Hyaluronan Facilitates Transforming Growth Factor-β1-mediated Fibroblast Proliferation*

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This study aims to understand the role of the matrix polysaccharide hyaluronan (HA) in influencing fibroblast proliferation and thereby affecting wound healing outcomes. To determine mechanisms that underlie scarred versus scar-free healing, patient-matched dermal and oral mucosal fibroblasts were used as models ofscarring and non-scarring fibroblast phenotypes. Specifically, differences in HA generation between these different fibroblast populations have been examined and related to differences in transforming growth factor-β1 (TGF-β1)-dependent proliferative responses and Smad signaling. There was a differential growth response to TGF-β1, with it inducing proliferation in dermal fibroblasts but an anti-proliferative response in oral fibroblasts. Both responses were Smad3-dependent. Furthermore, the two fibroblast populations also demonstrated differences in their HA regulation, with dermal fibroblasts generating increased levels of HA, compared with oral fibroblasts. Inhibition of HA synthesis in dermal fibroblasts was shown to abrogate the TGF-β1-mediated induction of proliferation. Inhibition of HA synthesis also led to an attenuation of Smad3 signaling in dermal fibroblasts. Microarray analysis demonstrated no difference in the genes involved in TGF-β1 signaling between dermal and oral fibroblasts, whereas there was a distinct difference in the pattern of genes involved in HA regulation. In conclusion, these two distinct fibroblast populations demonstrate a differential proliferative response to TGF-β1, which is associated with differences in HA generation. TGF-β1 regulates proliferation through Smad3 signaling in both fibroblast populations; however, it is the levels of HA generated by the cells that influence the outcome of this response.

Scarring is an important response to tissue injury that facilitates repair through replacement of damaged tissue with fibrous tissue. In the context of dermal injury, this restores tissue strength and aids in the repair of structural defects. However, in some injuries, deposition of scar tissue may have adverse consequences. Following injury to internal organs, repair by scarring can initiate a cascade of events that continue unabated leading to progressive accumulation of fibrous tissue and eventually resulting in loss of organ function (1, 2). End-stage organ dysfunction because of progressive fibrosis comprises a wide range of disorders, including congestive cardiac failure, chronic kidney disease, pulmonary fibrosis, and liver cirrhosis, well-known causes of worldwide death and disability (3–8). Fibroblasts are the most abundant cell type in connective tissue, and they play a central role in extracellular matrix (ECM) remodeling and wound contraction during tissue repair. In response to tissue injury, they proliferate, migrate to the site of injury, and differentiate into their active form, myofibroblasts (2, 9, 10). These myofibroblasts are then involved in wound contraction and ECM synthesis and turnover (11). They are also considered to be the key effector cells in fibrotic disease, and increased activity and proliferation of resident fibroblasts are central to fibrosis in all tissues (2). The cytokine transforming growth factor-β1 (TGF-β1) is a known mediator of fibroblast-myofibroblast differentiation, and it mainly elicits its effects through the Smad signal transduction pathway (12). It also influences a range of other cellular processes, including migration and proliferation, and its release initiates a sequence of events that are crucial in tissue repair, including chemotaxis of inflammatory cells, induction of angiogenesis, and regulation of inflammatory mediators (13–17). However, aberrant expression of TGF-β1 has been demonstrated in virtually every type of fibrotic disease, and several reports have established that increased TGF-β1 expression directly correlates with progressive tissue fibrosis and disease progression (16, 18–20). In this context, a better understanding of factors involved in TGF-β1 regulation and its influence on fibroblast behavior is crucial in determining the pathogenic mechanisms underlying progressive fibrosis and may provide novel insights into developing new therapeutic strategies.

In comparison with other tissues, wounds in the oral mucosa are clinically distinguished in that they heal without notable scar formation resulting in complete regeneration of tissue structure and restoration of function. Several lines of evidence indicate that although the oral mucosal environment (in comparison to other environments) demonstrates differences in

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3 The abbreviations used are: ECM, extracellular matrix; TGF-β1, transforming growth factor-β1; FITC, fluorescein isothiocyanate; HA, hyaluronan; PI, propidium iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; QPCR, quantitative PCR; FBS, fetal bovine serum; PBS, phosphate-buffered saline; HABP, HA-binding protein; ELISA, enzyme-linked immunosorbent assay; AMU, 4-methylumbelliflorone; FACs, fluorescence-activated cell sorter; siRNA, short interfering RNA; TE, trypsin extract; CE, cell extract; TBS, Tris-buffered saline; Bistris, 2-[b(is-2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; IQR, inter-quartile range; MAPK, mitogen-activated protein kinase.
inflammatory cell infiltrate and cytokine gene expression, tissue from the oral mucosa also possesses intrinsic differences that can account for its differential wound healing profile (21–25). In an attempt to delineate the mechanisms that differentiate between scarring versus scar-free repair, we have used patient-matched oral mucosal and dermal fibroblasts, as models of non-scarring and scarring fibroblast phenotypes, respectively, to attempt to identify the mechanisms that could be exploited to prevent scarring following tissue injury.

We have previously demonstrated clear differences between oral mucosal and dermal fibroblasts that can account for the differential wound healing profiles of the two tissues. Fibroblasts derived from the oral mucosa have been shown to demonstrate increased migration, experimental wound repopulation, and extracellular matrix re-organization as compared with fibroblasts derived from the dermis of the same individual (24, 25). We have also shown recently that these same fibroblast populations demonstrate intrinsic differences in their ability to differentiate in response to the pro-fibrotic cytokine TGF-β1. Although dermal fibroblasts readily differentiate in response to TGF-β1, oral mucosal fibroblasts are resistant to TGF-β1-driven myofibroblastic differentiation. Furthermore, we demonstrated that the matrix polysaccharide hyaluronan (HA) plays a pivotal role in regulating TGF-β1-driven cellular differentiation in that it facilitates fibroblast-myoﬁbroblast transition (21, 22). The purpose of this study was to expand these observations by comparing differences in other TGF-β1-mediated cell responses in scarring and non-scarring fibroblast phenotypes. Furthermore, this work aimed to test the general applicability of the hypothesis that intrinsic differences in HA generation in the two fibroblast populations is the key to determining cellular responses and influencing wound healing outcomes.

The data presented here show that fibroblasts derived from the dermis and patient-matched oral mucosa demonstrate a differential proliferative response to TGF-β1. Although TGF-β1 stimulates proliferation in dermal fibroblasts, it inhibits DNA synthesis and induces an anti-proliferative response in oral mucosal fibroblasts. The two fibroblast populations also demonstrate differences in their HA regulation, with dermal fibroblasts generating increased levels of HA as compared with oral fibroblasts. Furthermore, the data demonstrate that TGF-β1 regulates proliferation through Smad3 signaling in both fibroblast populations. However, the proliferative response to TGF-β1 in dermal fibroblasts appears to be directly linked to the increased levels of HA generated by these cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were from Sigma unless otherwise stated. Reverse transcription and QPCR reagents and primers were purchased from Invitrogen and Applied Biosystems (Cheshire, UK). Radioisotopes were purchased from Amersham Biosciences.

**Cell Culture**—Four donor-matched samples of dermal and oral mucosal fibroblasts were obtained by biopsy from consenting adults undergoing routine minor surgery, and ethical approval for the biopsies was obtained from the South Wales Research Ethics Committee. The cells were isolated as described previously (25) and cultured in Dulbecco’s modified Eagle’s medium and F-12 medium containing 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin supplemented with 10% fetal bovine serum (FBS) (Biologic Industries Ltd., Cumbernauld, UK). The cells were maintained at 37 °C in a humidified incubator in an atmosphere of 5% CO2, and fresh growth medium was added to the cells every 3–4 days until confluent. The cells were incubated in serum-free medium for 48 h before use in experiments, and all experiments were done under serum-free conditions unless otherwise stated. All experiments were undertaken using cells at passage 6–10 and performed on confluent cultures except for those examining proliferation. These experiments used subconfluent cells to allow for cell growth.

**Analysis of Cell Proliferation**

[^3H]Thymidine Proliferation Assay—Fibroblast proliferation was first assessed by the incorporation of d[^3H]thymidine into DNA. Cells were grown in 35-mm dishes and assessed at subconfluence. Metabolic labeling was performed by incubation with 1 µCi/ml d[^3H]thymidine for 24 h. The medium was then discarded, and the cells were washed repeatedly with PBS containing 1 mM thymidine prior to fixing with 500 µl of 5% trichloroacetic acid containing 1 mM thymidine at 4 °C for 1 h. The cell layer was extracted by incubation with 1 ml of 0.1 M NaOH at 20 °C for 24 h and neutralized with 0.1 M HCl. The radioactivity was determined by β-counting on a Packard Tri-Carb 1900 liquid scintillation analyzer, and the results are represented as disintegrations/min.

alamarBlue™ Proliferation Assay—Analysis of cell growth was also assessed by the commercial alamarBlue™ assay (BIOSOURCE), which is designed to measure cell viability and number. The assay utilizes an oxidation-reduction indicator that fluoresces in response to chemical reduction of growth medium resulting from cell growth and metabolism and demonstrates a linear relationship between the magnitude of fluorescence and cell number and viability. For this assay cells were grown in 35-mm dishes and assessed at subconfluence following a 48-h period of growth arrest. The cells were then stimulated with 10 ng/ml TGF-β1. Following 24 h, 10% alamarBlue was added to the medium for 1 h at 37 °C. 100-µl aliquots of the conditioned medium were removed and added to a clear 96-well plate. Subsequently fluorescence was measured in a Fluostar optima fluorescence meter (BMG Lab Technologies) with excitation wavelength at 540 nm and emission wavelength at 590 nm; and the results are expressed as arbitrary fluorescence units.

**Analysis of Cell Apoptosis**

Fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide were used to confirm the presence of apoptotic cells in in vitro cultures. Cells were grown to confluence in 35-mm dishes, washed in PBS, and then harvested using trypsin/EDTA. The cells were then resuspended in 100 µl of the reaction mix made up of 90 µl of distilled water, 10 µl of 10× binding buffer (100 mM HEPES, 1.5 mM NaCl, 50 mM KCl, 10 mM MgCl2, and 18 mM CaCl2, pH 7.4), 5 µl of FITC-annexin-V (Pharmingen), and 5 µl of propidium iodide (PI) (Pharmingen). The cells were incubated in the dark for 20 min at room tem-
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perature, centrifuged, washed in PBS, and resuspended in 1× binding buffer. The samples were analyzed immediately by flow cytometry (FACSCalibur). The viable cells were annexin-V/PI-negative, and cells in early apoptosis were annexin-V-positive and PI-negative, and cells in late apoptosis/necrosis were annexin-V/PI-positive.

Cell Cycle Analysis

The distribution of cells within the cell cycle was determined by propidium iodide incorporation into DNA. Cells were grown in 35-mm dishes and assessed at subconfluence. Briefly, cells were harvested by treatment with trypsin/EDTA, pelleted by centrifugation, and then washed in PBS. The cells were then resuspended in cold 70% ethanol and maintained at 4°C for 16 h. Subsequently, the cells were centrifuged, washed in PBS, and then resuspended in 300 μl of binding buffer and 10 μl of 50 μg/ml PI solution along with 5 μl/sample RNase (10 mg/ml stock). The samples were then incubated at 37°C for 30 min prior to FACS analysis. The percentage of cells in the different phases of the cell cycle were quantified using Cylchred version 1.0.2 software (Terry Hoy, University Hospital of Wales, Cardiff, UK).

Immunoblotting/Western Analysis

Western blot analysis was used to assess expression of phosphorylated Smad2 and Smad3. Cells were grown to confluence in 35-mm dishes and rinsed with cold PBS. Cells were then lysed using 1% protease inhibitor mixture, 1% phenylmethylsulfonyl fluoride, and 1% sodium orthovanadate in RIPA lysis buffer (Santa Cruz Biotechnology). The samples were scraped, collected, and centrifuged at 2500 rpm for 10 min. The supernatant was collected, and protein concentrations were determined by Bradford assay, and the samples were stored at −70°C until use. Equal amounts of protein were mixed with equal volumes of reducing SDS sample buffer and denatured for 5 min at 95°C before loading onto 10% SDS-polyacrylamide gels. Electrophoresis was carried out under reducing conditions at 150 V for 1 h, and the separated proteins were then transferred at 150 V over 90 min to a nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with Tris-buffered saline (TBS) containing 5% nonfat powdered milk for 1 h and then incubated with the primary antibody (anti-phosphorylated Smad3 or anti-phosphorylated Smad2, 1:500 dilution in TBS, host:rabbit) at 4°C overnight (Cell Signaling Technology, Danvers, MA). Expression of GAPDH was eluted in 8 μl urea buffer containing 0.3 M NaCl. Each sample was split into two, and the HA was precipitated with 1% potassium acetate in 95% ethanol in the presence of 50 μg/ml of each HA, heparin, and chondroitin sulfate as co-precipitants. The first half of each sample was resuspended in 500 μl of 4 M guanidine buffer and analyzed on a Sephacryl S-500 column equilibrated with 4 M guanidine buffer. To confirm that the chromatography profile generated was the result of radiolabeled HA, the second half of each sample was digested at 37°C overnight with 1 unit of Streptomyces hyalurolyticus hyaluronidase (ICN Pharmaceuticals Ltd.) in 200 μl of 20 mM sodium acetate, pH 6.0, containing 0.05% sodium azide and 0.15 M sodium chloride. The sample was then mixed with an equal volume of 4 M guanidine buffer and analyzed on the same Sephacryl S-500 column equilibrated with 4 M guanidine buffer. To produce the chromatography profile the ³H activity for each half of the sample was normalized and corrected for dilution, and then the hyaluronidase-resistant counts were subtracted. The chromatography profiles depict only the hyaluronidase-sensitive activity in each fraction plotted against fraction number. The column was calibrated with [³H]glucosamine hydrochloride, Mₙ 215; [³⁵S]chondroitin sulfate glycosaminoglycans, Mₙ 25 × 10³; decorin, Mₙ 10 × 10³; and [³⁵S]versican, Mₙ 1.3 × 10⁶. The radioactivity was determined by β-counting on a Packard Tri-Carb 1900 liquid scintillation analyzer, and the results are represented as disintegrations/min.

Determination of HA Concentration

Cells were grown to confluence in 35-mm dishes, and the HA concentration in the cell culture supernatant was determined using a commercially available enzyme-linked HA-binding protein assay (HA “Chuagu” quantitative test kit; Congenix, Petersborough, UK). The assay used microwells coated with a highly specific HA-binding protein (HABP) from bovine cartilage to capture HA and an enzyme-conjugated version of HABP to detect and measure HA in the samples. Briefly, diluted samples and HA reference solutions were incubated in HABP-coated microwells allowing binding of the HA in the samples to the immobilized HABP. The wells were then washed, and HABP conjugated with horseradish peroxidase was added to the wells forming complexes with bound HA. Following a sec-
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Determination of TGF-β1 Production

TGF-β1 concentration in the culture supernatant was determined by specific enzyme-linked immunosorbent assay (ELISA) (R & D Systems Europe Ltd.). This assay has less than 1% cross-reactivity with TGF-β2 and TGF-β3. Briefly, high protein-binding 96-well plates were coated with TGF-β capture antibody (2 μg/ml in PBS) overnight at room temperature. Wells were washed, and the plate was incubated for 1 h at room temperature with block buffer (5% Tween 20, 5% sucrose in PBS) and then washed before addition of TGF-β standards and cell culture supernatant samples. Cells were grown to confluence in 35-mm dishes and growth-arrested in serum-free medium for 48 h. Subsequently the medium was changed to medium containing 0.5 mM 4MU or serum-free medium alone, and the incubations were continued for a further 24 h. 20 μl of 1 M HCl was then added to 100 l cell supernatant and the incubations were continued for 24 h. Analysis of cell growth was performed using the alamarBlue™ assay as described under "Experimental Procedures," and the results are expressed as median ± IQR of four donor-matched oral and dermal fibroblasts, each in triplicate. Statistical analysis was performed using the Friedman test (p = 0.001) followed by the Wilcoxon Signed-Rank test and statistical significance was taken as p < 0.05.

FIGURE 1. Proliferative response of oral mucosal and dermal fibroblasts in response to TGF-β1. Subconfluent monolayers of oral mucosal and dermal fibroblasts were growth-arrested in serum-free medium for 48 h. A, medium was removed, and the cells were metabolically labeled with 1 μCi/ml [3H]thymidine for 24 h in serum-free medium alone (control) or containing 10 ng/ml TGF-β1. DNA synthesis was estimated as described under "Experimental Procedures." The results are presented as median ± IQR of four donor-matched oral and dermal fibroblasts, each in triplicate. For each box plot, median values are represented by the line within the box. The box represents 50% of the values (the 25th and 75th centiles), with the bars presenting the highest and lowest values, excluding outliers (o). Statistical analysis was performed using the Friedman test (p < 0.001 as shown) followed by the Wilcoxon Signed-Rank test, and statistical significance was taken as p < 0.05. B, medium was removed and replaced with either serum-free medium alone (control) or serum-free medium containing 10 ng/ml TGF-β1, and the incubations were continued for 24 h. Analysis of cell growth was performed using the alamarBlue™ assay as described under "Experimental Procedures," and the results are expressed as median ± IQR of four donor-matched oral and dermal fibroblasts, each in triplicate. Statistical analysis was performed using the Friedman test (p = 0.04) followed by the Wilcoxon Signed-Rank test and statistical significance was taken as p < 0.05.

FIGURE 2. Apoptotic response of oral mucosal and dermal fibroblasts in response to TGF-β1. Confluent monolayers of oral mucosal (A) and dermal (B) fibroblasts were growth-arrested in serum-free medium for 48 h. The medium was removed and replaced with either serum-free medium alone (control) or serum-free medium containing 10 ng/ml TGF-β1, and the incubations were continued for 24 h. Apoptotic cells were identified by flow cytometry using annexin-V-FITC in conjunction with PI to distinguish early apoptotic cells (annexin-V-FITC-positive, PI-negative) from late apoptotic/necrotic cells (annexin-V-FITC-positive, PI-positive). Results are presented graphically as the % of total apoptotic cells and represent the mean of three individual experiments.

ond washing step, a chromogenic substrate (TMB:H2O2) was added to develop a colored reaction. Stopping solution was added to the wells, and the intensity of the resulting color was measured in optical density units using a spectrophotometer at 450 nm. HA concentrations were calculated by comparing the absorbance of the sample against a reference curve prepared from the reagent blank and five HA reference solutions (50, 100, 200, 500, and 800 ng/ml) included in the kit. The assay is sensitive to 10 ng/ml, with no cross-reactivity with other glycosaminoglycan compounds.
2 h at room temperature and washed again. Streptavidin-horse-radish peroxidase (0.5% in reagent diluent) was added to each well, the plate was incubated in the dark for 20 min and then washed. Substrate solution (TMB/H2O2) was added to the wells, and the plate was incubated in the dark for 20 min before the addition of stop solution. The intensity of the resulting color was measured in optical density units using a spectrophotometer at 450 nm. TGF-β1 concentration was calculated by comparing the absorbance of the sample against a reference curve prepared from the reagent blank and the standards.

Cytotoxicity Assay

Cytotoxicity of 4MU was assessed by the alamarBlue™ assay. Subconfluent dermal fibroblasts were incubated for 24 h in serum-free medium alone or serum-free medium containing 1, 0.5, 0.4, 0.3, or 0.2 mM 4MU. Following 24 h 10% alamarBlue was added to the medium for 1 h at 37 °C. 100-µl aliquots of the conditioned medium were removed and added to a clear 96-well plate. Subsequently fluorescence was measured in a Fluostar optima fluorescence meter (BMG Lab Technologies) with excitation wavelength at 540 nm and emission wavelength at 590 nm; and the results are expressed as arbitrary fluorescence units.

Smad3 siRNA Transfection

Transient transfection of dermal and oral fibroblasts with specific siRNA nucleotides targeting Smad3 expression was performed using siPORT amine transfection reagent (Ambion Ltd., Huntington, UK) in accordance with the manufacturer’s protocol. Briefly, 8 µl of transfection agent was diluted in 100 µl of Opti-MEM reduced growth medium (Invitrogen) and left to incubate at room temperature for 10 min. Meanwhile, the specific Smad3 siRNA oligonucleotides were diluted in Opti-MEM reduced growth medium to give a final concentration of 20 µM in a total volume of 100 µl. The transfection agent mix and siRNA mix were then combined and incubated at room temperature for a further 10 min. The newly formed transfection complexes (200 µl) were dispensed into empty wells of 6-well culture plates. To each well, 2.3 × 10^5 cells were then added so that the total volume in each well was 2500 µl. As a control, cells were transfected with negative control siRNA (a scrambled sequence that bears no homology to the human genome). The cells were then incubated at 37 °C with 5% CO2 for 24 h in medium supplemented with 10% FBS followed by a 24-h incubation in serum-free medium prior to experimentation.

Reverse Transcription (RT) and Quantitative PCR (QPCR)

RT-QPCR was used to assess Smad3, HAS2, versican, and aggregan mRNA expression in oral mucosal and dermal fibroblasts.
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The cells were grown to confluence in 35-mm dishes and washed with PBS prior to lysis with tri-reagent and RNA purification according to the manufacturer’s protocol. RT was performed using the random hexamer method. 1 µg of RNA was added to 1 µl of 100 µM random hexamers, 2 µl of 10× PCR buffer, and 2 µl of 0.1 M dithiothreitol. The solution was heated to 95 °C for 5 min followed by 4 °C for 2 min. 1 µl of (40 units/µl) ribonuclease inhibitor RNasin (Promega) and 1 µl of (200 units/µl) Superscript were added to each sample and mixed. The solution was incubated at 20 °C for 10 min, 42 °C for 40 min, and then 95 °C for 5 min on a GeneAmp PCR System 9700. As a negative control RT was performed with sterile H₂O replacing the RNA sample.

QPCR was performed using the 7900HT fast real time PCR system from Applied Biosciences. PCR was carried out in a final volume of 25 µl/sample as follows: 1 µl of RT product, 1.25 µl of target gene primers and probe (commercially designed and purchased from Applied Biosciences), 12.5 µl of Taqman Universal PCR mastermix, and 10.25 µl of sterile H₂O. Amplification was carried out using a cycle of 95 °C for 1s and 60 °C for 20 s for 40 cycles. As a negative control, PCR was performed with sterile H₂O replacing the cDNA sample. PCR was simultaneously done for ribosomal RNA (primers and probe commercially designed and purchased from Applied Biosciences) as a standard reference gene.

The comparative CT method was used for relative quantification of gene expression. The CT (threshold cycle where amplification is in the linear range of the amplification curve) for the standard reference gene (ribosomal RNA) was subtracted from the target gene CT to obtain the ΔCT. The mean ΔCT for similar samples were then calculated. The expression of the target gene in experimental samples relative to expression in control samples was then calculated using Equation 1,

\[ 2^{-\Delta CT(1) - \Delta CT(2)} \]  

where ΔCT(1) is the mean ΔCT calculated for the experimental samples and ΔCT(2) is the mean ΔCT calculated for the control samples.

**Microarray Analysis**

Gene expression profiles of oral mucosal and donor-matched dermal fibroblasts were assessed using Affymetrix GeneChip® technology. The detailed protocol for sample preparation and microarray processing is available from Affymetrix.

Briefly, total RNA was isolated from unstimulated oral mucosal and dermal fibroblasts, and RNA extraction was performed as above. 5 µg of total RNA was then reverse-transcribed using a T7-oligo(dT) promoter primer in the first strand cDNA synthesis reaction. Following second strand cDNA synthesis in the presence of RNase H, the double-stranded cDNA was purified, which served as a template in the subsequent in vitro transcription reaction. The in vitro transcription reaction was carried out in the presence of T7 RNA polymerase and a biotinylated nucleotide analog ribonucleotide mix for cRNA amplification and biotin labeling. The biotinylated cRNA targets were then fragmented and hybridized to the Affymetrix U133A GeneChip, which contains 25K 500 sequences derived from the GenBank™ data base. After 16 h of hybridization, the chips were washed and stained with streptavidin-phycocerythrin in a fluids station and then scanned in the GeneArray® scanner. The MAS 5.0 software package was used to generate expression intensity values for each probe set on the GeneChip® (CEL files). The .cel file data were processed using robust multarray average
to generate expression values relative to the mean expression of all probes across all the chips used in the experiment.

**Statistical Analysis**

The results are expressed as the means ± S.D. when normally distributed and median ± inter-quartile range (IQR) when not normally distributed. For nonparametric data the Friedman test was used for global comparison of different groups followed by the Wilcoxon Signed-Rank test for subgroup analysis. For normally distributed data, the unpaired t-test was used for statistical analysis. All data were analyzed using software (SPSS 14.0 Chicago), and p < 0.05 was considered significant.

**RESULTS**

**TGF-β1 Differentially Regulates the Proliferation of Oral and Dermal Fibroblasts and This Is Mediated through Smad3 Signaling in Both Fibroblast Phenotypes** — The effect of TGF-β1 on fibroblast proliferation was assessed by the incorporation of [3H]thymidine into cellular DNA (Fig. 1A). In addition, assessment of cellular growth and cell counts was performed using the alamarBlue™ assay (Fig. 1B). The results from both proliferation assays demonstrated that TGF-β1 exerted a differential effect on human dermal and oral mucosal fibroblasts. In particular, it strongly inhibited DNA synthesis and cellular growth in fibroblasts derived from the non-scarring oral mucosa, whereas it stimulated DNA synthesis and cellular growth in fibroblasts derived from the dermis of the same individuals. These results were consistent for all four of the donor-matched samples that were assessed. The effect of TGF-β1 on cellular survival was then assessed using FACS analysis. The results demonstrated no significant apoptosis in response to TGF-β1 in either oral mucosal (Fig. 2A) or dermal fibroblasts (Fig. 2B). These observations were subsequently followed up with cell cycle analysis using propidium iodide staining and FACS analysis. The results revealed that ~18% of unstimulated oral mucosal fibroblasts were in the S phase of the cell cycle. This decreases to ~10% of oral mucosal cells in the S phase following TGF-β1 stimulation (Fig. 3). In unstimulated dermal fibroblasts, only 15% of cells are found in the S phase of the cell cycle. However, following stimulation with TGF-β1, this increases to ~40% of cells in the S phase. This was associated with a corresponding reduction in the percentage of cells in G0/G1 phase of the cell cycle from 65 to 45% following TGF-β1 stimulation (Fig. 3).

Smad2 and Smad3 are the main intracellular receptor-regulated Smads involved in the TGF-β1 signal transduction pathway. In light of the above results, we subsequently assessed the ability of the two fibroblast populations to activate Smad2 and Smad3 in response to TGF-β1 receptor activation. Western blot analysis illustrated that oral and dermal fibroblasts both strongly phosphorylated Smad2 and Smad3 in response to TGF-β1 stimulation (Fig. 4, A and B).

Smad3 signaling has been demonstrated previously to be important in TGF-β1-mediated regulation of cell proliferation (26). To assess the role of Smad3 phosphorylation in the regulation of fibroblast proliferation, we used siRNA to down-regulate Smad3 levels. QPCR was used to confirm down-regulation of Smad3 expression. Both oral mucosal (Fig. 5A) and dermal (Fig. 6A) fibroblasts transfected with Smad3 siRNA demonstrated potent down-regulation in Smad3 expression as compared with fibroblasts transfected with scrambled controls. In oral mucosal fibroblasts, Smad3 down-regulation resulted in stimulation of DNA synthesis and loss of the TGF-β1-mediated anti-proliferative response. In comparison, oral mucosal fibroblasts transfected with scrambled siRNA responded in a similar manner as nontransfected cells and demonstrated an anti-proliferative response to TGF-β1 (Fig. 5B). In dermal fibroblasts, transfection with scrambled siRNA resulted in a proliferative
response to TGF-β1, in a similar manner to nontransfected cells. Interestingly, however, the TGF-β1-mediated induction in proliferation was abrogated in dermal fibroblasts with reduced Smad3 expression (Fig. 6B).

**Dermal Fibroblasts Generate More HA than Oral Fibroblasts**—Labeling of fibroblasts with [3H]glucosamine for 24 h under serum-free conditions demonstrated that dermal fibroblasts have an increased base-line synthesis of HA compared with donor-matched fibroblasts derived from the non-scarring oral mucosa. Analysis of HA by size exclusion chromatography indicated that there was ∼3-fold more HA present in the conditioned medium of dermal fibroblasts compared with oral mucosal fibroblasts (Fig. 7A). Dermal fibroblasts also had 3-fold more cell-associated (CE) HA (Fig. 7B) but had similar amounts of HA associated with the trypsin-accessible cell-surface extract (TE), however, showed an increase in HA in both oral and dermal fibroblasts (data not shown). These results were further confirmed by measurement of extracellular HA concentration in the conditioned medium for all four donor-matched samples using ELISA. In contrast, oral fibroblasts demonstrated a significant reduction in extracellular HA concentration in response to TGF-β1 (Fig. 8C). The implications of the increased extracellular HA concentration in response to TGF-β1

(Fig. 8B). These changes were confirmed following normalization for cell numbers (Fig. 8, D and E).

**Increased HA Generation in Dermal Fibroblasts Is Causally Related to TGF-β1-mediated Stimulation of Fibroblast Proliferation**—The implications of the increased extracellular HA generation in dermal fibroblasts were investigated by inhibiting HA synthesis in these cells. Depletion of the UDP-glucuronic acid pool using 4MU has been shown to inhibit HA synthesis and HA synthase enzyme activity in a number of cell types, including fibroblasts (27–31). Metabolic radiolabeling and size exclusion chromatography were used to establish 0.5 mm as the optimal effective concentration of 4MU, leading to a greater than 50% inhibition of total HA production (Fig. 9A). In addition, 0.5 mm was determined to be a nontoxic 4MU dose as assessed by the alamarBlue cytotoxicity assay indicating over 90% cell survival at this dose (data not shown).

[3H]Thymidine incorporation demonstrated that stimulation with TGF-β1 alone resulted in an induction of dermal fibroblast proliferation as demonstrated previously. In contrast, following inhibition of HA synthesis, addition of TGF-β1 significantly inhibited DNA synthesis and resulted in an anti-proliferative response (Fig. 9B) (p = 0.002). These results were reproduced following assessment of cellular growth and cell counts using the alamarBlue proliferation assay (data not shown). Cell cycle analysis revealed an increase in the percentage of dermal fibroblasts in the S phase of the cell cycle follow-
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To investigate the mechanism underlying the above HA-mediated effect of inhibiting HA synthesis on the TGF-β1-mediated anti-proliferative response, we assessed the influence dermal fibroblast apoptosis as assessed by FACS analysis (Fig. 10). The results indicated that incubation of oral mucosal fibroblasts with 4MU did not significantly influence the TGF-β1-mediated Smad2/3 signaling (data not shown). However, Western blot analysis of phosphorylated Smad3 in dermal fibroblasts demonstrated a consistent attenuation of Smad3 signaling in the presence of 4MU in all four patients (Fig. 11A). This was confirmed with densitometric analysis demonstrating a statistically significant attenuation of phosphorylated Smad3 at 30 min in the presence of 4MU (p = 0.03). Densitometric analysis also demonstrated an attenuation of phosphorylated Smad3 at 30 min in the presence of 4MU, although this was not statistically significant (p = 0.06). Subsequently the effect of 4MU on total and active TGF-β1 production in dermal fibroblasts was assessed using ELISA. The results demonstrate that incubation of dermal fibroblasts with 4MU for 24 h did not influence either active or total TGF-β1 production (Fig. 11C).

To confirm that these effects were as a result of inhibiting HA synthesis rather than the influence of 4MU itself, we repeated the above experiments using oral mucosal fibroblasts. The results indicated that incubation of oral mucosal fibroblasts with 4MU did not significantly influence Smad2 signaling, nor did it interfere with TGF-β1-induced Smad2/3 signaling (data not shown).

Transcription Profiles of Genes Involved in HA Regulation and TGF-β1 Signaling in Oral Mucosal and Dermal Fibroblasts—As the results indicated no differences in stimulation with TGF-β1 alone. However, stimulation of dermal fibroblasts with TGF-β1 in the presence of 4MU resulted in a reduction in the percentage of cells in the S phase of the cell cycle from ~30 to 10% and an increase in the percentage of dermal fibroblasts in G0/G1 phase of the cell cycle from 60 to 80% (Fig. 9C). In addition, 4MU did not appear to influence dermal fibroblast apoptosis as assessed by FACS analysis (Fig. 10).

To investigate the mechanism underlying the above HA-dependent proliferative fibroblast response, we assessed the effect of inhibiting HA synthesis on the TGF-β1-mediated dermal and oral mucosal fibroblast populations with respect to Smad2/3 activation, we subsequently used Affymetrix GeneChip® technology to assess potential differences in transcription profiles of other components of the TGF-β signaling pathway in these cells. Although the two fibroblast populations demonstrated a differential response to TGF-β1 stimulation, the microarray analysis did not demonstrate a differential regulation of genes involved in the TGF-β1 signaling pathway (Fig. 12A). Furthermore, there was no statistically significant difference in the expression of these genes (Table 1).
In addition, we assessed the transcription profiles of genes involved in the regulation of HA and demonstrated a differential expression of genes involved in HA synthesis and packaging into pericellular aggregates (Fig. 12B). In particular, dermal fibroblasts demonstrated an increased base-line expression of one of the HA synthase enzymes (HAS2) \((p = 0.03)\), an increased base-line expression of aggrecan \((p = 0.02)\), and a reduced base-line expression of the chondroitin sulfate proteoglycan, versican \((p = 0.009)\) (Table 2). These observations were confirmed by QPCR demonstrating increased base-line expression of HAS2 and aggrecan in dermal fibroblasts and increased base-line expression of versican in oral mucosal fibroblasts (Fig. 12C).

**DISCUSSION**

The cytokine TGF-\(\beta_1\) plays an important role in regulating the wound healing and tissue repair process, and aberration of its action has been implicated in several pathological processes, including cancer, autoimmune disease, and fibrosis (15, 32–34). It mainly elicits its effects through the Smad signal transduction pathway; however, TGF-\(\beta\) receptors can also activate non-Smad pathways, including MAPK, Rho GTPases, and Protein kinase B (35–39). Its actions are wide ranging and vary depending on biological context and target cell type. For example, it has been reported that it can inhibit epithelial, endothelial, and hematopoietic cell growth, whereas it stimulates the growth of mesenchymal cells (34, 40). Cell proliferation as well as epithelial mesenchymal transition and differentiation are TGF-\(\beta_1\)-regulated responses important in both cancer and fibrotic disease. Therefore, any mechanisms that are involved in regulating the outcome of these responses may be potentially used in the development of therapies to target these disease processes.
Our previous work has demonstrated that fibroblasts derived from the adult dermis and oral mucosa exhibit intrinsic differences in their ability to differentiate in response to TGF-β1 (21, 22). In this study we have demonstrated that these distinct fibroblast populations also demonstrate a differential proliferative response to TGF-β1. TGF-β1-induced proliferation in adult dermal fibroblasts and increased the proportion of cells in the S phase of the cell cycle, whereas it inhibited proliferation and DNA synthesis in fibroblasts derived from the non-scarring oral mucosa. Furthermore, we have demonstrated that although the two fibroblast populations demonstrated a differential proliferative response to TGF-β1, they both demonstrated a minimal apoptotic response to TGF-β1 confirming that the differences in cell numbers and DNA synthesis observed were as a result of a differential regulation of cell proliferation rather than cell survival. These results led us to examine the ability of the two fibroblast populations to activate the TGF-β1 signaling machinery. Both fibroblast populations demonstrated strong Smad2 and Smad3 activation in response to stimulation. Furthermore, microarray analysis using Affymetrix GeneChip technology did not demonstrate a significant difference in the transcription profiles of genes involved in the main TGF-β1 signaling pathway between the two fibroblast populations. Recently Nicolas et al. (26) demonstrated that epithelial mesenchymal transition in Madin-Darby canine kidney cells resulted in gradual down-regulation of Smad3 expression and was associated with loss of the ability of the cells to undergo growth arrest in response to TGF-β1. They further demonstrated that responsiveness to TGF-β1-mediated growth arrest was restored when Smad3 was re-expressed in these cells, suggesting a critical role for Smad3 in the control of cell proliferation. Given these reports we assessed the role of Smad3 in TGF-β1-dependent fibroblast proliferation and found that it appears to be involved in the regulation of both the proliferative response in dermal fibroblasts as well as the anti-proliferative response in oral mucosal fibroblasts. This therefore suggests the involvement of other factors in the control of the Smad3-dependent proliferative response. We and others have previously demonstrated that the linear polysaccharide, HA, plays a pivotal role in regulating in the process of TGF-β1-mediated fibroblast differentiation (21, 22, 41). This led to the hypothesis that it may also be involved in the regulation of the proliferative response to TGF-β1.

HA is a ubiquitous component of extracellular matrix known for its role in maintaining matrix stability and tissue hydration. In addition, it has a major role in regulating cell functions through interaction with cell-surface receptors (principally CD44 and RHAMM) and also by generation of cell-surface HA aggregates in association with HA-binding proteins (hyaladherins) (41–48). As a result it is an important regulator of tissue remodeling and has been implicated in a number of biological and pathological processes, including wound healing, embryonic development, tumor growth, and inflammation (49–54). It is a prominent component of the stroma surrounding many tumors, and its presence has been associated with tumor progression and demonstrated to be a negative predictor of patient survival (50, 51). In addition, increased expression of HA has been detected in numerous fibrotic conditions associated with organ dysfunction (55–59). However, the mechanism by which HA influences these disease processes has not been clear. Our work has shown that the differential proliferative response to TGF-β1 in our two fibroblast populations was associated with differences in HA generation. Dermal fibroblasts generated increased levels of HA in comparison with oral mucosal fibroblasts, likely related to the increased expression of the HA synthase 2 enzyme (HAS2). To investigate the functional consequences of the increased HA generation, we studied the effect of the HA synthase enzyme inhibitor, 4MU, on TGF-β1-mediated proliferation in dermal fibroblasts as well as the anti-proliferative response in oral mucosal fibroblasts. This therefore suggests the involvement of other factors in the control of the Smad3-dependent proliferative response. We and others have previously demonstrated that the linear polysaccharide, HA, plays a pivotal role in regulating in the process of TGF-β1-mediated fibroblast differentiation (21, 22, 41). This led to the hypothesis that it may also be involved in the regulation of the proliferative response to TGF-β1.

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![Graph showing the effect of 4MU on dermal fibroblast apoptosis](Image)

**FIGURE 10. Effect of 4MU on dermal fibroblast apoptosis.** Confluent monolayers of dermal fibroblasts were growth-arrested in serum-free medium for 48 h. The medium was removed and replaced with either serum-free medium alone (control), serum-free medium containing 0.5 mM 4MU, serum-free medium containing 10 ng/ml TGF-β1, or serum-free medium containing both 0.5 mM 4MU and 10 ng/ml TGF-β1, and the incubations were continued for 24 h. Apoptotic cells were identified by flow cytometry using annexin-V-FITC in conjugation with PI to distinguish early apoptotic cells (annexin-V-FITC-positive, PI-negative) from late apoptotic/necrotic cells (annexin-V-FITC-positive, PI-positive). Results are presented graphically as the % of total apoptotic cells and represent the mean of three individual experiments.
blasts. Depletion of the UDP-glucuronic acid pool by 4MU has been shown previously to inhibit HA synthesis and pericellular HA coat formation in a number of cell types, including fibroblasts (27–30). The results indicated that dermal fibroblasts with lower levels of HA generation had a reduced proportion of cells in the S phase of the cell cycle and demonstrated an anti-proliferative response to TGF-β1, similar to the response seen in oral mucosal fibroblasts. In addition, inhibiting HA synthesis with 4MU resulted in attenuation of Smad3 signaling in these cells. It was also clear that 4MU did not achieve this by influencing the levels of TGF-β1 generated by the dermal fibroblasts. These data suggest that HA is essential for stimulation of DNA synthesis by TGF-β1, and it promotes fibroblast proliferation through Smad3 signaling.

HA is thought to influence biological actions in part through the formation of HA pericellular aggregates or "coats." In several cell types the pericellular HA coat has been associated with cell proliferation and migration and is prominent during processes such as inflammation, wound healing, and tumor invasion (45–47, 52, 60). Our recent data have shown that TGF-β1 induces HA pericellular coat assembly in dermal fibroblasts but not in oral fibroblasts. Furthermore, these recent data suggest that HA coat assembly in fibroblasts promotes fibrosis by facilitating TGF-β1-mediated fibroblast differentiation (22). Therefore, regulation of HA pericellular coat formation may be important in the regulation of TGF-β1-dependent responses. There is a current focus on the role of hyaladherins such as inter-α inhibitor, tumor necrosis factor-α stimulating gene-6 (TSG-6), and versican in regulating HA pericellular coat formation. Recent data suggest that changes in the balance of these hyaladherins regulates the ability of cells to assemble HA coats (44, 61). Our data have shown that the expression of one of these hyaladherins, versican, is differentially expressed in dermal and oral mucosal fibroblasts; demonstrating increased expression in oral fibroblasts. Increases in hyaladherin expression are seen in different lesions in a variety of diseases. For example, the accumulation of hyaluronan in early atherosclerotic lesions is often accompanied by increases in versican (62, 63). Treatment of arterial smooth
FIGURE 12. A, microarray analysis of genes involved in TGF-β1 signaling. Gene expression profiling of unstimulated oral mucosal and dermal fibroblasts was performed using Affymetrix GeneChip® technology. Results are demonstrated for four donor-matched samples of fibroblasts. Red squares indicate increased expression, and green squares indicate decreased expression relative to the mean expression level. Statistical analysis was performed using Student’s t test (see tables). B, microarray analysis of genes involved in HA regulation. Gene expression profiling of unstimulated oral mucosal and dermal fibroblasts was performed using Affymetrix GeneChip® technology. Results are demonstrated for four donor-matched samples of fibroblasts. Red squares indicate increased expression, and green squares indicate decreased expression relative to the mean expression level. Statistical analysis was performed using Student’s t test (see tables). C, versican, aggrecan, and HAS2 mRNA expression in unstimulated oral mucosal and dermal fibroblasts. Confluent monolayers of oral mucosal and dermal fibroblasts were growth-arrested in serum-free medium for 48 h. RT-QPCR was used to assess versican, aggrecan, and HAS2 mRNA expression as described under “Experimental Procedures,” and ribosomal RNA expression was used as an endogenous control. The comparative Ct method was used for relative quantification of gene expression, and gene expression was assessed relative to oral fibroblast samples. The results are expressed as the median ± IQR of for all four donor-matched samples of oral mucosal and dermal fibroblast, and statistical analysis was performed using the Wilcoxon Signed-Rank test, with statistical significance was taken as p < 0.05.
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It serves as a primary receptor for hyaluronan, providing cells with a mechanism for attachment to hyaluronan and thereby mediating cell-cell and cell-matrix interactions (73, 74). It also serves as an anchor for other components of the pericellular matrix and is involved in the activation of a number of signaling pathways (including tyrosine kinase, MAPK, and protein kinase C) (75–77). Our previous work has shown that fibroblast to myofibroblast differentiation resulted in an accumulation of HA (27). Furthermore, this was associated with re-localization of CD44 from a punctate distribution to a more diffuse staining pattern.⁴ The functional consequences of these observations have not yet been determined; however, segregation of CD44 into distinct compartments may be involved in regulation of intracellular signaling pathways, thereby influencing cell behavior. CD44 can also function as a co-receptor, and previous reports have demonstrated that it can localize with both TGF-β type I and type II receptors and thereby facilitate modulation of both Smad and non-Smad-dependent TGF-β₁-mediated events (78, 79).

In this study, we have shown that both dermal and oral mucosal fibroblasts respond to TGF-β₁. However, the levels of HA generated by these cells impact on the outcome of this response and may thereby affect the wound healing profiles of tissues and ultimately influence processes such as progressive fibrosis. These results may also provide increased insight into the mechanisms underlying the role of TGF-β₁ in tumor growth. TGF-β₁ plays a paradoxical role in cancer where it has an anti-proliferative effect on normal epithelial cells (from which most human tumors are derived) and acts as a tumor suppressor in early disease, yet functions as a promoter of cancer progression and metastasis in later stages of disease (80). We propose that in early disease, in the presence of minimal HA levels, TGF-β₁ induces an anti-proliferative response facilitating tumor suppression, whereas in advanced disease, the presence of a HA-rich matrix promotes cell proliferation thereby facilitating tumor progression.

⁴ R.H. Jenkins, G. J. Thomas, J. D. Williams, and R. Steadman, unpublished data.

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**TABLE 1**

| Gene symbol | Gene name | p value (t test) |
|-------------|-----------|-----------------|
| TGFB1       | Transforming growth factor β₁ | 0.63 |
| TGFB1R1     | Transforming growth factor receptor I | 0.91 |
| TGFB1R2     | Transforming growth factor receptor II | 0.69 |
| TGFB1R3     | Transforming growth factor receptor III | 0.75 |
| CTNBR1      | Catenin (cadherin-associated Protein), β₁ | 0.63 |

**TABLE 2**

| Gene symbol | Gene name | p value (t test) |
|-------------|-----------|-----------------|
| HAS1        | Hyaluronan synthase 1 | 0.29 |
| HAS2        | Hyaluronan synthase 2 | 0.03 |
| HLY1        | Hyaluronoglucosaminidase 1 | 0.72 |
| HLY2        | Hyaluronoglucosaminidase 2 | 0.52 |
| HLY3        | Hyaluronoglucosaminidase 3 | 0.45 |
| HMMR        | Hyaluronan-mediated motility receptor (RHAMM) | 0.45 |
| CD44        | CD44 antigen (HA receptor) | 0.98 |
| AGC1        | Aggrecan 1 (chondroitin sulfate proteoglycan 1, large aggregating proteoglycan, antigen identified by monoclonal antibody A0122) | 0.02 |
| CSPG2       | Chondroitin sulfate proteoglycan 2 (versican) | 0.009 |
| AMBP        | α1-Microglobulin/bikunin precursor | 0.57 |
| TNFAIP6     | Tumor necrosis factor, α-induced protein 6 | 0.35 |

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**Gene expression and regulation**

Muscle cells in culture with growth factors implicated in both fibrosis and atherosclerotic disease increased the expression of versican (64). Interleukin-1, however, a cytokine central to inflammation, decreased the expression of versican (65). Versican expression also correlates with the metastatic potential and invasiveness of a variety of tumor cells (66, 67). Furthermore, the ECM of tumors is enriched in both versican and other HA-binding proteoglycans (68). Some recent reports have suggested that increased versican expression hampers HA pericellular coat formation (61). However, others have indicated that versican may promote pericellular matrix assembly (67). Mechanisms that regulate versican expression therefore may be cell-specific but may also be a potential therapeutic target for the control of TGF-β₁-dependent cell responses and for the phenotypic fate of cells. Aggrecan, another chondroitin sulfate proteoglycan, was also found to be differentially expressed in dermal and oral fibroblasts, demonstrating increased expression in dermal fibroblasts. This hyaladherin is mainly present in articular cartilage, and the significance of its increased expression in dermal fibroblasts is poorly understood but may be associated with the increased viscoelastic properties of dermal tissue compared with oral mucosal tissue.

Assembly and retention of the HA pericellular matrix has also been demonstrated to be dependent on hyaluronan-CD44 interactions (69, 70). CD44 is a transmembrane receptor that is encoded by a single gene but is expressed as multiple isoforms, the result of alternative RNA splicing (71, 72).
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In conclusion, TGF-β1 differentially regulates the proliferation of fibroblasts with scarring and non-scarring phenotypes in a Smad3-dependent manner. It is, however, the levels of HA generated by the different fibroblast phenotypes that affect the outcome of this response.

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