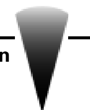

Differential effects of hyaluronan and its fragments on fibroblasts: Relation to wound healing

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ABSTRACT

Hyaluronan (HA) is involved in wound healing and its biological properties depend on its molecular size. The effects of native HA and HA-12 and HA-880 saccharide fragments on human fibroblast proliferation and expression of matrix-related genes were studied. The three HA forms promoted cell adhesion and proliferation. Matrix metalloproteinase-1 and -3 mRNA were increased by all HA forms, whereas only HA-12 stimulated the expression of the tissue inhibitor of metalloproteinase 1. HA-12 enhanced type I collagen and transforming growth factor-β (TGF-β) 1 expression. Interestingly, HA-12 and native HA stimulated type III collagen and TGF-β3. HA and its fragments activated Akt and extracellular-regulated kinases 1/2 and p38. Inhibition of these signaling pathways suggested their implication in most of the effects. Only native HA activated nuclear factor-κB and activating protein 1. Use of CD44 siRNA suggests that this HA receptor is partly implicated in the effects, although it does not rule out the involvement of other receptors. Depending on its size, HA may exert differential regulation on the wound-healing process. Furthermore, the HA up-regulation of type III collagen and TGF-β3 expression suggests that it may promote a fetal-like cell environment that favors scarless healing.

Hyaluronan (HA) is a nonsulfated, linear glycosaminoglycan (GAG), consisting of repeating units of (β, 1–4) glucuronic acid-(β, 1-3)-*N*-acetyl glucosamine. HA is present in most living tissues as a high molecular mass polymer ($> 10^6$ Da) and in significant quantities in the skin (dermis and epidermis), brain, and central nervous system.[1] Besides its function in the viscoelasticity of joint synovial fluid and the organization of cartilage extracellular matrix (ECM),[2] HA has a crucial role in tissue repair, including wound healing.[3–6] HA is involved in dynamic cellular processes such as cell migration and cell–cell recognition[7,8] during wound healing and inflammation (review in Chen and Abatangelo[3]). It can act as a promoter of early inflammation, an important step of the wound-healing process, by enhancing cellular infiltration[9] and production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-8.[10] HA may also play a role in the control of angiogenesis during tissue repair. High molecular weight HA has been shown to inhibit angiogenesis[11,12] whereas low molecular weight HA oligosaccharides promote angiogenesis in several experimental models.[13,14] In addition, it has been proposed that HA may protect granulation tissue from oxygen free radical damage by its ability to scavenge reactive oxygen species (ROS), a characteristic of large polyanionic polymers,[15,16] and that it could reduce the deleterious effect of oxygen free radical on wound healing,[16] although no clear experimental proof of a direct effect of HA on the role of ROS has been provided.

Several studies have shown that exogenous HA exerts beneficial effects on the wound-healing process. Topically applied HA has been shown to accelerate skin wound healing in rats[17] and hamsters.[18] Furthermore, some data suggest that HA could reduce scarring,[19,20] probably by reducing collagen deposition.[21]

Under physiological conditions, HA is a high average molecular mass polymer in excess of 10^6 Da. However, following tissue injury, HA fragments of lower molecular mass are produced as a result of hyaluronidases or oxidation.[22] A potential functional role for HA fragments has been suggested by both in vitro and in vivo studies.[23–25] However, the detailed mechanisms of how they function are not clear and are only beginning to be elucidated. It is known that native HA interacts with cell surfaces in at least two ways. It can bind to specific cell surface receptors, such as CD44 and receptor for hyaluronic acid-mediated motility (RHAMM), and induces the transduction of several intracellular signals that influence cell proliferation, survival, and motility, either directly or by activating other receptors.[26] HA can also be retained at the cell surface by membrane interactions with its synthases.[1] Both means of retention can generate a voluminous pericellular matrix that incorporates several other HA-binding molecules. Although it is clear that CD44 and RHAMM can participate in proliferative and migratory phenomena, their relative contribution to any given event has not been fully resolved in most cases, nor is the influence of interactions with other pericellular components on their signaling properties. Several groups have addressed the possibility that HA breakdown products may play a key role in the sequential phases of wound healing but the cellular mechanisms underlying their action are not clearly defined. These HA

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fragments are probably able to interact with CD44 and RHAMM but the resulting effect on intracellular signaling and gene expression is likely to be highly dependent on the cell type and the composition of the pericellular matrix.[1] The limited studies have so far failed to identify the respective effects of native HA and HA-derived fragments on dermal fibroblast signaling and gene expression in the context of wound recovery responses.

In this study, we have examined the comparative effects of native HA and HA fragments on cell adhesion, proliferation, and expression of matrix components and metalloproteinases (MMPs) in cultured dermal fibroblasts. In addition, we have investigated the rapid up-regulation of signaling proteins elicited by HA and its fragments. Although hyaluronidases can degrade native HA to tetrasaccharides under in vitro optimal conditions[27] it is likely that a family of fragments of various sizes appears during the wound-healing process. In this regard, it is noteworthy that hyaluronidase-derived small fragments of sizes similar to those used in our study were found in an injured arterial wall,[28] although it has not been proven that this can also apply to skin lesions. In the latter case, the actual molecular range of these fragments still remains to be determined. Therefore, we arbitrarily decided to use a small fragment (HA-12) and an intermediary one (HA-880).

MATERIALS AND METHODS

Reagents

Streptococcal HA (MW: 1.7×10^6 Da) was purchased from Fluka (Sigma-Aldrich Co., Saint Quentin-Fallavier, France) and bovine testicular hyaluronidase (H3884) from Sigma. Rabbit anti-type I collagen and mouse monoclonal anti-type III collagen were a generous gift from Pr. Daniel Hartmann (Novotec, Lyon, France). Goat anti-transforming growth factor-β3 (TGF-β3) was from R & D Systems (Lille, France). Mouse monoclonal anti-phospho extracellular-regulated kinase 1/2 (ERK1/2) and rabbit anti-ERK1/2 antibodies were provided by Upstate Biotechnology Inc. (Lake Placid, NY). Mouse monoclonal anti-β-actin, rabbit anti-phospho Akt1, mouse monoclonal anti-Akt1, and secondary antibodies (horseradish peroxidase labeled) were from Santa Cruz Biotechnology Inc. (Tebu-Bio SA, Le Perray-en-Yvelines, France). The ECL+Plus Western blot detection kit was purchased from Amersham Biosciences (Orsay, France). CD44 siRNA was purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-CD44 (J-173) was from Immunotech (Marseille, France).

Preparation of HA fragments

The HA fragments used in this study were HA-12 and HA-880 (the numeral indicates the number of saccharide units). These fragments were prepared as described previously[29] with modifications introduced by Tranchepain et al.[30] in order to produce HA fragments with any molar mass. Briefly, 90 mg of native HA were dissolved in 25 mL of 5 mM H_2PO_4 pH 4. Hyaluronidase was added at 0.25 g/L to obtain the optimal amounts of HA-12 and at 0.01 g/L to obtain the maximum of HA-880. The reaction was stopped by heating the solution at 100 °C and the fragments were separated by low-pressure chromatography on glass columns filled with Ultrogels (BioSepra, Pall Life Scienceo, St Germain-en-Lay, France) AcA 202 for HA-12 and AcA 22 for HA-880, eluted with 0.25 M glacial acetic acid /0.28 M pyridine. Fractions containing each fragment were collected, freeze-dried, and redissolved in Milli-Q (Millipore, St Quentin en Yvelineo, France) water. The total content of uronic acid in native HA and in HA fragment solutions was determined using the carbazol method.[31] The molar mass of the HA fragments was determined using size-exclusion high-pressure liquid chromatography (HPLC). In brief, in the case of HA-12, the size-exclusion chromatography used for purification showed a discrete separation of the fragments. The sample used for the study was obtained from the fraction corresponding to the top of the chromatographic peak whose analysis by mass spectrometry has shown that it did not contain any HA fragment of other sizes. For the HA fragments of the size range including HA-880, all the collected fractions were analyzed by size-exclusion HPLC and the polydispersity index was < 1.25 in all cases. More precisely, the polydispersity index of the HA-880 fraction was 1.22.

Cell culture and treatment

Human dermal fibroblasts (HDFs) were derived from foreskin explants. Tissue samples were obtained with informed consent of the donors and approval from the hospital Ethical Committee (Department of Pediatric Surgery, Caen Universitary Hospital). The cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), penicillin (100 IU/mL), streptomycin (100 μg/mL), and fungizone (0.25 μg/mL). Culture flasks were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were used between passages 5 and 10. For a proliferation assay, cells were seeded in 24-well plates at 4×10^3 cells/cm^2 in medium containing 10% FCS and allowed to adhere for 24 hours, before incubation with HA in serum-free medium. For protein and RNA extraction, cells were seeded in Petri dishes at 6×10^4 cells/cm^2 and allowed to attach for 24 hours. Preconfluent cells were then serum starved for 24 hours before HA addition.

Cell adhesion assay

Ninety-six–well plates were incubated with 100 μL of HA solution (2 mg/mL in phosphate-buffered saline [PBS]) for 24 hours at 4 °C. Control wells were incubated with 2% bovine serum albumin (BSA). The wells were washed three times with PBS and unspecific binding was blocked by 2% BSA for 30 minutes before addition of cells at 2×10^4/well in DMEM. Cells were allowed to adhere for 2 hours at 37 °C. Nonadherent cells were removed by washing three times with DMEM. The remaining adherent cells were fixed in 1% glutaraldehyde for 15 minutes and stained with 0.1% crystal violet. The stain was eluted from the cells with 10% acetic acid and the absorbance was read at 600 nm.

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Cell proliferation assay

Cells were incubated with HA for 24 hours. During the last 4 hours of incubation, [^{3}H]-thymidine was added in 10 μL of medium to a final concentration of 1 Ci/mL. At the end of incubation, culture medium was removed and cells were rinsed three times with ice-cold 5% trichloracetic acid. The precipitated material was then solubilized with 0.1 N NaOH/0.1% sodium dodecylsulfate (SDS). [^{3}H]-thymidine radioactivity was determined in a liquid scintillation counter.

RNA extraction and real-time reverse transcription polymerase chain reaction (RT-PCR) analysis

After a 48-hour incubation with HA, total RNA was extracted using the guanidinium isothiocyanate method.[32] Two micrograms of total RNA were DNase treated and reverse transcribed for 1 hour at 37 °C in the presence of Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Cergy Pontoise, France) and random hexamers (Applied Biosystems, Foster City, CA). The cDNA samples generated were diluted and used for real-time PCR analysis. Briefly, 5 μL of diluted cDNA was mixed with both forward and reverse primers and 2× SYBR Green PCR Master mix (Applied Biosystems) in 15 μL final volume. The amplification was performed using the ABI Prism 7000 sequence detection system (Applied Biosystems) under the following PCR conditions: initial incubation at 95 °C for 10 minutes, followed by 50 cycles at 95 °C for 10 seconds and 60 °C for 1 minute. The relative expression of each gene was obtained by normalizing the amount of gene product of interest to that of 18-second RNA. The relative quantity of cDNA was calculated from the number of cycles corresponding to 100% polymerization efficiency, using the $2^{-\Delta\Delta C_T}$ method.[33] The primers used in this study (Table 1) were designed from the human sequence of each gene using Primer Express software (Applied Biosystems).

Enzyme-linked immunosorbent assay (ELISA) for MMP1, MMP3, and TGF-β3 detection

After a 48-hour incubation with HA, culture media were harvested for ELISA detection. The amounts of MMP1 were determined using the Fluorokine® E kit and those of MMP3 and TGF-β3 using the Quantikine® kit (R & D Systems) according to the manufacturer's instructions.

Protein extraction and Western blotting

After cells' incubation with HA (30 minutes for signaling analysis [Akt, ERK, and p38] and 48 hours for collagen types I and III proteins and TGF-β3), total cellular proteins were extracted using radioimmunoprecipitation (RIPA) buffer. Briefly, after washing with ice-cold PBS, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% IGEPAL, 1 mM ethyleneglycolaminoethyl ether tetraacetic acid (EGTA), 1 mM NaF, 0.25% Na-deoxycholate, 10 μg/mL phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4 and 1 μg/mL leupeptin, pepstatin A, and aprotinin). The protein concentration of cell lysates was determined using the Bradford assay (Bio-Rad, S.A., Ivry sur Seine, France). Equal amounts of protein were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto a polyvinylidene difluoride membrane (NEN Life Sciences Products, Zawentem, Belgium). The membranes were blocked for 1 hour at room temperature in tris-buffered saline (pH 7.6) with 0.1% Tween 20 (TBS-T) and 10% (w/v) de-fatted milk, before incubation with a primary antibody in TBS-T overnight at 4 °C. After washing in TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature and proteins were visualized using the ECL chemiluminescent detection kit. To reprobe the blots, the membranes were incubated in stripping buffer (100 mM 2 mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 7.6) for 30 minutes at 50 °C.

Table 1. Oligonuleotide primers for real-time reverse transcription polymerase chain reaction (RT-PCR)

Primer (5′ → 3′)	Sequence
18S F	CGGCTACCACATCCAAGGAA
18S R	GCTGGAATTACCGCGGCT
MMP1 F	GAAGCTGCTTACGAATTTGCCG
MMP1 R	CCAAAGGAGCTGTAGATGTCCT
MMP3 F	TAAAGACAGGCACTTTTGGCGC
MMP3 R	TTGGGTATCCAGCTCGTACCTC
TIMP1 F	GTGTCTGCGGATACTTCCACAG
TIMP1 R	AGCTAAGCTCAGGCTGTTCCAG
Type I collagen F	CACCAATCACCTGCGTACAGAA
Type I collagen R	CAGATCACGTCATCGCACAAC
Type III collagen F	TCTTGGTCAGTCCTATGCGGATA
Type III collagen R	CATCGCAGAGAACGGATCCT
TGF-β1 F	TGACAAAGAGCAACACGGGTTCAGGTA
TGF-β1 R	GTTCAAGCAGAGTACACACA
TGF-β3 F	CAATTACTGCTTCCGCAACTTG
TGF-β3 R	GATCCTGTCGGAAGTCAATGTAGA
CD44 F	TGACCTCTGCAAGGCTTTCA
CD44 R	TCCGATGCTCAGAGCTTTCTC

TGF-β, transforming growth factor.

Use of signaling pathway inhibitors

PI3-kinase inhibitor LY294002 (20 μM, Calbiochem, VWR International, Straobourg, France), MEK1/2 inhibitor U0126 (10 μM, Calbiochem), and p38 inhibitor SB203580 (10 μM, Calbiochem) were used as specific inhibitors. The concentrations used were checked beforehand to ensure that they had no toxic effects on the fibroblasts. Cells were preincubated for 2 hours with each inhibitor before the addition of HA molecules. The inhibitors were dissolved in dimethyl sulfoxide (Me_2SO) and controls received the same amount of vehicle. The maximal Me_2SO concentration in the cultures was 0.1% (v/v).

Electrophoretic mobility shift assay (EMSA)

Cells were treated with HA for 24 hours to prepare nuclear protein extracts. At the end of the incubation, cells were

rinsed with ice-cold PBS and lysed with a low-salt buffer (10 mM Hepes pH 7.9, 0.1% IGEPAL, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM EGTA, 0.5 mM dithiothreitol (DTT), 0.5 mM PMSF, 10 μg/mL pepstatin, 10 μg/mL leupeptin, and 10 μg/mL aprotinin). Cell lysates were then centrifuged and the resulting pellets were resuspended in a high-salt buffer (20 mM Hepes, 25% glycerol, 420 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 μg/mL pepstatin, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) and kept at 4 °C with gentle shaking for 4 hours. The suspensions were centrifuged and the supernatants containing nuclear proteins were collected. Oligonucleotide probes containing the specific DNA-binding domain for nuclear factor-κB (NF-κB) (5′-AG TTGAGGGGACTTTCCCAGGC-3′) and activating protein 1 (AP1) (5′-CGCTTGATGAGTCAGCCCG-GAA-3′) were end-labeled with [$\tilde{\gamma}^{32}$P]dATP using T4 polynucleotide kinase. Nuclear extracts were incubated with a labeled probe in binding buffer (20 mM Hepes [pH 7.5], 50 mM KCl, 4 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.05% IGEPAL, 20% glycerol, 1 mg/mL BSA, and 0.025 mM poly[dI-dC]) for 15 minutes at room temperature and then resolved by 5% PAGE in 0.5×TBE (45 mM Tris pH 7.8, 45 mM boric acid, and 1 mM EDTA). Gels were dried and visualized by autoradiography. In DNA-binding competition studies, excess of unlabeled consensus and mutant oligonucleotide was added 15 minutes before addition of the radiolabeled probe. Supershift experiments were performed by preincubating the nuclear extracts with an antibody against the p65 subunit for NF-κB and with an anti C-jun B antibody for AP1 before addition of a radiolabeled probe.

siRNA experiments

For siRNA experiments, 1×10^6 cells were transfected with 1 μg of CD44 siRNA according to the optimized protocol used for HDFs (Nucleofector solution, Amaxa, Köln, Germany). Scramble siRNA was used under the same conditions, as a negative control. After transfection, cells were seeded in six-well plates in 10% FCS/DMEM and allowed to adhere for 12 hours. Culture medium was then replaced with fresh 10% FCS/DMEM for another 12-hour period. Cells were serum starved for 24 hours before incubation with HA molecules as described above.

Statistical analysis

The results are given as mean ± SD of three experiments. Statistical difference between the groups was determined using the Bonferroni multiple comparison test. The difference was considered to be as significant at $p < 0.05$.

RESULTS

Fibroblast adhesion to native HA and HA-derived fragments

We first tested the hypothesis that the molecular size of HA could influence the ability of dermal fibroblasts to bind the GAG. Multiwell plates were coated either with native HA or with the HA-880 and HA-12 fragments and the number of cells adhering to these surfaces was determined. From the 2×10^4 cells seeded, 72% were found to attach to native HA-coated wells (Figure 1A). The level of cell adhesion to HA-880 or to HA-12–coated wells was, respectively, 51 and 62%. For each molecular form studied, cell adhesion to HA-coated wells was significantly higher than that of control BSA-coated wells.

Differential effect of HA and its fragments HA-12 and HA-880 on fibroblast proliferation

To determine whether fibroblast adhesion resulted in cell growth, we then compared the effect of native HA and its fragments HA-12 and HA-880 on cell proliferation. [^{3}H]-thymidine incorporation was determined in response to increasing concentrations of each HA form. As shown in Figure 1B, HA-12 induced the stimulation of cell proliferation in a concentration-dependent manner. This effect was significant at 10 μg/mL and markedly increased at the highest concentration used (50 μg/mL). HA-880 also

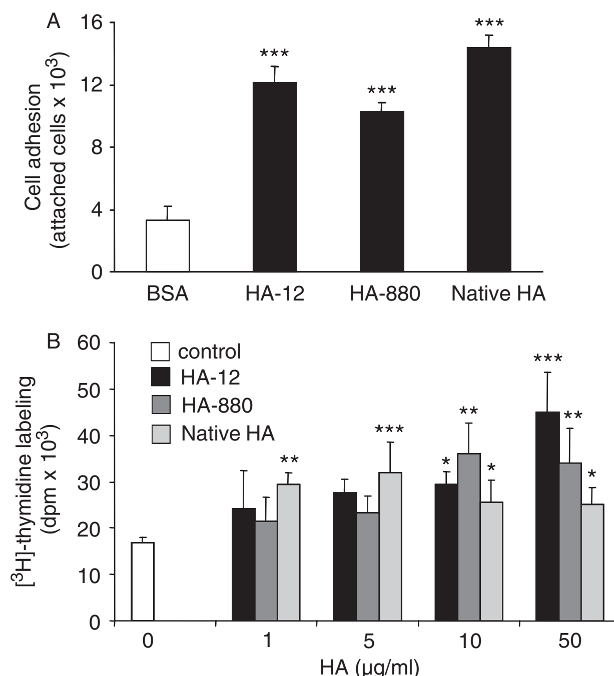

Figure 1. Effect of hyaluronan (HA) on fibroblast adhesion and proliferation. In the adhesion assay (A), cells were seeded on HA-coated wells or bovine serum albumin (BSA)-coated control wells and allowed to adhere for 2 hours at 37 °C. At the end of incubation, adherent cells were stained with crystal violet and the absorbance was read at 600 nm. In proliferation experiments (B), cells were incubated with native HA or the fragments HA-880 and HA-12 at 1, 5, 10, and 50 μg/mL for 24 hours. [^{3}H]-thymidine was added during the last 4 hours of incubation and incorporated radioactivity was determined as described in ''Materials and Methods.'' Results are expressed as means ± SD of at least three determinations. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ indicate the differences between control and HA-treated groups.

caused stimulation of cell proliferation but the cell response was maximum at 10 μg/mL and reached a plateau at this concentration, no further increase being observed at 50 μg/mL. Treatment of cells with native HA induced a significant increase of the proliferation at a concentration as low as 1 μg/mL. This effect was also present for 5 μg/mL and slowly decreased above this concentration.

Differential effect of native HA and its fragments HA-12 and HA-880 on the expression of genes related to wound healing

We next studied the effect of HA on the expression of genes involved in the ECM remodeling that is associated with wound healing, i.e., degradation and synthesis of connective tissue components. Serum-starved cells were incubated with increasing concentrations (1–50 μg/mL) of HA-12, HA-880, or native HA for 48 hours, and the steady-state mRNA levels of MMP1, MMP3, tissue inhibitor of metalloproteinase 1 (TIMP1), types I and III collagen, TGF-β1, and TGF-β3 were determined using real-time PCR.

Treatment of cells with the three HA molecular forms resulted in an increase of MMP1 and MMP3 mRNA steady-state levels (Figure 2A and B). The effect of HA-12 reached its maximum at the lowest concentration tested (1 μg/mL) for both MMPs. The effect on MMP1 mRNA was still present at higher concentrations, whereas the effect of HA-12 on MMP3 mRNA levels decreased with increasing concentrations of the GAG. The stimulatory effect of HA-880 and native HA on the expression of both MMPs was dose dependent, with a maximum effect at 50 μg/mL. This was more marked with native HA than with HA-880. The up-regulation of MMPs by different HA molecules led us to determine whether the GAG could also modulate TIMP1 expression in fibroblasts. As shown in Figure 2C, only the fragment HA-12 was found to enhance TIMP1 mRNA levels, with a significant effect for 10 μg/mL.

We used the concentrations of HA that exerted the maximum effect on MMP mRNA expression to determine MMP production by ELISA. As shown in Figure 2D and E, native HA induced the maximum increase of both MMP1 and MMP3 (+26 and +18%, respectively). The effect of HA-12 and HA-880 resulted in a mild increase of both MMPs, with a significant effect of A-880 on MMP3 (+15%). It must be noted that the stimulation of MMP1 and MMP3 protein expression was smaller than the corresponding increase of their mRNA levels.

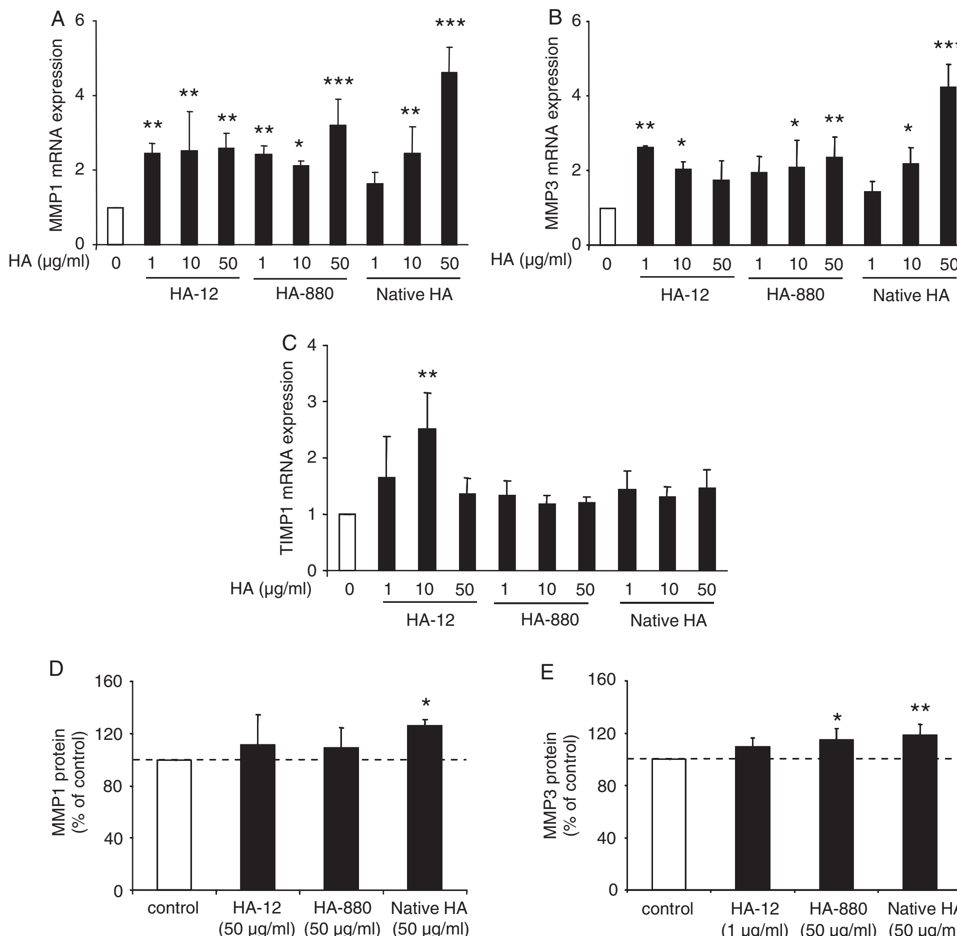

Figure 2. Effect of hyaluronan (HA) on matrix metalloproteinase 1 (MMP1) (A), MMP3 (B), and tissue inhibitor of metalloproteinase 1 (TIMP1) (C) mRNA expression. Serum-starved cells were cultured in the presence of native HA or the fragments HA-12 and HA-880 at the concentration indicated. At the end of the experiment, total RNA was extracted and real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as described in "Materials and Methods." Results are normalized to 18S RNA. Proteins levels of MMP1 (D) and MMP3 (E) were assayed in conditioned medium by an enzyme-linked immunosorbent assay (ELISA). Control without HA treatment (9.4 ± 1.52 ng/mL for MMP1 and 18 ± 5.5 ng/mL for MMP3) was designated as 100%. Values are means ± SD of three experiments. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ indicate the differences between control and HA-treated groups.

In addition to its stimulating effect on MMPs and TIMP1 expression, HA-12 appeared to increase the levels of types I and III collagen mRNAs (Figure 3A and B). The maximum effect was reached at 10 μg/mL for type I collagen mRNA, whereas the effect on type III collagen mRNA was similar for every HA-12 concentration (1–50 μg/mL). The treatment of cells with HA-880 resulted in a small increase of type I collagen mRNA expression. In contrast, this HA form caused a marked increase in type III collagen expression. This is true particularly at the concentration of 50 μg/mL. Native HA did not affect type I collagen at any concentration used (1–50 μg/mL). In contrast, it produced significant elevation of type III collagen mRNA levels, with the maximum effect at 10 μg/mL (Figure 3A and B).

The up-regulation of type I collagen mRNA levels by HA-12 was correlated by increase of protein expression as shown by Western blot (Figure 3C). Protein expression analysis of type III collagen also showed data in agreement with those observed for mRNA expression. Indeed, the three HA molecules were found to enhance type III collagen and expression at the protein level. However, native HA and HA-880 were the most potent (Figure 3D).

Because TGF-β1 and TGF-β3 have been shown to exert differential effects on skin collagen deposition and wound healing, it was of interest to determine the potential influence of HA molecules on the expression of these isoforms in our system. Our results show that HA-12 caused an increase of both TGF-β1 and TGF-β3 mRNA expression in a dose-dependent manner (Figure 4A and B). For both genes, the maximum stimulation occurred at 10 μg/mL and then the effect of HA-12 decreased at 50 μg/mL to almost the control levels. This observation suggests a biphasic effect of this HA form. HA-880 did not significantly affect TGF-β1 and TGF-β3 mRNA expression (Figure 4A and B). Interestingly, native HA caused a moderate but significant specific increase of TGF-β3 mRNA levels (Figure 4B). As HA-12 yielded the maximum increase of TGF-β1 mRNA level at 10 μg/mL, we used this concentration to test its effect on the protein level, using ELISA. As expected, HA-12 significantly stimulated TGF-β1 protein secretion by cells (+40%, Figure 4C). To determine the expression of TGF-β3 protein, we used Western blot analysis because no ELISA kit for TGF-β3 was available. We found that the HA-12 was more potent to increase TGF-β3 protein expression than native HA, which was in agreement with the expression of corresponding mRNA (Figure 4D).

Effect of HA molecules on Akt, ERK1/2, and p38 kinases

To gain insights into the mechanisms whereby HA molecules exert their effect on gene expression in dermal fibroblasts, we studied the activation state of signaling kinases that might be elicited by the GAG forms. We focused on the activation of ERK1/2, p38, and PI3-kinase/Akt, because HA has been reported to stimulate some of these signaling pathways.[34,35] Serum-starved cells were treated for 30 minutes with increasing concentrations of native HA, HA-880, and HA-12, and the phosphorylation state of Akt, ERK1/2, and p38 kinases was determined by West-

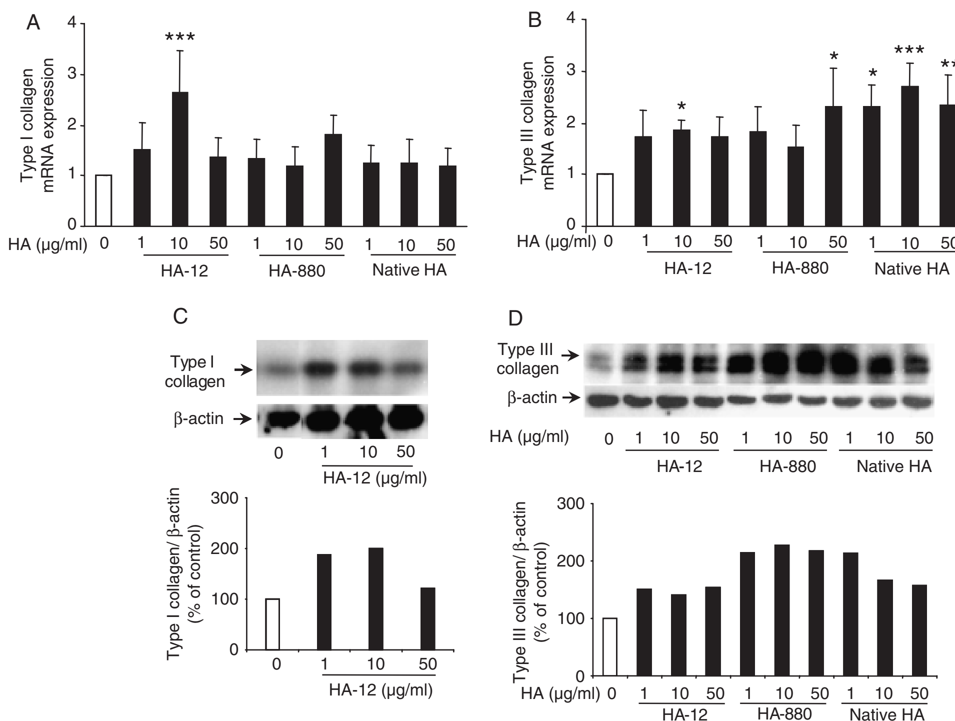

Figure 3. Effect of hyaluronan (HA) on type I collagen (A) and type III collagen (B) mRNA expression. Cells were cultured and treated as described in ''Materials and Methods.'' At the end of the experiment, total RNA was extracted and real-time reverse transcription polymerase chain reaction was performed. Results are normalized to 18S RNA. Values are means ± SD of three experiments. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ indicate the differences between control and HA-treated groups. Protein expression of types I and III collagen was determined by Western blot (C, D). Cells were cultured and treated as described in ''Materials and Methods.'' Total cell proteins were extracted using radioimmunoprecipitation buffer, subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis, and then immunoblotted with anti-collagen types I and III as well as anti-β-actin antibodies. The amounts of types I and III collagen protein (normalized to β-actin) were semi-quantitated by densitometry scanning. The data are presented as percent of control.

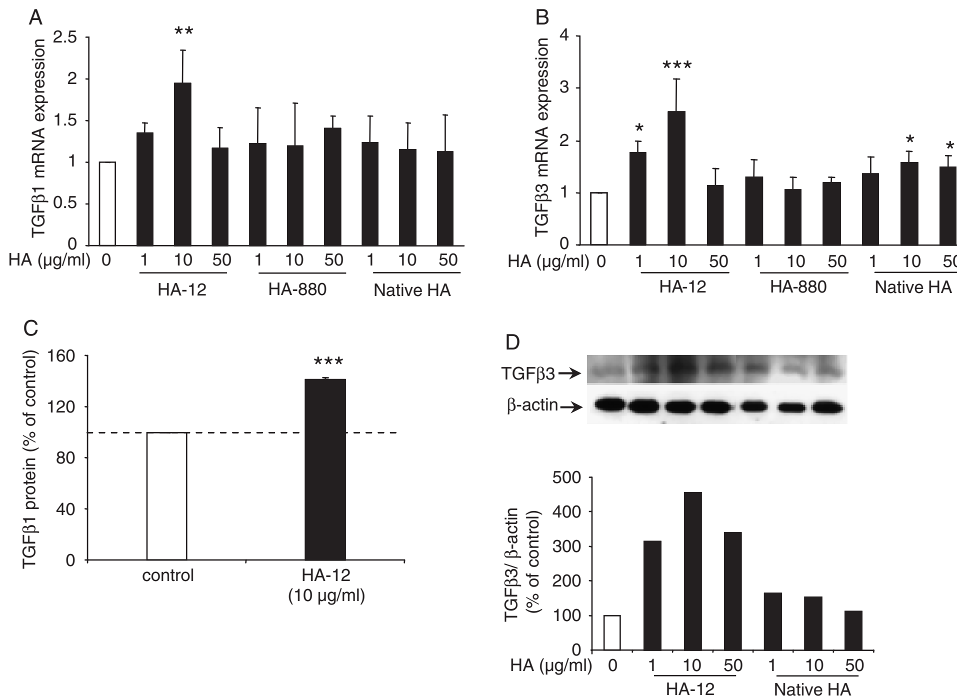

Figure 4. Effect of hyaluronan (HA) on transforming growth factor-β1 (TGF-β1) (A) and TGF-β3 (B) mRNA expression. At the end of the incubation, total RNA was extracted and real-time reverse transcription polymerase chain reaction was performed as described. Results are normalized to 18S RNA. Protein levels of TGF-β1 were determined in conditioned medium by an enzyme-linked immunosorbent assay (C). Control without HA-12 treatment (57 ± 12 ng/mL) was designated as 100%. Values are means ± SD of three experiments. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ indicate the differences between control and HA-treated groups. Protein expression of TGF-β3 was analyzed by Western blot (D). Cells were cultured and treated as described in ''Materials and Methods.'' Total cell proteins were extracted using radioimmunoprecipitation buffer, subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis, and then immunoblotted with anti-TGF-β3 and anti-β-actin antibodies. The amount of TGF-β3 protein (normalized to β-actin) was semi-quantitated by densitometry scanning. The data are presented as percent of control.

ern blotting of cellular extracts. We found that both HA-12 and HA-880 induced the phosphorylation of Akt, with a greater effect at 10 μg/mL (Figure 5A). Native HA also enhanced Akt phosphorylation; however, in contrast to the fragments, its effect was similar whatever the concentration used. Regarding the activation of ERK1/2, our results showed that the three HA molecular forms studied induced strong phosphorylation of this protein at the three concentrations used (Figure 5B). Similar results were observed for p38 phosphorylation; the effect of HA was, however, less important than for ERK1/2 (Figure 5C).

Role of Akt and ERK1/2 pathways in HA-induced proliferation

To determine the potential implication of HA-induced Akt and ERK1/2 activation in the effects on cell proliferation, we performed [^{3}H]-thymidine incorporation experiments in the presence of a PI3-kinase/Akt inhibitor (LY294002), and an MEK/ERK1/2 inhibitor (U0126). The two inhibitors were found to suppress HA-880 and native HA stimulation of cell proliferation, indicating that both Akt and ERK1/2 are involved in this stimulation (Figure 6). Thus, despite the mild activation of Akt by native HA, this signaling pathway is required for its effect on proliferation. Interestingly, whereas the stimulatory effect of HA-12 was also abolished by U0126, LY294002 only reduced this effect (Figure 6). This suggests that ERK1/2 plays a more important role than Akt in HA-12–induced fibroblast proliferation.

Differential involvement of Akt, ERK1/2, and p38 in the effect of HA molecules on the expression of matrix genes

In an attempt to determine which signaling pathway was involved in the effect of HA and its fragments on the expression of matrix genes, we measured the mRNA steady-state levels that were produced in cultures treated with specific inhibitors of these pathways. Treatment of cells with LY294002 (Akt inhibitor) or SB203580 (p38 inhibitor) reduced the native HA-induced MMP1 mRNA level, whereas U0126 completely abolished this effect (Figure 7A). U0126 also prevented an increase in MMP1 due to HA-12 or HA-880 treatment. The stimulatory effect of both native HA and its fragments on MMP3 and type III collagen expression was suppressed when cells were incubated in the presence of Akt, ERK1/2, or p38 inhibitor, indicating that these signaling pathways are likely to be involved in the effect of both native HA and its fragments on MMP3 and type III collagen expression (Figure 7B and D). Similarly, the HA-12–induced increase of type I collagen mRNA levels was completely prevented by incubation of cells with inhibitors of the three signaling molecules (Figure 7C). On the other hand, the stimulatory effect of HA-12 on TGF-β1 and TGF-β3 was suppressed by the inhibition of Akt and ERK1/2,

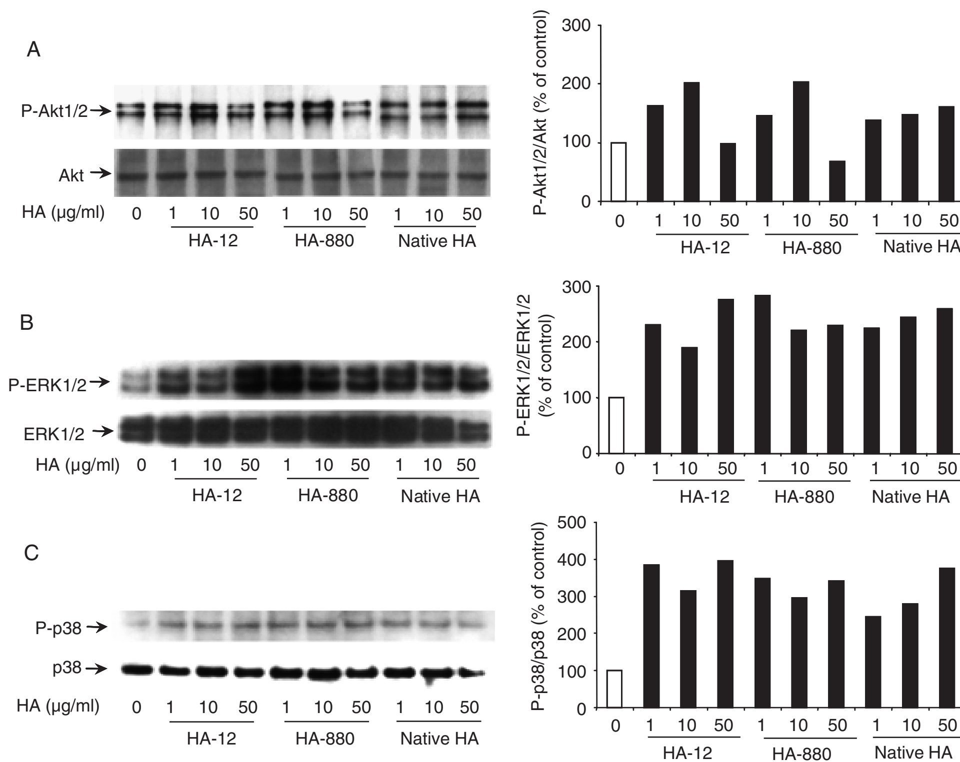

Figure 5. Effect of hyaluronan (HA) on phosphorylation of extracellular-regulated kinase 1/2 (ERK1/2), Akt, and p38. Serum-starved cells were treated with native HA or the fragments HA-12 and HA-880 at 1, 10, and 50 µg/mL for 30 minutes. Cell lysates were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis and then immunoblotted with anti-phospho Akt and anti-AKt, anti-phospho ERK1/2 and anti-ERK1/2, and anti-phospho p38 and p38. Kinase phosphorylation was semi-quantitated by densitometry scanning of the phosphorylated and the total form. The ratio of phosphorylated to total form was calculated. The data are presented as percent of control.

whereas the inhibition of p38 reduced but did not completely suppress HA-12 increase of TGF-β1 expression (Figure 7E and F).

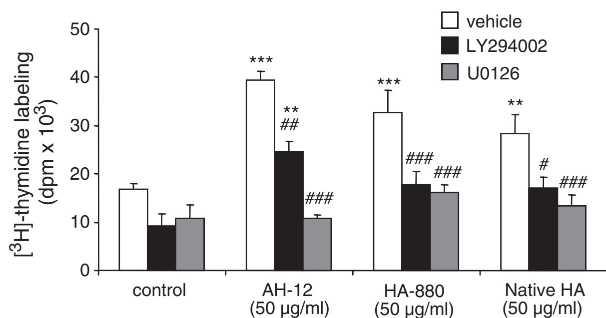

Figure 6. Effect of Akt and extracellular-regulated kinase 1/2 inhibition on a hyaluronan (HA)-induced effect on cell proliferation. Cells were incubated with LY294002 (20 µM) or U0126 (10 µM) in the presence of native HA or the fragments HA-880 and HA-12 at 50 µg/mL for 24 hours. $[^3H]$-thymidine was added during the last 4 hours of incubation and incorporated radioactivity was determined as described in ''Materials and Methods.'' Results are expressed as means ± SD of three experiments. $**p < 0.01$ and $***p < 0.001$ indicate the differences between control and HA-treated groups. $\#p < 0.05$; $\#\#p < 0.01$; and $\#\#\#p < 0.001$ indicate the differences between vehicle and inhibitor treated groups.

Activation of NF-κB and AP1 DNA binding by native HA

To further investigate the signaling mechanism induced by HA in dermal fibroblasts, we studied the DNA-binding activity of the transcription factors NF-κB and AP1 because these nuclear factors were found to be activated by HA in other cell types. Cells were treated with HA (1–50 µg/mL) for 24 hours and nuclear extracts were subjected to EMSA using radiolabeled oligonucleotides containing NF-κB and AP1 consensus sequences as specific probes. As shown in Figure 8, only native HA was found to enhance significantly the binding activity of NF-κB, the maximum increase being observed at the concentration of 10 µg/mL. In contrast to native HA, HA-12 and HA-880 did not affect the DNA-binding activity of NF-κB. AP1 DNA-binding activity was found to be mainly enhanced by native HA, especially at the concentration of 10 µg/mL (Figure 8). HA-880 induced a weak increase while HA-12 did not affect the binding activity of AP1. The protein–DNA complexes induced by native HA were specific to NF-κB and AP1, as suggested by efficient competition produced by its respective wild-type consensus sequences but not by mutant unlabeled probes. Supershift analysis using anti-p65 and anti-Jun B antibodies for, respectively, NF-κB and AP1 confirmed the identity of the complexes activated by native HA (Figure 8).

Down-regulation of CD44 expression by siRNA

To investigate the possible involvement of the major HA receptor, CD44, in the effects of HA observed in this

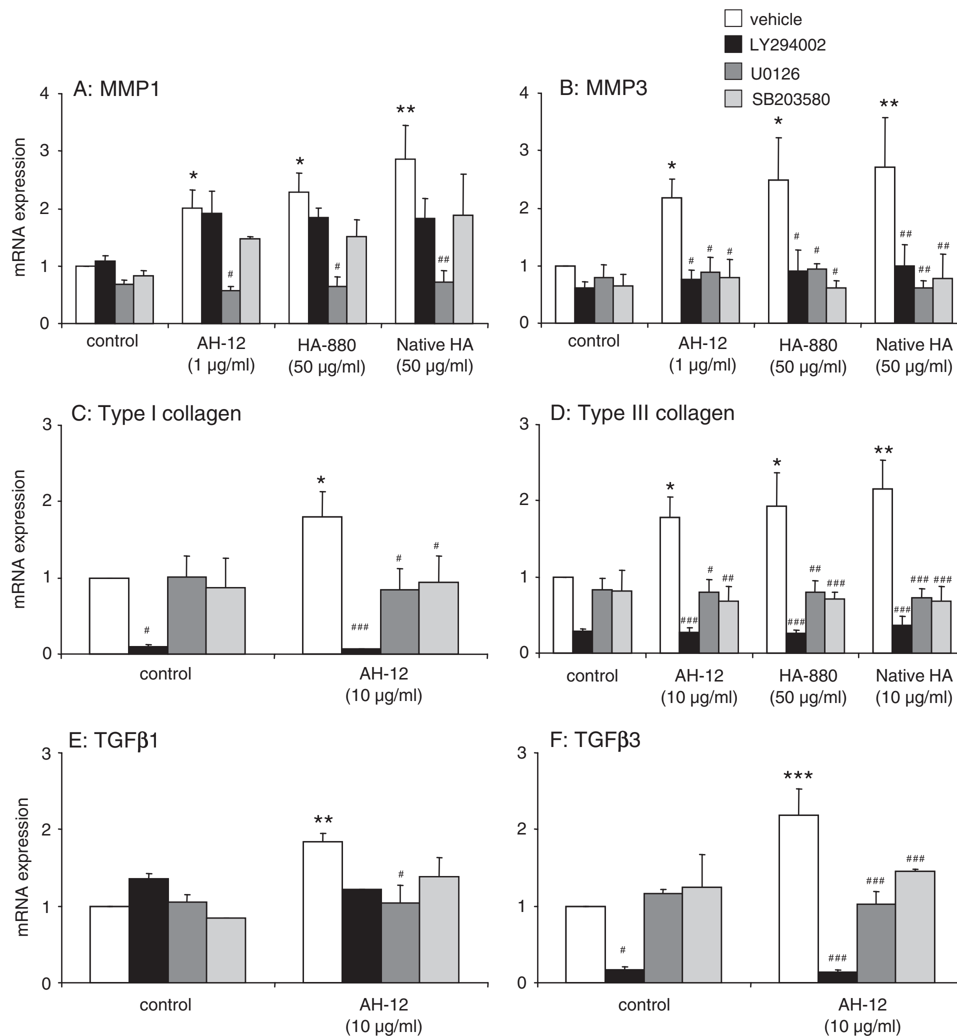

Figure 7. Effect of Akt, extracellular-regulated kinase 1/2, and p38 inhibition on matrix genes' mRNA expression. Serum-starved cells were cultured in the presence of native hyaluronan (HA) or the fragments HA-12 and HA-880. At the end of the experiment, total RNA was extracted and a real-time reverse transcription polymerase chain reaction was performed as described in ''Materials and Methods.'' Results are normalized to 18S RNA. Values are means ± SD of three experiments. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$ indicate the differences between control and HA-treated groups. #$p < 0.05$; ##$p < 0.01$; and ###$p < 0.001$ indicate the differences between vehicle and inhibitor-treated groups.

study, fibroblasts were transfected with CD44 short interfering RNA (siRNA). CD44 inhibition was verified at both mRNA and protein levels. As shown in Figure 9A, cell treatment with CD44 siRNA resulted in 65 and 40% inhibition of CD44 mRNA, respectively, after 48 hours (HA treatment starting time point) and 96 hours (the end of the incubation), as compared with the control scramble siRNA. The down-regulation of CD44 transcripts was associated with a significant reduction of CD44 protein production, as shown by Western blotting (Figure 9B).

Effect of CD44 siRNA on fibroblast adhesion and proliferation

To determine whether CD44 played a role in the HA-mediated cell adhesion and proliferation, cells transfected with CD44 siRNA were used in these experiments. CD44 inhibition did not affect fibroblast adhesion to the HA-coated surface whatever the molecular weight of the GAG (Figure 10A). CD44 down-regulation did not affect HA-12–induced thymidine incorporation by fibroblasts while it significantly reduced the effect of HA-880 and native HA (Figure 10B), suggesting the involvement of the receptor in their proliferative effect.

Effect of CD44 siRNA on HA-induced matrix gene expression

CD44 siRNA treatment markedly reduced the stimulatory effect of HA-12 on type I collagen but not on type III collagen (Figure 11A and B). Similarly, HA-12–induced TGF-β1 mRNA expression was suppressed by CD44 siRNA while TGF-β3 was not (Figure 11C and D). These observations suggest an involvement of the HA receptor, CD44, in HA-12–induced stimulation of type I collagen and TGF-β1. The increased expression of type III collagen in the presence of CD44 siRNA was reduced for native HA whereas it persisted for HA-880. Our results concerning type III collagen suggest that CD44 plays a role in the effect of native HA, whereas the effect of the fragments could involve another receptor. CD44 inhibition did not change the effect of HA molecules on both MMP1 and MMP3 transcript levels (data not shown).

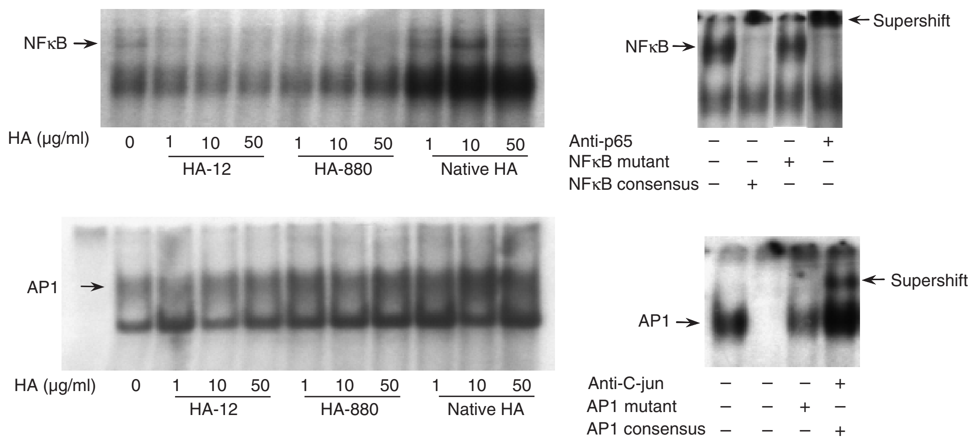

Figure 8. Effect of native hyaluronan (HA) and HA fragments on nuclear factor-κB (NF-κB) and AP1 activation. Serum-starved cells were cultured in the presence of native HA or the fragments HA-12 and HA-880 at 1, 10, and 50 μg/mL for 24 hours. Nuclear extracts were prepared and used for determination of NF-κB and AP1-DNA–binding activity as described in ''Materials and Methods.'' For competition studies, nuclear extracts were preincubated with 100-fold molar excess of the consensus or mutant unlabeled NF-κB and AP1 probes and subjected to an electrophoretic mobility shift assay (EMSA). For a supershit assay, nuclear extracts were preincubated with an anti-p65 (for NF-κB) or with an anti-C-jun (for AP1) antibody and then subjected to EMSA.

DISCUSSION

Besides its structural role in the organization of the ECM, HA also modulates cell behavior and metabolism, especially during the course of development, in the early phases of inflammation and wound repair,[3] as well as in tumors.[1] Given that the HA level increases in the early phases of wound repair, it is of interest to investigate its interactions with dermal fibroblasts and its potential role in mechanisms involved in wound healing, such as cell adhesion, proliferation, and matrix deposition and remodeling.

In this study, we first found that HDFs are able to bind to both native HA and HA-derived fragments, suggesting that these cells could respond to HA-induced stimuli through specific binding sites. This property is likely to promote fibroblast adhesion to the wound site. Indeed, provisional matrix formation is one of the early responses to tissue injury, which provides a substratum to which recruited fibroblasts can adhere to the wound site.

HA and its fragments were found to exert a mitogenic effect on several cell types, but the results are controversial and depend on cellular type and origin, as well as the size and concentration of HA. In endothelial cells, native HA was found to inhibit proliferation, whereas 3–10 disaccharide HA fragments stimulated proliferation.[36] Investigations on fibroblasts also led to contradictory data, showing either inhibition or stimulation of cell growth. High molecular weight HA was found to decrease the cell proliferation of fetal rabbit and embryogenic chick skin fibroblasts, at 1–100 and 50–500 μg/mL, respectively.[37,38] The discrepancy between these results and our findings, despite the use of a comparable HA concentration range, could be due to the fact that we used human juvenile fibroblasts whereas fetal non human fibroblasts were used in those studies. In another study,[39] a dose-dependent inhibition of human adult fibroblast proliferation was found, using an HA concentration at least 10-fold greater than that in the present study. It was suggested that high concentrations of HA prevent cells from entering the cell cycle rather than influencing the cell cycle as such.[39] Our data are consistent with previous reports on human dermal and NIH 3T3 fibroblasts, which showed a stimulatory effect of HA on cell proliferation.[40,41]

The mechanism of this stimulatory effect is not clearly defined. It has been suggested that HA, especially at a high concentration, could exert a mechanical effect, causing the detachment of cells from their matrix and then facilitating mitosis.[42,43] Alternatively, and consistent with our findings, HA can induce signal transduction and activate intracellular pathways related to cell cycle machinery. In this study, native HA and its fragments were found to activate two signaling pathways, Akt and ERK1/2, known to support cell survival and proliferation. In addition, these

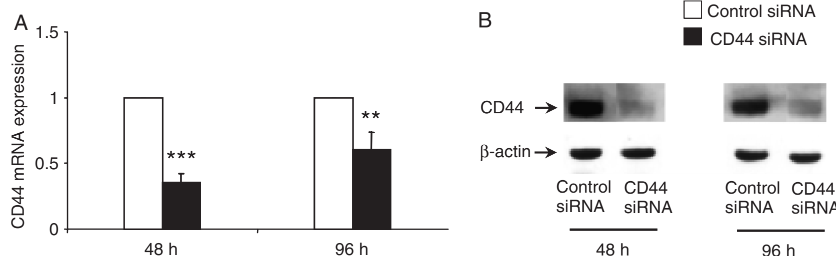

Figure 9. CD44 down-regulation by siRNA. Cells were incubated with CD44 siRNA as described in ''Materials and Methods.'' Total RNA was extracted and real-time reverse transcription polymerase chain reaction was performed to test CD44 inhibition at mRNA levels (A). Values, normalized to 18S RNA, are means ± SD of three experiments. ** $p < 0.01$ and *** $p < 0.001$ indicate the differences between the control siRNA and the CD44 siRNA group. A CD44 Western blot was performed to check the protein inhibition by siRNA (B).

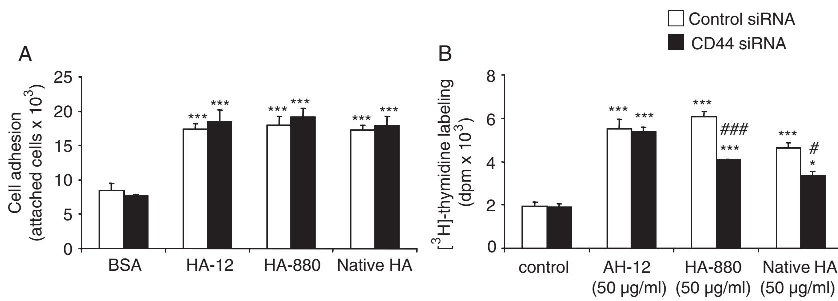

Figure 10. Effect of CD44 down-regulation on fibroblast adhesion (A) and proliferation (B). Cells treated with control or CD44 siRNA were subjected to adhesion or a proliferation assay as described in ''Materials and Methods.'' Results are expressed as mean ± SD of at least three determinations. $***p < 0.001$ indicates the differences between control and HA-treated groups. $\#p < 0.05$ and $\#\#\#p < 0.001$ indicate the differences between control and CD44 siRNA-treated groups.

signaling pathways are closely involved in HA-induced fibroblast proliferation.

Besides their proliferative activity, dermal fibroblasts play a pivotal role in the synthesis and remodeling of ECM components in damaged tissue. We therefore investigated the potential action of HA on the expression of MMP1 and MMP3. Several studies have reported regulation of MMP expression and activity by HA in malignant cells while little is known about the effect on normal cells, including fibroblasts. In our study, the increase of steady-state levels of MMP1 and MMP3 mRNA was always greater than that of the corresponding proteins. We do not have a clear explanation for this discrepancy. However, although ample evidence indicates that MMP gene expression is, to a large extent, regulated at the transcriptional level, recent evidence suggests that post-transcriptional mechanisms are also involved in the control of MMP expression in response to certain cues (review in Yan and Boyd[44]). Both histone modifications and chromatin-remodeling motors have been shown to play a role in controlling the expression of multiple MMPs. In addition to transcriptional control, mRNA stability and protein translation also contribute to the MMP product amount. In a study on the role of nucleolin in the post-transcriptional control of MMP9 expression, up to seven potential steps in the control of this MMP have been proposed: transcription, processing, transport, localization and translation of mRNA, as well as vesicle transport, secretion and activation of pro-peptide, and interaction with inhibitors.[45] We found that both native HA and HA fragments induced stimulation of MMP1 and MMP3 mRNA expression. The effect of native HA correlated with a mild but significant increase of MMP protein levels while the effect of the fragments did not. These data are consistent with a previous observation that high molecular weight HA modulates the expression of other MMPs (MMP2 and MMP9). In fibroblast cultures, HA was found to increase the expression of MMP9, whereas in skin explant cultures, HA increases the conversion of inactive MMP2 and MMP9 to their active form.[46] Interestingly, native HA induced the maximum increase of MMPs mRNA expression, suggesting that the stimulation induced by the fragments was not sufficient to induce subsequent increase of proteins.

To further investigate the involvement of HA in the wound-healing mechanisms, we examined the expression of types I and III collagens in HA-treated fibroblasts. Together with types IV and VII collagens, they are the main types present in the skin and the most involved in wound healing.[47] Because types IV and VII collagens are present

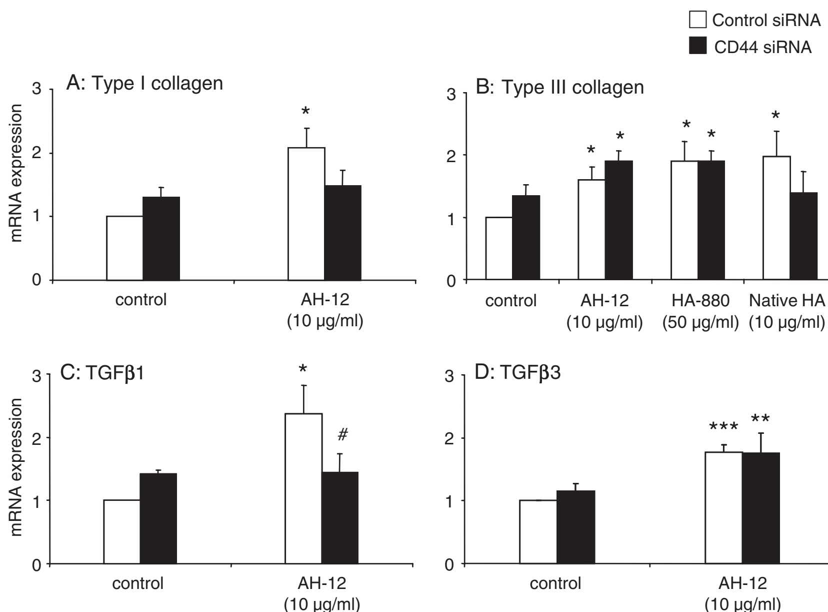

Figure 11. Effect of CD44 down-regulation on matrix genes' mRNA expression. Cells were transfected with control or CD44 siRNA 48 hours before hyaluronan (HA) treatment. The incubation was continued for another 48 hours and total RNA was extracted and real-time reverse transcription polymerase chain reaction performed. Results are expressed as mean ± SD of three experiments. $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$ indicate the differences between control and HA-treated groups. $\#p < 0.05$ indicates the differences between control and CD44 siRNA-treated groups.

in the basement membrane that anchors the epidermis to the dermis, we focused on types I and III collagens. Type I collagen is the predominant form in the adult normal skin and its accumulation is enhanced in scar and fibrosis, whereas type III is more present during fetal development and in the early stages of wound healing. We found that native HA, and its fragments to a lesser extent, stimulated the expression of type III collagen whereas the fragment HA-12 also stimulated type I collagen. Concerning native HA, this result is in agreement with a previous study that reported stimulation of total collagen production by native high molecular weight HA in fetal fibroblasts.[37] Our results are divergent, with a report showing that high molecular weight HA decreased total collagen production by fibroblasts without a change in the ratio types I/type III collagens. However, these authors were using a rather high concentration of exogenous HA.[48] The present data appear to be consistent, to some extent, with an in vivo study in which degradation of fetal wound HA by *Streptomyces* hyaluronidase injection in a rabbit model resulted in fibroplasia and collagen deposition, i.e., a more adult-like phenotype.[25] It is difficult to compare such a study, where hyaluronidase was likely to induce a family of HA oligosaccharides with various chain sizes, with our experiments performed with isolated well-defined types of HA fragments. Nevertheless, as far as that tissue HA was degraded to small oligosacharides, we may suppose that increased deposition of collagen, probably of the fibrotic type I isoform, was caused by the smallest HA fragments, as demonstrated here in cultured dermal fibroblasts with HA-12. In contrast, native high molecular size HA, which had no in vitro effect on type I collagen synthesis in our model, but rather stimulated type III collagen production, would be responsible for the maintenance of a fetal-like phenotype. HA fragments of six to 18 disaccharides (a range that includes HA-12) were also found to stimulate the expression of types I and III collagens in lung fibroblasts, data in agreement with our results.[49]

TGF-βs are of critical importance in wound healing because of their ability to modulate ECM formation. TGF-β1 and TGF-β2 are known to promote scar formation, as their neutralization in adult wounds reduces scarring.[50,51] By contrast, exogenous addition of TGF-β3 to healing adult wounds markedly reduces scarring.[51] Interestingly, fetal wounds that heal without scarring express high levels of TGF-β3 and low levels of TGF-β1 and TGF-β2.[52] Conversely, adult scar-forming wounds contain predominantly TGF-β1 and TGF-β2. An interesting finding of this study was the ability of native HA and its fragments to enhance TGF-β3 expression by dermal fibroblasts. This result suggests that HA molecules could improve the wound-healing process. Fetal wound healing differs from that of adults, insofar as it occurs with moderate inflammation, mild proliferation, and collagen deposition, resulting in scarless repair. In comparison with the adult, fetal wound healing is characterized by persistently higher levels of HA at the wound sites.[21] Thus, our results concerning TGF-β3 expression suggest that HA could create a fetal-like environment that may favor scar-free wound healing. This hypothesis is supported by our observation showing an enhancement of type III collagen expression (a fetal-type collagen), which is rather more marked for the highest HA molecular forms (native HA and HA-880) than for the small fragment (HA-12).

HA-12 also increased TGF-β1 expression, data that correlate with its effect on type I collagen and TIMP1 expression and confers to this fragment a dual effect on cells. Indeed, HA-12 up-regulates mRNA expression of factors involved in both the accumulation and degradation of ECM.

The mechanisms by which HA affects gene expression are not completely understood. We searched for the signaling molecules that could be elicited by HA. Native HA and the HA fragments activated the MAPKs ERK1/2 and p38 kinase. However, while the activation of ERK1/2 is required for the stimulation of both MMP1 and MMP3 by HA, p38 seems to play a crucial role only for MMP3. ERK1/2 and p38 kinase have been shown to be involved in the TNF-α activation of MMP1 and MMP3 by human skin fibroblasts.[53] The induction of MMP1 and MMP3 by these pathways was mediated by two different mechanisms: AP1-dependent via ERK1/2 and AP1-independent via p38 kinase. In this study, we found that native HA and HA fragments activate AP1 DNA binding, with a greater effect for native HA. Taken together, these results suggest that in our system, the stimulation of MMP3 expression may be mediated by two distinct mechanisms: AP1 dependent and independent. The activation of MMP1 most likely involves only the AP1/ERK-dependent mechanism. Only native HA was able to activate NF-κB in fibroblasts. This may constitute another mechanism by which native HA enhances the expression of MMP1 and MMP3.

We tried to determine whether the effects induced by HA and its fragments could be receptor mediated. We focused on the major HA receptor, CD44, using siRNA to down-regulate its synthesis. Depending on the parameters and the HA forms, the results were variable. It was found that CD44 was not implicated in fibroblast adhesion to all the three HA sizes. In contrast, CD44 appeared to play a role in the proliferative effect of HA and HA-880, whereas it was not implicated in HA-12–induced proliferation. Curiously, the stimulatory effect of HA-12 on type I collagen and TGF-β1 expression was found to depend on CD44 while it was not so for type III collagen and TGF-β3. Finally, our results concerning type III collagen suggest that CD44 only plays a role in the effect of native HA. In conclusion, although we favor the idea that the CD44 cell surface receptor is partially responsible for the signal transduction leading to the effects on gene expression observed here, it is likely that other receptors (including RHAMM/IHABP, Toll-like receptor 4, or yet unknown molecules) may be implicated in these mechanisms, especially for the HA fragments effects. It may be surprising that some effects of HA-12 could be mediated, at least partially, by CD44. However, it has been demonstrated using CD44-null mice that CD44 is partly responsible for removing HA fragments from the inflamed lung,[54] suggesting that, in some contexts, CD44 might be involved directly or indirectly in the metabolism of HA degradation products. Future work will try to identify the receptors that could, besides CD44, be responsible for some of the effects induced by HA and its fragments.

In summary, this shows that HA and its fragments may play crucial roles in the skin wound-healing process, by modulating the expression of fibroblast genes involved in

remodeling and repair of ECM. By its ability to enhance type III collagen synthesis and TGF-β3 expression in dermal fibroblasts, high molecular weight HA promotes the creation of a fetal-like cell environment, which is known to favor scarless healing, whereas the HA-12 oligosaccharide might favor a fibrotic phenotype through stimulation of type I collagen. While the precise mechanisms through which HA, as well as its enzymatically derived fragments, affect the ECM metabolism during the sequential events of wound repair require further investigation, these results suggest that the relative proportion of HA molecular sizes exerts a fine regulation of matrix state, from the early inflammatory steps to the final tissue recovery.

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