatant was measured using a BCA kit. Then, samples (40 μg of protein/well) were mixed with 1 vol. of reducing sample buffer, denatured at 95 °C for 3 min, cooled down for 15 min before addition of 20 mM iodoacetamide and separation on SDS/PAGE using 10 % polyacrylamide gels, followed by transfer on to nitrocellulose membranes (Amersham Biosciences). Non-specific binding sites on the membrane were blocked with 5 % (w/v) defatted dried milk powder in TBS-T [Tris-buffered saline (50 mM Tris/HCl, pH 7.4, and 200 mM NaCl) supplemented with 0.1 % (v/v) Tween 20], at room temperature (20 °C) for 1 h, followed by two washes in TBS-T. Then, the membranes were incubated with anti-[phospho-p44/42 ERK MAPK (Thr^{202}/Tyr^{204})] rabbit monoclonal antibodies (p-ERK1/2; dilution 1:1000) (Cell Signaling Technology), phospho-Akt (Ser^{473}) rabbit antiserum (p-Akt; dilution 1:1000) (Cell Signaling Technology), rat monoclonal antibody 17E9 against HYAL1 (2 μg/ml) (kindly provided by Dr G. Frost, Sidney Kimmel Cancer Center, San Diego, CA, U.S.A.), polyclonal HYAL2 antiserum (1:300 dilution), or rabbit anti-HAS2 IgG (5 μg/ml; HiTrap protein G HP-purified HAS2 antiserum) in TBS-T containing 1 % BSA overnight at 4 °C. The endogenous levels of ERK1/2 and Akt were detected by incubating the membranes with rabbit polyclonal anti-ERK1/2 antiserum (1:250 dilution) (kindly provided by Charlotte Rorsman, Ludwig Institute for Cancer Research, Uppsala, Sweden) and Akt antiserum (1:1000 dilution) (Cell Signaling Technology) respectively. After five washes with TBS-T, the membranes were incubated either with HRP-conjugated anti-(rabbit IgG) (diluted 1:3000 in 5 % defatted dried milk powder in TBS-T) or with HRP-conjugated anti-rat IgG (diluted 1:1000 in 5 % defatted dried milk powder in TBS-T). Immunoreactive bands were detected by enhanced chemiluminescence, stripped with stripping buffer (Pierce,) and re-blotted with antibodies, as specified.

Real-time PCR

Total RNAs were obtained from human dermal fibroblast cultures (4×10^5 cells/10-cm-diameter dish) incubated in starvation medium alone or in medium supplemented with PDGF-BB (50 ng/ml), U0126 (10 μM) or LY294002 (20 μM), or combinations thereof, as well as 10 % FBS. After 1, 4 and 24 h of treatment, the cells were washed twice with PBS, and the total RNAs were extracted by using RNAqueous®-4PCR kit (Ambion). Each of the total RNAs were treated further with DNase I (Ambion), followed by DNase inactivation, in order to obtain DNA-free total RNA. The purity of the RNAs was verified by measurement of A_{260}/A_{280} values (ratio $\geqslant 2.0$), and their integrity was determined by separation in a 1 % agarose formaldehyde gel, followed by inspection under UV light. Then, each purified RNA (2 μg of RNA in 20 μl of total reaction volume) was reverse-transcribed into the corresponding cDNA by SuperScript™ II RNase H^- Reverse Transcriptase (Invitrogen), followed by real-time PCR using qPCR™ core kit for SYBR® Green I (Eurogenetec), according to the instructions of the manufacturer. The primers for the target genes (*HAS1*, *HAS2*, *HAS3*, *HYAL1* and *HYAL2*) (300 nM) and reference gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (100 nM) were designed by Primer Express® Software v 2.0 (Applied Biosystems) and synthesized by Applied Biosystems (Table 1). Each reaction mixture, in addition to the specific primer, contained 1 μl of cDNA from reverse-transcription reactions, 3.5 mM $MgCl_2$, 200 μM dNTPs, SYBR® Green I (diluted 1/66 000) and Hot Goldstar enzyme (0.025 units/μl); it was amplified for 40 cycles at an annealing temperature of 60 °C using an ABI PRISM 7000 Sequence Detection System (SDS Software; Applied Biosystems). For each

Table 1 Primer sequences and real-time PCR parameters used for the quantification of gene expression in human dermal fibroblasts

T_m is melting temperature, the maximum value for the dissociation curve, which is a useful tool to verify a specific amplicon product.

Target gene	Primer sequences	Amplicon (bp)	T_m (°C)	GenBank® accession number
HAS1	Forward: 5′-GGAATAACCTCTTGCAGCAGTTTC-3′ Reverse: 5′-GCCGGTCATCCCCAAAAG-3′	84	81	U59269
HAS2	Forward: 5′-TCGCAACACGTAACGCAAT-3′ Reverse: 5′-ACTTCTCTTTTTCCACCCCATTT-3′	77	77	U54804
HAS3	Forward: 5′-AACAAGTACGACTCATGGATTTCCT-3′ Reverse: 5′-GCCCGCTCCACGTTGA-3′	71	83	AF232772
HYAL1	Forward: 5′-GATGTCAGTGTCTTCGATGTGGTA-3′ Reverse: 5′-GGGAGCTATAGAAAATTGTCATGTCA-3′	79	82	U03056
HYAL2	Forward: 5′-CTAATGAGGGTTTTGTGAACCAGAATAT-3′ Reverse: 5′-GCAGAATCGAAGCGTGGATAC-3′	79	79	AJ000099
GAPDH	Forward: 5′-CCCATGTTCGTCATGGGTGT-3′ Reverse: 5′-TGGTCATGAGTCCTTCCACGATA-3′	145	84	NM_002046

optimized assay, a relative standard curve was run which resulted in a linear plot of threshold values (C_t, which reflects the cycle number at which statistically significant amplicon numbers have been accumulated above the baseline) against log input total RNA (0–100 ng); the slope values of each constructed standard curve were approximately equal (within 10 % of the theoretical value of the slope which should not be lower than -3.3 [32]), indicating a similar amplification efficiency of each target. A dissociation curve analysis was performed to verify the presence of correct product at predicted melting temperatures and absence of primer dimers. Using Excel, the relative expression level of each target normalized to the endogenous reference gene (*GAPDH*) and relative to a calibrator (sample exhibiting the lowest expression level, 1× sample) was measured by using the comparative C_t method (Applied Biosystems, User Bulletin #2).

Proliferation assay

Human dermal fibroblasts (1×10^4 cells/well in 24-well plates) were cultured for 24 h in complete medium followed by 48 h in starvation medium. At this time, fresh starvation medium was added in the absence or presence of PDGF-BB (3–10 ng/ml), hyaluronan (5 or 25 μg/ml) and monoclonal antibodies against the hyaluronan receptor CD44 (100 μl of Hermes-1 serum-free myeloma culture medium/ml which specifically inhibits the binding of hyaluronan to the CD44 or 50 μg of Hermes-3 IgG/ml, ammonium sulfate precipitate; generously provided by Professor Sirpa Jalkanen, MediCity Research Laboratory, Turku, Finland), or combinations thereof, and 16 h later the cells were treated with a 6 h pulse of 0.2 μCi of [^{3}H]thymidine/well. Media were then aspirated, and cells were washed with PBS and incubated with 5 % trichloroacetic acid for 20 min at 4 °C. Cell layers were washed twice with water followed by lysis in 400 μl of 1 M NaOH for 30 min at room temperature and neutralization with 500 μl of 1 M HCl. The precipitated DNA was then processed for scintillation counting.

RESULTS

PDGF-BB is the predominant stimulator of hyaluronan production in human dermal fibroblast cultures

Our previous studies revealed that PDGF-BB is a potent stimulator of hyaluronan synthesis in cells of mesenchymal origin, such as fibroblasts and mesothelial cells [20,33]. However, in order to investigate whether there are differences in the hyaluronan-synthesizing capacity of various fibroblast-derived cell lines, including primary fibroblast cultures derived from normal human dermis and from keloid-like dermal scars [34], as well as in a panel of epithelial cells, the PDGF-BB-mediated hyaluronan production by various connective tissue cell types was studied. The analysis revealed that untreated and PDGF-BB-treated NIH-3T3 mouse embryo fibroblasts and human adult lung fibroblasts synthesized about 650 ng and 750 ng of hyaluronan per 1×10^5 cells and 24 h respectively, independently of PDGF-BB stimulation. Notably, MEFs, HEK-293 cells and Cos-1 cells synthesized only minute amounts of hyaluronan (Figure 1). The hyaluronan content in 24 h-conditioned media from subconfluent cultures of human foreskin fibroblasts AG1518 and AG1523, as well as fibroblasts derived from normal dermis or dermal keloid scars, were increased 2–4-fold when cells were stimulated with PDGF-BB (Figure 1). Among the cells with an epithelial phenotype, primary human epidermal keratinocytes synthesized hyaluronan in a PDGF-BB-independent manner, consistent with the lack of PDGF receptors on these cells, whereas the synthesis of hyaluronan by the human epithelial cell line HaCat was stimulated by PDGF-BB. Notably, the mammary NMuMG cells did not synthesize any significant amounts of hyaluronan (Figure 1).

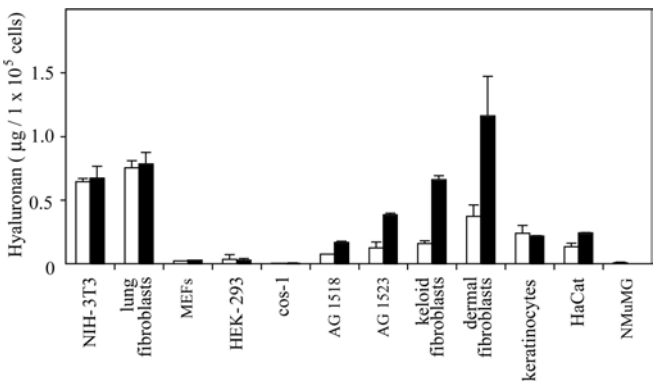

Figure 1 Effects of PDGF-BB on hyaluronan synthesis by different types of cells

Hyaluronan content in 24 h-conditioned media was determined in cells (3×10^4 cells/well in 12-well plates) stimulated or not by PDGF-BB (50 ng/ml), as described in the Materials and methods section. Results are representative of three separate experiments and are means ± variation of duplicate determinations. Open bars, control cells; closed bars, PDGF-BB-stimulated cells.

Thus the hyaluronan-synthesizing capacity of fibroblasts derived from various origins differs upon PDGF-BB stimulation; the

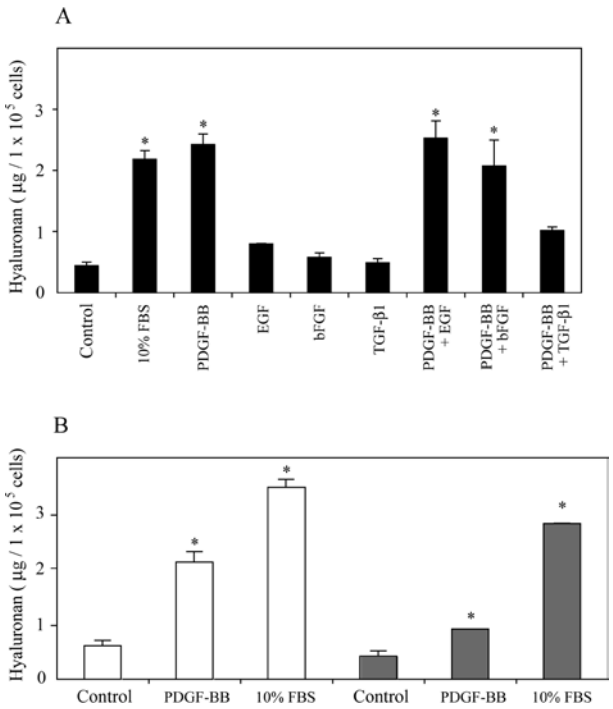

Figure 2 Stimulation of hyaluronan synthesis by growth factors in cultures of human dermal fibroblasts at different cell densites

(**A**) Quiescent human dermal fibroblasts (3×10^4 cells/well in 12-well plates) were incubated in the absence (medium containing 0.1 % FBS) or presence of 10 % FBS, PDGF-BB (50 ng/ml), EGF (10 ng/ml), bFGF (5 ng/ml) or TGF-β1 (5 ng/ml), or combinations thereof. (**B**) Subconfluent (1×10^4 cells/well in 12-well plates) or confluent (4×10^4 cells/well in 12-well plates) dermal fibroblast cultures were incubated in starvation medium, or in medium supplemented with 10 % FBS or 50 ng/ml PDGF-BB. Open bars, subconfluent cultures; closed bars, confluent cultures. The hyaluronan amount in the cell culture media was determined after 24 h. Results are representative of two separate experiments and are means $\pm$ variation of duplicate determinations. *$P < 0.05$ compared with non-stimulated cells.

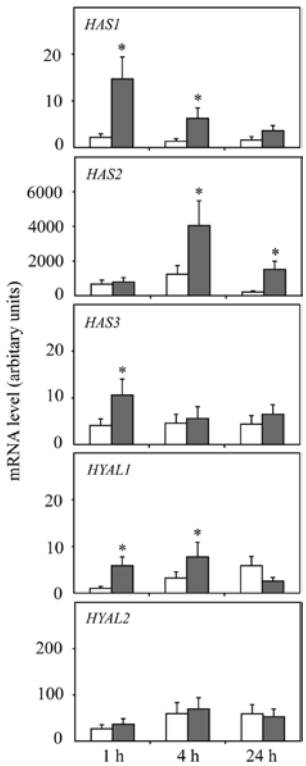

Figure 3 Time course of *HAS* and *HYAL* mRNA expression in response to PDGF-BB

Quiescent human dermal fibroblasts (4×10^5 cells/10-cm-diameter dish) were incubated in the absence (medium containing 0.1 % FBS) or presence of PDGF-BB (50 ng/ml) for 1, 4 and 24 h. The relative expression levels of *HAS* and *HYAL* mRNA were determined by real-time reverse transcription–PCR, as described in the Materials and methods section. Results are means $\pm$ S.D. of triplicate determinations, representative of two separate experiments. Open bars, non-stimulated cells; closed bars, PDGF-BB-stimulated cells. *$P < 0.05$ compared with non-stimulated cells.

primary cultures of human dermal fibroblasts exhibited the highest hyaluronan production in response to PDGF-BB, compared with other cells tested, and were thus chosen for further studies.

We next investigated the effects of other growth factors important for the biology of dermis on hyaluronan synthesis at concentrations found to give maximum effects (results not shown). PDGF-BB stimulated hyaluronan synthesis in human dermal fibroblasts even more efficiently than did 10 % FBS (Figure 2A). EGF (epidermal growth factor) slightly increased the hyaluronan content in the 24 h-conditioned media, whereas bFGF (basic fibroblast growth factor) and TGF-β1 had no effect. Addition of EGF together with PDGF-BB did not increase hyaluronan production appreciably over that synthesized in response to PDGF-BB alone, whereas addition of bFGF gave a slightly decreased effect. Interestingly, addition of TGF-β to PDGF-BB resulted in an approx. 72 % decrease in the amount of hyaluronan compared with stimulation with PDGF-BB alone (Figure 2A). These data support the notion that PDGF-BB has a major role in hyaluronan biosynthesis in dermis. The stimulatory effects of PDGF-BB were higher in primary subconfluent cultures compared with that of confluent cultures of human dermal fibroblasts (Figure 2B).

PDGF-BB and TGF-β1 affect differentially the enzymes involved in synthesis and degradation of hyaluronan

As a first step to elucidate further which of the three HAS isoforms accounts for the increased levels of hyaluronan in the conditioned media of dermal fibroblast cultures in response to PDGF-BB, we determined the mRNA levels of *HAS* genes in non-stimulated and PDGF-BB-stimulated cells using real-time PCR. Furthermore, to investigate whether the hyaluronan-degradative enzymes also contributed to the modulation of the levels of hyaluronan in the conditioned media, the levels of mRNAs for *HYAL1* and *HYAL2* were also determined. The analysis of non-stimulated and PDGF-BB-stimulated dermal fibroblasts, at 1, 4 and 24 h, revealed that dermal fibroblasts expressed only traces of *HAS1*, *HAS3* and *HYAL1* transcripts. PDGF-BB stimulation of the cells triggered an early and significant induction (after 1 h of stimulation) of mRNAs for *HAS1* (10-fold), *HAS3* (7-fold) and *HYAL1* (4-fold) compared with the corresponding basal transcript levels ($P < 0.05$). Longer treatments of cell cultures with PDGF-BB led to a decline in the expression levels of *HAS1* and *HAS3*, whereas the *HYAL1* transcript was high even after 4 h of stimulation, followed by a decline at 24 h. Independently of the degree of stimulation, the mRNA levels of *HAS1*, *HAS3* and *HYAL1* were very low (between 0.01 and 0.07 %) compared with that of *GAPDH*. Importantly, dermal fibroblasts exhibited a high basal expression of *HAS2* and *HYAL2* mRNA levels, approx. 900-fold and 40-fold higher compared with *HYAL1* (1 $\times$ sample) (Figure 3). The already high transcriptional activity of *HAS2* gene in non-stimulated cells, was induced approx. 3-fold after 4 h of stimulation with PDGF-BB; after longer treatment, *HAS2* gene expression still possessed transcriptional activity that was significantly higher compared

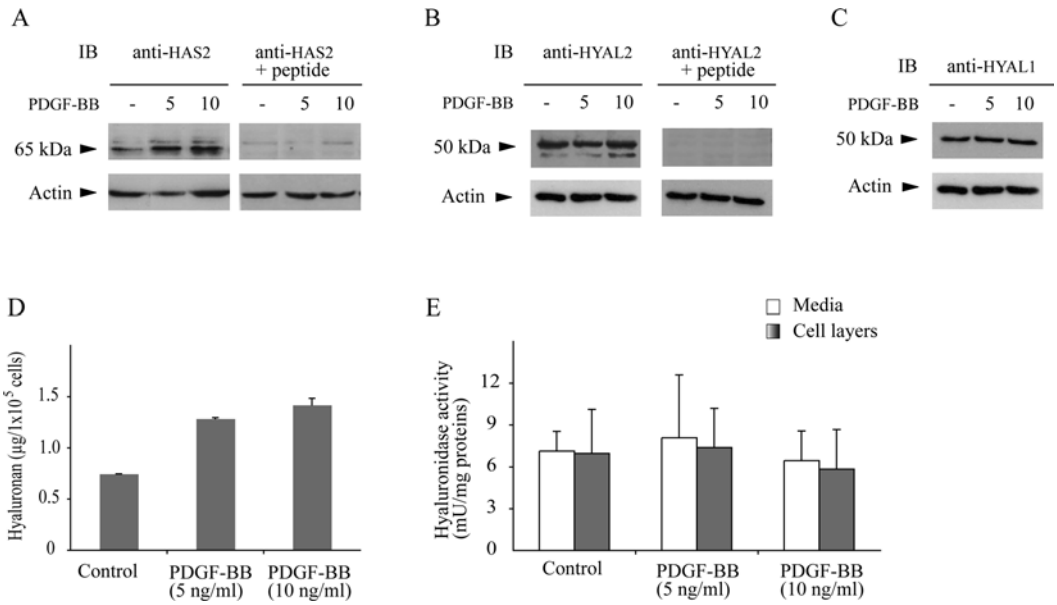

Figure 4 Effects of PDGF-BB on HAS2, HYAL1 and HYAL2 protein expression levels and activities

Quiescent dermal fibroblasts (4×10^5 cells/10-cm-diameter plate) were incubated in the absence (medium containing 0.1 % FBS) or presence of PDGF-BB (5–10 ng/ml) for 6 h, followed by lysis as described in the Materials and methods section. Cell lysates (40 μg/well) were subjected to SDS/PAGE using 10 % acrylamide gels followed by transfer on to nitrocellulose membranes. The blots were incubated with polyclonal antibodies against HAS2 protein (5 μg/ml) (**A**) or against HYAL2 protein (1:300 dilution) (**B**), in the absence or presence of a 200-fold molar excess of the corresponding blocking peptides respectively. In (**C**), the membrane was blotted with the monoclonal antibody 17E9 against HYAL1 (2 μg/ml). (**D**) Quantification of hyaluronan content in conditioned media. (**E**) HYAL activities in conditioned media (open bars) and cell layers (closed bars) respectively. Results are means $\pm$ variation for two determinations. Results are representative of two separate experiments. IB, immunoblot.

with control levels (Figure 3). Notably, the endogenous high transcriptional activity of *HYAL2* did not significantly change from 1 h to 24 h in response to PDGF-BB. Comparing the mRNA levels of the *HAS2* and *HYAL2* isoforms with those of *GAPDH* revealed that the *HAS2* and *HYAL2* transcript levels were approx. 36 % and 0.6 % in PDGF-BB-stimulated cells respectively of those of *GAPDH*. Thus it is likely that the induction of the *HAS2* gene and, to a lesser extent, the *HAS1* and *HAS3* genes, rather than the suppression of *HYAL1* and *HYAL2* genes, accounts for the increased hyaluronan levels in response to PDGF-BB in dermal fibroblast cultures.

We next investigated, using immunoblotting, the expression of HAS2, HYAL1 and HYAL2 proteins in 6 h PDGF-BB-stimulated and quiescent fibroblast cultures (Figure 4). Bands of $\sim$65 kDa (consistent with the size of HAS2) and $\sim$50 kDa (consistent with the size of HYAL2) were seen in lysates of dermal fibroblasts when blots were incubated with polyclonal anti-HAS2 or anti-HYAL2 antibodies respectively, but not when the antiserum was pre-incubated with a 200-fold excess of the respective peptides used for immunization. Furthermore, a 50 kDa component, consistent with the size of HYAL1 [35,36], was detected using the 17E9 monoclonal antibodies against HYAL1 [37]. Despite the low amount of *HYAL1* mRNA (Figure 3), an appreciable amount of HYAL1 protein was expressed. PDGF-BB-stimulated cells expressed more HAS2 protein (Figure 4A) and synthesized more hyaluronan (Figure 4D) compared with unstimulated cells, whereas neither HYAL2 nor HYAL1 protein expression changed significantly (Figures 4B and 4C). In addition, PDGF-BB-stimulated dermal fibroblasts exhibited similar activity of HYAL(s) present in cell-layer extracts and conditioned media as the non-stimulated cells (Figure 4E). Thus PDGF-BB-mediated stimulation of hyaluronan synthesis is due to up-regulation of HAS2 expression.

Because treatment of dermal fibroblasts with TGF-β suppressed the stimulatory effect of PDGF-BB on hyaluronan production (Figure 2A), we investigated the effects of TGF-β on the expression of HAS2, HYAL1 and HYAL2, as well as on HYAL activity present in cell-layer extracts and conditioned media. As shown in Figure 5, TGF-β stimulation did not have any effect on HAS2 protein expression or hyaluronan amounts in 24 h-conditioned media, neither did it affect HYAL1 and HYAL2 expression. Interestingly, however, stimulation with TGF-β increased, in a dose-dependent manner, the HYAL activity approx. 3–4-fold in conditioned media and approx. 2–3-fold in cell-layer extracts (Figure 5E). These data suggest that, whereas TGF-β does not affect the amount of HYAL1 and HYAL2, it is a powerful stimulator of HYAL activity in dermal human fibroblasts.

PDGF-BB-induced stimulation of hyaluronan synthesis requires active ERK1/2 and PI3K signalling pathways

In order to explore which signalling pathways are important for stimulation of hyaluronan biosynthesis in response to PDGF-BB, we used specific inhibitors of downstream signalling molecules at non-cytotoxic concentrations, as determined by the exclusion of Trypan Blue (results not shown). The PDGF-BB-induced 2.5-fold increase in the hyaluronan synthesis was completely prevented when the dermal fibroblasts were cultured in the presence of the MEK1/2 inhibitor U0126 that inhibits MEK, an activator of the MAPK ERK1/2, or in the presence of the PI3K inhibitor LY294002 (Figure 6A). Notably, addition of the specific Src inhibitor SU6656 and p38 inhibitor SB203580, only slightly suppressed hyaluronan production (less than 20 % inhibition), indicating that these signalling pathways do not have a major role in PDGF-BB-induced hyaluronan synthesis. Thus the powerful

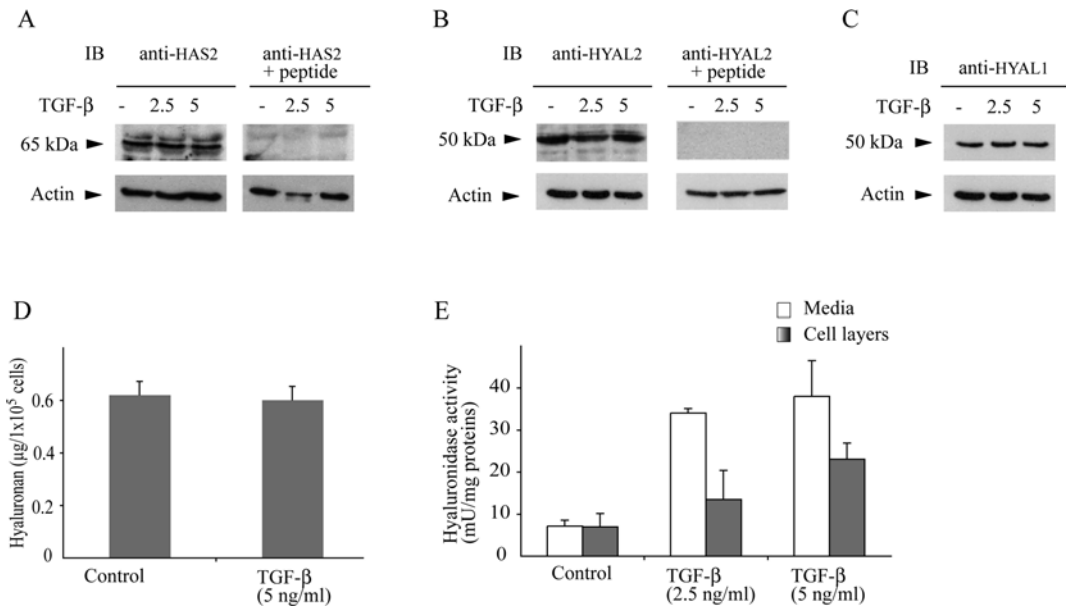

Figure 5 Effects of TGF-β1 on HAS2, HYAL1 and HYAL2 protein expression levels and activities

Lysates (40 μg/well) from quiescent or TGF-β1-stimulated cells were subjected to SDS/PAGE using 10 % acrylamide gels followed by transfer on to nitrocellulose membranes. The blots were incubated with polyclonal antibodies against HAS2 protein (5 μg/ml) (**A**) or against HYAL2 protein (1:300 dilution) (**B**), in the absence or presence of a 200-fold molar excess of the corresponding blocking peptides, or against the monoclonal antibody 17E9 against HYAL1 (2 μg/ml) (**C**). (**D**) Quantification of hyaluronan content in conditioned media. (**E**) HYAL activity in conditioned media (open bars) and cell layers (closed bars). Results are means $\pm$ variation for two determinations. Results are representative of two separate experiments. IB, immunoblot.

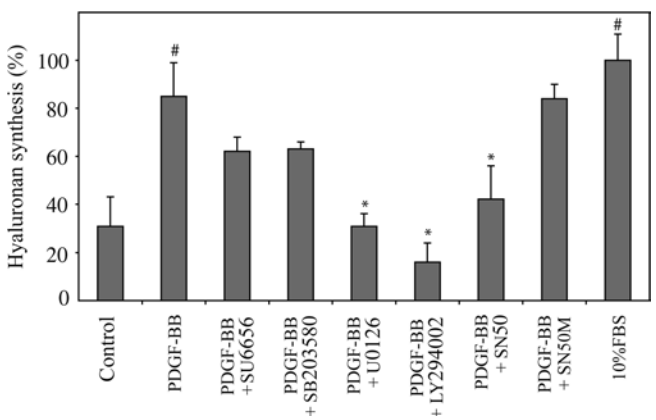

Figure 6 Effects of inhibitors on hyaluronan synthesis by dermal fibroblasts

Quiescent dermal fibroblasts (3×10^4 cells/well in 12-well plates) were cultured in the absence (medium containing 0.1 % FBS) or presence of PDGF-BB (50 ng/ml), the Src inhibitor SU6656 (2 μM), the p38 inhibitor SB203580 (10 μM), the MEK1/2 inhibitor U0126 (10 μM), the PI3K inhibitor LY294002 (20 μM), the NF-κB inhibitor SN50 (16 μM) and its control inhibitory peptide SN50M (16 μM), and 10 % FBS or combinations thereof for 24 h. Hyaluronan content in conditioned media was measured as described in the Materials and methods section. #$P < 0.01$ compared with non-stimulated control cells; *$P < 0.01$ compared with PDGF-BB-stimulated cells. Results are values relative to the stimulation given by 10 % FBS and are means $\pm$ S.D. of duplicate determinations, representative of three separate experiments.

stimulatory effect of PDGF-BB on hyaluronan biosynthesis is dependent on activation of PI3K and ERK1/2 signalling pathways.

Further studies on the more downstream signalling molecules involved in PDGF-BB-mediated hyaluronan synthesis revealed an important role of NF-κB. This was demonstrated by using the SN50 peptide inhibitor which is cell-membrane-permeant and contains the nuclear localization sequence of NF-κB and thereby inhibits the translocation of its active complex into the nucleus. PDGF-BB stimulation of hyaluronan synthesis was decreased approx. 80 % by the SN50 peptide. In contrast, the control SN50M peptide, which has mutations in the nuclear localization sequence of NF-κB and thus does not prevent translocation of the NF-κB active complex into the nucleus, did not significantly inhibit the hyaluronan produced by PDGF-BB stimulation. These observations support the notion that NF-κB has a central role in the activation of hyaluronan synthesis.

Also at the transcriptional level, PDGF-BB-induced *HAS2* gene expression was completely prevented if the fibroblasts were cultured in the presence of the MEK inhibitor U0126, or in the presence of the PI3K inhibitor LY294002; the effects of the inhibitors on PDGF-BB-induction of *HAS1*, *HAS3* and *HYAL2* were less apparent (Figure 7). Furthermore, the levels of mRNAs for *HYAL1* were also suppressed when these inhibitors were added to the cultures. These studies indicate an involvement of the ERK MAPK and PI3K signalling pathways in *HAS2* and *HYAL1* transcriptional activities. A 4 h stimulation of the cells with 10 % FBS led to increases in *HAS1*, *HAS2* and *HAS3* transcripts; the increase in *HAS2* transcriptional activity was similar to that detected after PDGF-BB stimulation. However, in addition, 10 % FBS caused powerful induction of *HYAL1* and *HYAL2* transcripts (Figure 7).

Inhibition of hyaluronan binding to CD44 inhibits PDGF-BB-mediated proliferation of human dermal fibroblasts

In order to investigate whether hyaluronan is of importance for the PDGF-BB-stimulated growth stimulation of dermal fibroblasts, we subjected the cells to a [^{3}H]thymidine incorporation assay. As shown in Figure 8(A), PDGF-BB induced [^{3}H]thymidine incorporation in a dose-dependent manner. When unstimulated or PDGF-BB-stimulated fibroblasts were cultured in the presence of 5 or 25 μg/ml hyaluronan, an increase in the PDGF-BB-mediated cell growth was observed (Figure 8B). Interestingly, this growth-promoting effect of hyaluronan on PDGF-BB-stimulated

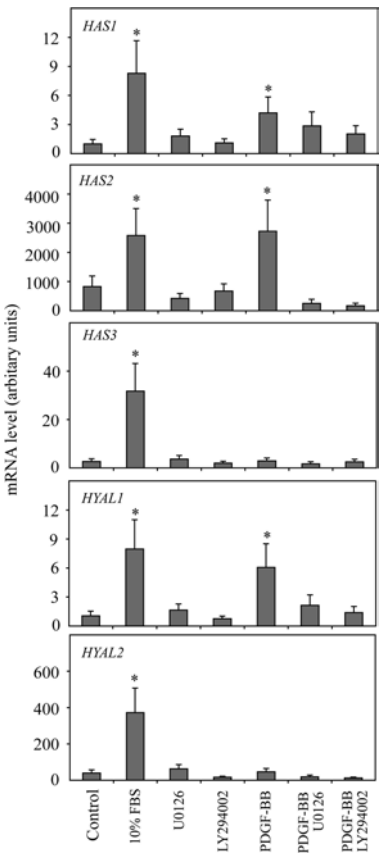

Figure 7 *HAS* and *HYAL* mRNA expression in response to PDGF-BB and inhibitors

Quiescent dermal fibroblasts (4×10^5 cells/10-cm-diameter dish) were treated with PDGF-BB (50 ng/ml), MEK1/2 inhibitor U0126 (10 μM) and PI3K inhibitor LY294002 (20 μM), alone or in combinations, for 4 h. The relative expression levels of *HAS* and *HYAL* mRNA were determined by real-time PCR, as described in the Materials and methods section. Results are means $\pm$ S.D. of triplicate determinations, representative of two separate experiments. $^*P < 0.01$ compared with control.

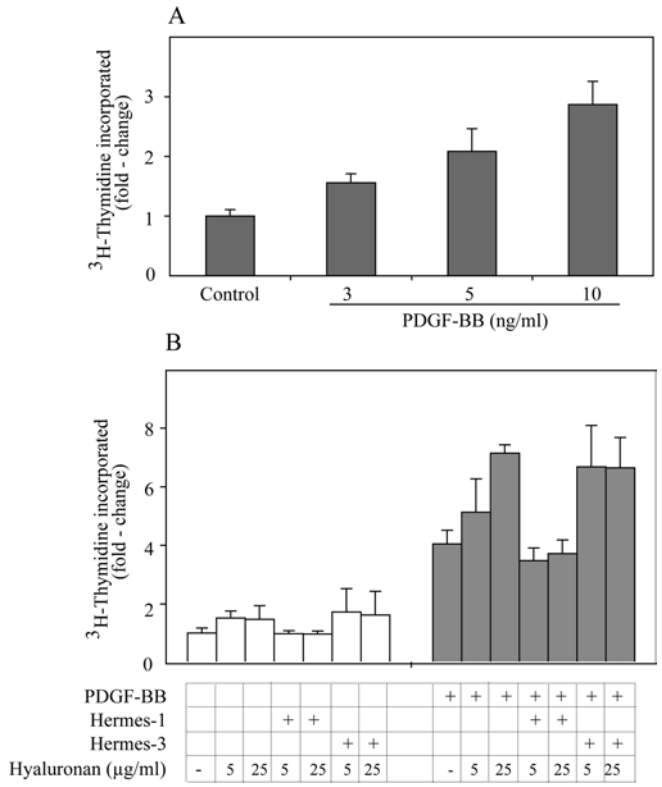

Figure 8 PDGF-BB-mediated incorporation of [^{3}H]thymidine into fibroblast cultures is promoted by hyaluronan binding to CD44

Cells (1×10^4/well in 12-well plates) after 48 h of starvation were cultured for an additional 16 h in starvation medium alone or in medium containing PDGF-BB (3–10 ng/ml), hyaluronan (5 or 25 μg/ml), Hermes-1 (100 μl/ml), Hermes-3 (50 μg/ml) or combinations thereof. Then, [^{3}H]thymidine was added and the cultures were incubated for 6 h at 37 °C, as described in the Materials and methods section. (**A**) PDGF-BB-induced [^{3}H]thymidine incorporation is dose-dependent. Results are means $\pm$ S.D. of triplicate determinations, representative of two separate experiments. (**B**) Hyaluronan addition alone promoted PDGF-BB-induced [^{3}H]thymidine incorporation, and its addition together with Hermes-1, which blocks the binding of hyaluronan to CD44, suppressed PDGF-BB growth-promoting capacity. Open bars, unstimulated cells; closed bars, PDGF-BB-stimulated fibroblasts (3 ng/ml). Results are means $\pm$ S.D. of triplicate determinations, representative of two separate experiments.

cells was inhibited in the presence of Hermes-1 antibodies that specifically block the binding of hyaluronan to CD44, but not in the presence of Hermes-3 antibodies that do not effect the binding of hyaluronan to CD44. Thus binding of hyaluronan to CD44 receptor is important for the proliferative effect of PDGF-BB in dermal fibroblasts in culture.

DISCUSSION

In the present study, we show that, among various epithelial and fibroblast cells of different origins, the adult human dermal fibroblasts synthesize the highest amounts of hyaluronan. We used primary cultures of dermal fibroblasts for a thorough analysis of the effects of PDGF-BB and TGF-β1 on the mRNA and protein levels of HAS and HYAL isoforms. PDGF-BB potently stimulated hyaluronan synthesis (Figures 1 and 2), *HAS2* gene transcriptional activity (Figures 3 and 7), and HAS2 protein induction (Figure 4A).

Importantly, we found that inhibition of hyaluronan binding to its receptor CD44 prevents PDGF-BB-induced [^{3}H]thymidine incorporation, suggesting that induction of hyaluronan is an important part of the mitogenic PDGF-BB response. Interestingly, we recently found that higher concentrations of hyaluronan (100 μg/ml) inhibits PDGF β-receptor activation in a tyrosine phosphatase-dependent mechanism [25]. Thus hyaluronan appears to affect PDGF stimulation in a biphasic manner: low concentrations of hyaluronan enhances PDGF stimulation, whereas high concentrations inhibits PDGF stimulation.

Previous studies revealed that PDGF-BB and TGF-β1 induce hyaluronan synthesis in cultures of human foreskin fibroblasts in an additive manner [38]. In contrast, we report in the present paper that, in human dermal fibroblast cultures, TGF-β1 suppresses the PDGF-BB-induced hyaluronan levels (Figure 2A). Most likely, the stimulatory effect of TGF-β1 on HYAL activity in dermal fibroblasts (Figure 5E) contributes to the lowering of the hyaluronan levels. Interestingly, the increase in HYAL activity occurred without an increase in the amount of HYAL protein. It is possible that TGF-β1 induces proteolytic activation of HYAL proteins or induces the synthesis of an activation cofactor. Furthermore, TGF-β1 stimulation of normal human mesothelial cell cultures led to suppression of *HAS2* gene expression, but induction of *HAS1* transcript [33]. Thus the differential effects of TGF-β1 on hyaluronan production and degradation is cell-type-specific and involves regulation of both hyaluronan-synthesizing and -degrading enzymes. These differences may have functional implications since hyaluronan fragments have different effects

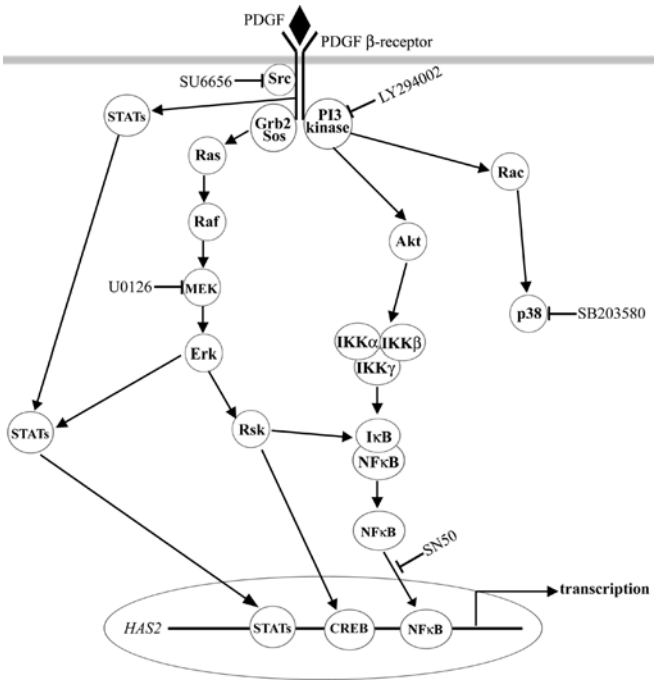

Figure 9 Signalling pathways downstream of the PDGF β-receptor that are involved in *HAS2* gene regulation

on cells compared with high-molecular-mass hyaluronan, and, for example, stimulates angiogenesis in a CD44- and chemokine CXCL1-dependent manner [39].

Following activation of the PDGF β-receptor by PDGF-BB, several signalling pathways are activated. Two of the best defined pathways, supporting cell survival and proliferation, are the ERK MAPK and PI3K pathways (Figure 9). Activation of ERK MAPK leads to activation of the 90 kDa RSKs (ribosomal S6 kinases) that are responsible for the phosphorylation of several substrates, such as the transcription factor CREB (cAMP-response-element-binding protein) and the transcriptional inhibitor IκB (inhibitor of NF-κB) that, upon phosphorylation, is targeted for ubiquitination and degradation leading to activation of NF-κB and its translocation to the nucleus and binding to specific promoter elements [40]. The PI3K pathway is also important in the PDGF-mediated anti-apoptotic signalling through Akt and IKK (IκB kinase) leading to active NF-κB complex [41]. As shown in Figures 6 and 7, inhibition of either of these pathways using specific inhibitors results in inhibition of both *HAS2* transcriptional activity and hyaluronan synthesis, to a similar level as for the non-stimulated cells. Notably, the proximal promoter region of *HAS2* gene has putative binding sites for the CREB and NF-κB transcription factors [42] (Figure 9), which are downstream of the ERK MAPK and PI3K signalling pathways. Our finding that inhibition of either of the ERK MAPK or PI3K pathways is sufficient to inhibit PDGF-BB-induced hyaluronan synthesis, suggests that these pathways are necessary for induction of hyaluronan synthesis in dermal fibroblasts. More recent studies on the *HAS2* promoter have also identified a functional STAT (signal transducer and activatory of transcription)-responsive element [43]; STAT isoforms are also effector molecules downstream of the PDGF β-receptor.

The composition of the extracellular matrix strongly influences the phenotypes of cells [44], and thus has important pathobiological implications. Hyaluronan and hyaluronan–CD44 complexes have been shown to be involved in the progression of inflammation and cancer [45,46]. Thus, given the many important functions of hyaluronan and its tight regulation in response to growth factors and cytokines released in rapidly remodelling tissues, further studies on the molecular mechanisms that regulate each *HAS* and *HYAL* gene expression in response to external stimuli are needed.

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