During the initiation and progression of fibrosis there is extensive differentiation of cells to a myofibroblastic phenotype. Because the synthesis of hyaluronan (HA) was recently linked to oncogenic epithelial-mesenchymal transformation, the present study investigated whether increased HA synthesis was also associated with myofibroblastic differentiation. HA synthesis and size were measured by incorporation of [3H]glucosamine, ion exchange, and size exclusion chromatography. Hyaluronan synthase (HAS) or hyaluronidase (HYAL) mRNA levels were assessed by reverse transcription-PCR. HYAL was detected by immunoblotting and the degradation of [3H]HA. Between 2- and 3-fold more HA appeared in the conditioned medium and became associated with the cells upon myofibroblastic differentiation. Inhibition of HAS and examination of HAS mRNA expression demonstrated that this was not the result of increased synthesis of HA or the induction of HAS 2. After differentiation, however, myofibroblasts metabolized exogenously supplied [3H]HA at a slower rate than fibroblasts and expressed lower levels of both HYAL 1 and HYAL 2 mRNA. Immunoblotting revealed more HYAL 1 and 2 in the myofibroblast conditioned medium. After acidification, however, there was no difference in HA degradation. This suggests that much of the released HYAL is inactive and that the observed differences in HA degradation are caused by cell-associated rather than secreted activity. This was confirmed by immunohistochemical staining for HYAL 1 and HYAL 2. This finding indicates the potential importance of the HYAL enzymes in controlling fibrotic progression and contrasts HA synthesis as a mediator of oncogenic transformation with that of HA degradation controlling fibrogenic differentiation.

Changes in cellular phenotype and differentiation occur in response to localized alterations in the cellular environment. At sites of inflammation and tissue damage these alterations may have fundamental effects on the phenotype of the resident cells, particularly fibroblasts. Fibroblasts, characteristically, are able to undergo a range of phenotypic conversions between distinct but related cell types, and their phenotypic heterogeneity is well described. At sites of tissue damage and wound healing, fibroblasts with a contractile phenotype are essential for the synthesis of the collagen-rich scar and for providing the force for wound contraction. In such circumstances, these “myofibroblasts” are normally a transient cell population. In contrast to wound healing, however, a persistent accumulation of myofibroblasts is associated with the pathological reorganization and expansion of the extracellular matrix. This fibrotic response includes the synthesis and accumulation of extracellular matrix components that may not normally be present or are present at only low levels in normal tissue. One of the earliest of these to be deposited is hyaluronan (HA) in fibrotic tissue.

HA is a negatively charged, linear, nonsulfated glycosaminoglycan consisting of repeating disaccharide units of glucuronic acid and N-acetylgalactosamine. Unlike other glycosaminoglycans its synthesis does not take place in the Golgi. Rather it is extruded from the plasma membrane as a chain, which may reach a size of as much as 10^7 Da. Three vertebrate HA synthase (HAS) genes have been described. The HAS enzymes they transcribe are bound to the inner, cytoplasmic face of the plasma membrane. The expression of each of these is cell type-specific, and each is regulated differentially in response to extracellular mediators. Under normal conditions there is a tightly regulated equilibrium between the synthesis of HA and its turnover. Different tissues, however, turn over HA at different rates, varying from a half-life of less than 5 min in blood to between 1 and 3 weeks in cartilage. HA normally exists as a high molecular weight molecule that is involved in matrix stability and maintenance of cell homeostasis. In contrast, lower molecular weight oligosaccharides of HA stimulate gene expression and protein synthesis of a variety of proinflammatory molecules and mediators.

Many of the responses initiated by HA binding to its receptors have been investigated, although a detailed understanding of the mechanisms whereby HA influences specific aspects of cellular function is lacking. Recently Zoltan-Jones et al. demonstrated that overexpression of HAS 2 leading to the increased synthesis of HA, initiated the acquisition of mesenchymal and transformed characteristics in Madin-Darby canine kidney and MCF-10A epithelial cells. Because an increase in myofibroblast numbers in fibrotic tissue is associated with the accumulation of HA, we examined the hypothesis that a change in the synthesis and catabolism of HA is also a major factor contributing to the profibrotic differentiation of fibroblasts to myofibroblasts.

**EXPERIMENTAL PROCEDURES**

**Materials**—All general reagents were from Sigma, and all tissue culture reagents were purchased from Invitrogen unless stated otherwise.

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‡ To whom correspondence should be addressed. Tel.: 44-292-074-8389; Fax: 44-292-074-8470; E-mail: steadmanr@cf.ac.uk.
Reduced Hyaluronan Turnover in Myofibroblasts

| Primer sequences | Cycles | Product size |
|------------------|--------|-------------|
| F-GGGACATGATCTTGATCTTT | 26 | 204 |
| R-CCTTCCTGGGCACTGAGTCCTT | | |
| \(5'\) | 28 | 369 |
| F-ATCTGGCACCACCTTTCTTACA | Up to 40 | 395 |
| R-CTCCACGCCTGACAGTATA | | |
| HAS 1 | 36 | 313 |
| F-TCTACGGGGCTCCTGTCAGC | | |
| R-CTCCACGCCTGACAGTATA | | |
| HAS 2 | 36 | 453 |
| F-GCAGCGGAGAAGGGGAGCAAC | | |
| R-TGAGTGTAAGATGGCAGAGAT | | |
| HYAL 1 | 36 | 400 |
| F-CAGCCGTGAGCTTGAGTGGAGA | | |
| R-GTATGGTCAACAGCGTGGGC | | |
| HYAL 2 | 32 | 446 |
| F-GAAGGCAGCAGCAGCTTIC | | |
| R-CACCCAGAGGATGACACAGCAG | | |
| HYAL 3 | 36 | 150 |
| F-GATCCAGACGATGGAGACG | | |
| R-CCTCAGCTTCTACTGCTTGGCC | | |

**Semi-quantitative Reverse Transcription (RT)-PCR**—Confluent monolayers of cells grown in 35-mm dishes were washed once with PBS and then lysed in 1 ml of TRI-Reagent and RNA purified 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 (hexadeoxyribonucleotides, pd[N]6, Amersham Biosciences), 5 μl of 10 μM dNTPs (Amersham Biosciences), 2 μl of 10× PCR buffer (Applied Biosystems), and 2 μl of 1 M Tris-HCl, pH 8, 0.05% sodium azide for 24 h. The solutions were incubated at 94°C for 5 min followed by 4°C for 2 min. 1 μl of (40 units/ml) ribonuclease inhibitor RNasin (Promega) and 1 μl of (200 units/ml) Superscript (Rnase H-reverse transcriptase) (Invitrogen) were added to each sample and mixed. The solutions were 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 (Applied Biosystems). As a negative control (-RT) reverse transcription was performed with sterile H2O replacing the RNA sample. The cDNA products were stored at -20°C.

The PCR was carried out in a final volume of 50 μl/sample, 2 μl of RT product, 37.5 μl of sterile H2O, 2.5 pmol of each primer (Table I), 3 μl of 10 μM dNTPs, 5 μl of 10× PCR buffer, and 0.25 unit of AmpliTaq Gold polymerase (Applied Biosystems) per sample, on a GeneAmp PCR system 9700. Amplification was carried out using a cycle of 94°C for 40 s, 55°C for 60 s, and 72°C for 60 s. A negative control (-PCR) was included with H2O replacing the cDNA sample. 5 μl of each PCR product plus 3 μl of loading buffer (40% sucrose and 0.1% bromphenol blue) were then separated on 1.5% agarose/gel (Seakem Gold) at 200 V for 1 h. 3 μl of a 123-bp ladder (Invitrogen) plus 3 μl of loading buffer were also loaded onto the gel. The gels were visualized and photographed under UV light using a ChemiDoc system (Bio-Rad Laboratories). The ratio of each product to that of the \(\beta\)-actin housekeeping gene was calculated by scanning densitometry and expressed as the mean ± S.E.M for each group of 5. The results were analyzed using the Mann-Whitney U test, and statistical significance was taken as a p value < 0.05.

**Metabolic Labeling and Analysis of HA**—Confluent monolayers of cells grown in T75 flasks were incubated in serum-free medium containing 20 μCi/ml \([3H]\)glucosamine for 24 h. The medium was removed, and the cells were washed with PBS. The wash and medium were combined to form the conditioned medium extract (CM). The CM was then treated with an equal volume of 200 μg/ml Pronase in 100 mM Tris-HCl, pH 8, 0.05% sodium azide for 24 h. To remove any surface/CD44-associated HA, the cells were treated with 10 μg/ml trypsin in PBS for 10 min at room temperature; this was designated the trypsin extract. The trypsin extract was then treated with an equal volume of 100 μg/ml Pronase (Vela 24 h). The cell remaining in the culture flask were incubated directly with 100 μg/ml Pronase for 24 h. The supernatant was decanted and designated the cell extract.

Each extract was passed over DEAE-Sephalac ion exchange columns (Amersham Biosciences), equilibrated with 8 M urea in 20 mM BisTris buffer, pH 6, containing 0.2% Triton X-100. This removed any low molecular weight peptides and unincorporated radiolabel. HA was eluted in 8 M urea buffer containing 0.3 M NaCl. Sulfated glycosaminoglycans remained bound and were subsequently eluted in 4 M guanidine buffer (4 M guanidine HCl, 50 mM sodium acetate, 0.5% Triton X-100, and 0.05% sodium azide). Each sample was split into two, and the HA was precipitated with 3 volumes of 1% potassium acetate in 85% ethanol in the presence of 50 μg/ml of each HA, heparin, and chondroitin sulfate as coprecipitants. 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 radionabeled HA, the second half of each sample was digested at 37°C overnight with 1 unit of Streptomyces hyalurolyticus hyaluronidase (HYAL) (ICN Pharmaceuticals Ltd.) in 200 μl of 20 mM sodium acetate, pH 6, 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 \(^1^H\) activity (dpm) for each half of the sample was normalized, corrected for dilution, and then the HYAL-resistant counts were subtracted. The chromatography profiles only depict HYAL-sensitive activity in each fraction plotted against fraction number. The column was calibrated with \((a)\) \([3H]\)glucosamine hydrochloride, M, 215; \((b)\) \([35S]\)Sodium chloride, M, 25 × 10^2; \((c)\) \([35S]\)Sodium chloride, M, 10 × 10^4; \((d)\) \([35S]\)Sodium chloride, M, 1.5 × 10^5.

**Purification of \([3H]HA**—The HK2 cell line was used to prepare large quantities of \([3H]HA. Confluent monolayers of HK2 cells grown in T75 flasks were incubated with \([3H]glucosamine for 72 h in serum-free medium. The CM was removed, \([3H]HA\) isolated, and each extract was processed and analyzed as used “Metabolic Labeling and Analysis of HA” above. After size separation on Sephacryl S-500, the fractions corresponding to \([3H]HA\) with a size greater than 1,000 kDa were pooled. Triton X-100 was removed from the sample by passing over DEAE-Sephasel and washing the bound \([3H]HA\) extensively with water. The \([3H]HA\) was eluted with 4 M guanidine buffer without Triton
Fig. 1. Expression of cytokeratin, vimentin, α-smooth muscle actin (SMA), and ED-A fibronectin by fibroblasts and TGF-β1-differentiated myofibroblasts. Cells were grown to near confluence and then growth arrested for 72 h. The medium was replaced with serum-free medium alone or containing 10 ng/ml TGF-β1 and the incubations continued for a further 72 h. Magnification, ×250.

X-100. Before addition of exogenous [3H]HA to confluent monolayers of cells it was dialyzed repeatedly against serum-free medium to remove the 4 M guanidine buffer. For the estimate of cell-free HYAL activity, [3H]HA (50 × 10^3 dpm) was dialyzed against water and lyophilized.

Addition of Exogenous [3H]HA to Confluent Monolayers of Cells—Serum-free medium containing 100,000 dpm of [3H]HA/ml was added to confluent monolayers of cells grown in T25 flasks and incubated at 37 °C in a humidified incubator in an atmosphere of 5% CO2 for 24 and 72 h. Controls consisted of serum-free medium containing 100,000 dpm/ml of [3H]HA/ml incubated for the appropriate time points. The CM was removed, freeze-dried, and then reconstituted in 4 M guanidine buffer and analyzed by size exclusion chromatography on a Sephacryl S-500 column equilibrated with 4 M guanidine buffer. The recovery of HA-specific counts varied between 91 and 95% in the CM and 3 and 5% in the cell layer in these experiments compared with 100% recovery of the cell-free control.

Assay of Cell-free HYAL Activity—The CM decanted from confluent monolayers of cells grown in T25 flasks was passed over DEAE-Sephacel ion exchange columns in 50 mM Tris-HCl, pH 7.8. The flow-through (containing HYAL) was then dialyzed against water and freeze-dried. The samples were reconstituted in 0.1 M sodium formate buffer, pH 3.7, and incubated with 50 × 10^3 dpm [3H]HA for 72 h at 37 °C. HYAL activity was not detectable at neutral pH (not shown). Controls consisted of 0.1 M sodium formate buffer, pH 3.7, containing 50 × 10^3 dpm [3H]HA incubated for 72 h. The HA size distribution was then examined by size exclusion chromatography on a Sephacryl S-500 column equilibrated with 4 M guanidine buffer.

HA Turnover by Pulse-Chase—Confluent monolayers of cells grown in T25 flasks were incubated in serum-free medium containing 20 μM [35S]glucosamine for 24 h. The medium was then removed at times up to 12 h, and the cells were washed with PBS, and fresh medium without radiolabel was added. The wash and medium were combined to form the CM as above. The CM was then treated with an equal volume of 200 μg/ml Pronase in 100 mM Tris-HCl, pH 8, 0.05% sodium azide for 24 h. The remaining cell/matrix layer was incubated directly with 100 μg/ml Pronase for 24 h. The supernatant was decanted and designated the cell layer extract. Each extract was processed and analyzed as under “Metabolic Labeling and Analysis of HA,” above.

Particle Exclusion Assay—The exclusion of horse erythrocytes was used to visualize the HA pericellular coat. Subconfluent layers of cells grown in 35-mm dishes were washed repeatedly in PBS. Formalized horse erythrocytes were washed in PBS and centrifuged at 1,000 × g for 7 min at 4 °C. The pellet was resuspended in serum-free medium, approximate density 1 × 10^9/ml. 500 μl of formalized horse erythrocyte suspension was added to each 35-mm dish and swirled gently to distribute the cell suspension evenly. The dishes were incubated at 37 °C for 15 min to allow the formalized horse erythrocyte to settle around the cell layer. Controls cells were incubated with 200 μg/ml HYAL from bovine testes in serum-free medium for 60 min prior to the addition formalized horse erythrocytes. Zones of exclusion were visualized on a Zeiss Axiosvert 135 inverted microscope (Carl Zeiss; Light Microscopy, Gottingen, Germany) with a Hamamatsu C5885 chilled CCD camera (Hamamatsu Photonics UK Ltd., Hertfordshire, UK) and OpenLab software 3.0.8 (Improvision, Coventry, UK). Because of the elongated shape of many of the cell processes, the exclusion zone at some areas of the cell was not visible, making calculation of areas difficult. Therefore, the width of the exclusion zone was calculated at the widest point of the cell (usually at the nucleus). Nonparametric statistical analysis (Mann-Whitney U test) was then carried out on the measurements from each cell type.

SDS-PAGE and Immunoblotting—The CM decanted from confluent monolayers of cells grown in T25 flasks was passed over DEAE-Sephacel ion exchange columns in 50 mM Tris-HCl, pH 7.8. The flow-through (containing HYAL) was then dialyzed against water, freeze-dried, and reconstituted in 0.5 mM Tris-HCl, pH 6.8. The sample was added to an equal volume of loading buffer, and proteins were separated on discontinuous 7.5% polyacrylamide gels followed by transfer to nitrocellulose. After transfer, blots were blocked with 5% skimmed milk, 0.5% Tween
20 in 50 mM Tris-HCl-buffered saline for 1 h. Rabbit polyclonal anti-
HYAL 1 and rabbit polyclonal anti-HYAL 2 (a kind gift from Dr. Robert
Stern, Department of Pathology, University of California, San Fran-
cisco) diluted 1:200 were added to the blots in 0.1% Tween 20 and 1%
BSA in Tris-HCl-buffered saline and incubated at 4°C overnight. After
washing, the blots were incubated with horseradish peroxidase-conju-
gated goat anti-rabbit secondary antibody. Antibody binding was visu-
alized by ECL (Amersham Biosciences). Negative controls consisted of
incubation with normal rabbit serum (diluted 1:1,000) in place of the
primary antibody.

RESULTS

HA Synthesis after Myofibroblast Differentiation—Exposure
of human lung fibroblasts to TGF-β1 resulted in their differen-
tiation to a myofibroblast phenotype with the induction of
β-smooth muscle actin and ED-A fibronectin, which was max-
imal by 72 h at concentrations above 10 ng/ml (Fig. 1). As
reported previously (14, 16), this was not reversed by removal
of TGF-β1 (not shown), indicative of stably differentiated cells.
Both cell types stained positively for vimentin and were nega-
tive for cytokeratin. Labeling the cells with [3H]glucosamine
for 24 h after their differentiation resulted in an augmented
accumulation of HA compared with fibroblasts. Analysis of HA
by size exclusion chromatography indicated that there was
between 2- and 3-fold more HA present in the CM of myofibro-
blasts compared with fibroblasts (Fig. 2A). Myofibroblasts also
had more HA associated with the cell surface (Fig. 2B) and with
the cell layer (Fig. 2C) than fibroblasts. In addition, although
the majority of the HA from each of these three compartments
had a high molecular weight, both the MC and the cell layer
also contained lower molecular weight forms, which were not
found in the trypsin-accessible cell surface extract.

Effect of Manipulation of HA Synthesis and Turnover on Cell
Phenotype—The increased synthesis of HA through the induc-
tion of HAS 2 has been described previously as a stimulus-
ininitiating epithelial-mesenchymal transformation (13). To in-
vestigate the possibility that a similar mechanism was involved
after fibroblast to myofibroblast differentiation, the expression
of each of the HAS isoforms was examined by semiquantitative
RT-PCR. mRNA was extracted from five separate cultures of fibroblasts or myofibroblasts and cDNA prepared
by RT-PCR. The products were separated by agarose gel electrophoresis
and the intensity of the bands quantitated by densitometry as de-
scribed. The results were analyzed using the Mann-Whitney U test, and
statistical significance was taken as a p value < 0.05. Myofibroblasts
expressed significantly more mRNA for α-smooth muscle actin but
significantly less for both HAS 2 and HAS 3 (α-smooth muscle actin,
\( p = 0.009 \), HAS 2, \( p = 0.028 \); HAS 3, \( p = 0.009 \)).

Fig. 3. Expression of α-smooth muscle actin (SMA), HAS 2, and
HAS 3 mRNA measured by RT-PCR. mRNA was extracted from five
separate cultures of fibroblasts or myofibroblasts and cDNA prepared
by RT-PCR. The products were separated by agarose gel electrophoresis
and the intensity of the bands quantitated by densitometry as de-
scribed. The results were analyzed using the Mann-Whitney U test, and
statistical significance was taken as a p value < 0.05. Myofibroblasts
expressed significantly more mRNA for α-smooth muscle actin but
significantly less for both HAS 2 and HAS 3 (α-smooth muscle actin,
\( p = 0.009 \), HAS 2, \( p = 0.028 \); HAS 3, \( p = 0.009 \)).

Fig. 4. Inhibition of HA synthesis in fibroblasts by the optimal
concentration of 4-MU. Fibroblasts were metabolically labeled for
24 h with [3H]glucosamine in the absence (○) or presence (●) of 0.2 mM
4-MU. The labeled HA was isolated from the CM (A), the trypsin extract
(B), and the cell layer extract (C) and subjected to size exclusion chro-
matography on a Sephacryl S-500 column as described under “Experi-
mental Procedures.” HMW, MMW, and LMW, as in Fig. 2.
Reduced Hyaluronan Turnover in Myofibroblasts

The HA pericellular coat was visualized using the exclusion of formalized erythrocytes (Fig. 5). Myofibroblasts had a well established coat of HA that was 3-fold thicker than that of fibroblasts (Fig. 5B compared with 5A). Taking measurements of the coat thickness at the widest point of 30 randomly chosen cells of each phenotype gave a mean thickness for the myofibroblast coat of 1.61 ± 0.72 µm and for the fibroblast coat of 0.52 ± 0.15 µm (mean ± S.D.) (Z -6.661, p < 0.0001 Mann-Whitney U test). Incubation with 4-MU inhibited coat assembly in both cell types (Fig. 5, C and G) without affecting cell morphology or viability (not shown). Control cells incubated with bovine testicular HYAL (Fig. 5, D and H) had no pericellular coats.

**HYAL Activity in Fibroblasts and Myofibroblasts**—The observation that there was lower expression of HAS 2 and HAS 3 in myofibroblasts, combined with the metabolic labeling data, suggested that the greater accumulation of HA by myofibroblasts may be the result of decreased HA degradation. The mRNA level for each of the HYAL enzymes was, therefore, examined by RT-PCR. The mRNA for HYAL 1, HYAL 2, and HYAL 3 was detected in each of five separate cultures of each cell type. Although there was no difference between the levels of expression of HYAL 3, both HYAL 1 and HYAL 2 were expressed at lower levels in myofibroblasts than in fibroblasts. This was statistically significant, however, only for HYAL 2 (Table II).

To investigate whether there was also a functional difference in the capacity for HA degradation between the cells, cell-generated, high molecular weight [3H]HA was incubated with the cells for times up to 72 h. There was a clear, reproducible difference in the resulting elution profiles of the HA in the CM on size exclusion chromatography. There was significantly more cleavage of the HA substrate by fibroblasts compared with myofibroblasts (Fig. 6). This supported the suggested mechanism by which myofibroblasts accumulated HA through decreased HA degradation.

Western blot analysis demonstrated significantly greater levels of both HYAL 1 and HYAL 2 in the CM of myofibroblasts when compared with fibroblasts cultured for times up to 72 h (Fig. 7). The HYAL enzymes were concentrated from the CM and their activity analyzed in the cell-free HYAL assay at pH 3.7. There were no significant differences between the two cell types (Fig. 8). These results demonstrated that the HYALs in the CM were active at an acidic pH. Furthermore, because there was more HYAL 1 and HYAL 2 identified by immunoblotting of the myofibroblast culture medium, a proportion of

**TABLE II**

**Expression of HYAL mRNA by fibroblasts and myofibroblasts**

| Isoform | Fibroblasts | Myofibroblasts | Significance* |
|---------|-------------|----------------|---------------|
| HYAL 1  | 1.14 ± 0.19 | 0.84 ± 0.24    | NS            |
| HYAL 2  | 0.97 ± 0.05 | 0.84 ± 0.06    | p = 0.008     |
| HYAL 3  | 1.07 ± 0.09 | 1.08 ± 0.16    | NS            |

* Mann-Whitney U test, n = 5. NS, not significant.

**Fig. 5.** Inhibition of the HA pericellular coat. Fibroblasts (A–D) or myofibroblasts (E–H) in 35-mm dishes were growth arrested, washed, and incubated in the absence (B and F) or presence (C and G) of 4-MU added to give a final concentration of 0.2 mM. After 72 h the cells were examined under brightfield illumination (A and E), or formalin-treated erythrocytes were added as under “Experimental Procedures.” Some wells were treated with bovine testicular HYAL (see “Experimental Procedures”) before the addition of erythrocytes (A and B). Arrows indicate the cell body; arrowheads show the extent of the pericellular matrix. Magnification, ×200.

**Fig. 6.** Addition of exogenous [3H]HA to confluent monolayers of cells. Purified [3H]HA was incubated with confluent cultures of fibroblasts (○) or myofibroblasts (●) in serum-free medium for 24 h (A) or 72 h (B). The CM was then decanted and lyophilized. The HA size distribution was then examined by loading volumes corrected for cell number on a Sephacryl S-500 column and comparing the elution profiles with that of the original [3H]HA preparation, incubated in serum-free medium in the absence of cells (□).

**Fig. 7.** Immunoblot of HYAL 1 and HYAL 2 in the CM of fibroblasts and myofibroblasts. HYAL from the CM of two separate fibroblast or myofibroblast cultures was concentrated and corrected for cell number, separated by SDS-PAGE, and immunoblotted as described under “Experimental Procedures.” Binding of anti-HYAL 1 or HYAL 2 antibody was visualized by anti-rabbit IgG-horseradish peroxidase-conjugated antibody and ECL. The gels shown are representative of three identical experiments.
Reduced Hyaluronan Turnover in Myofibroblasts

Fig. 9. HA degradation assessed by pulse-chase. Fibroblasts (A and B) or myofibroblasts (C and D) were pulsed with [3H]glucosamine for 24 h and then chased in medium without radiolabel for times up to 12 h. The cell layer (A and C) and CM (B and D) were prepared as described under “Experimental Procedures,” then made up to a volume appropriate to correct for cell numbers. The HA size distribution was then examined on a Sephacryl S-500 column. The profiles show the HA size distribution at 0 h (○), 2 h (●), 4 h (□), and 12 h (■) of chase. 0 h is the profile of the cell layer HA immediately before the chase began. The results are representative of two separate experiments.

Fig. 10. Fibroblasts or myofibroblasts, fixed, washed, and incubated with anti-HYAL 1 or HYAL 2 antibodies, were labeled with fluorescein isothiocyanate-conjugated secondary antibody as described. They were then viewed by confocal scanning laser microscopy. The results shown are representative of five separate preparations of each cell phenotype. Magnification, ×400.

The results showing the differences in degradation of exogenously added HA, these data confirm that a decrease in HA degradation is at least partly responsible for the observed accumulation of HA by myofibroblasts.

HYAL Localization—Confocal microscopy of cells incubated with anti-HYAL 1 or anti-HYAL 2 antibodies demonstrated a distinctly different pattern and intensity of HYAL expression between the two cell types (Fig. 10). HYAL 1 was visualized as distinct punctate staining in fibroblasts, whereas HYAL 2 was localized to the cell surface. In contrast, the staining for both enzymes in myofibroblasts was reduced and more diffuse. Together with the pulse-chase data, these results suggest a reorganization and loss of HYAL activity in the myofibroblasts, leading to reduced HA turnover.

DISCUSSION

Myofibroblasts express α-smooth muscle actin and the ED-A isoform of fibronectin and are phenotypically intermediate between smooth muscle cells and fibroblasts (20, 21). In pathological lesions, myofibroblasts may arise from the transdifferentiation of epithelial cells (22) or the differentiation of stromal fibroblasts under the influence of profibrotic growth factors, such as TGF, particularly TGF-β1. TGF-β1 has been shown to induce myofibroblastic differentiation of fibroblasts both in vivo and in vitro and is implicated in the progression of most forms of human fibrosis (4, 14, 16). The present study describes for the first time a change in HA turnover as a result of the differentiation of fibroblasts to myofibroblasts. The most obvious feature of this change was accumulation of HA in the myofibroblasts, on the cell surface, and associated with the cell layer/matrix extract. HAS 2 mRNA expression was, however, lower, as a result of myofibroblastic differentiation. This was in contrast to the reported effect on epithelial-mesenchymal transformation induced by adenoviral infection with HAS 2. In the current study it appeared, therefore, that the changes in HA

medium for both cell types. After the pulse (T0) there was 3-fold more HA associated with the myofibroblast cell layer compared with that of the fibroblast (Fig. 9, A compared with C). After 2 h of chase, fibroblasts had released ∼2-fold more HA into the medium than myofibroblasts (Fig. 9, B and D). By 12 h, ∼50% of the high molecular weight HA released into the fibroblast medium had been degraded, whereas the ratio of high to low molecular weight material in the myofibroblast medium had stayed relatively constant. Over the chase period, there was an increase in total HA in the system in both cells, indicating that reutilization of label from other rapidly turning over macromolecules was occurring. This was particularly prominent, however, in the fibroblast culture medium. The faster turnover of fibroblasts was confirmed by the increased degradation of high molecular weight HA with increased chase time. Together with the results showing the differences in degradation of exogenously added HA, these data confirm that a decrease in HA degradation is at least partly responsible for the observed accumulation of HA by myofibroblasts.

The released HYAL must have been in a form that could not be activated.

To confirm that myofibroblasts degraded HA less efficiently than fibroblasts, the cells were pulsed with [3H]glucosamine and the radiolabeled HA chased for times up to 12 h. Fig. 9 shows the elution profiles of the HA from the cell layer and the released HYAL must have been in a form that could not be activated.

The reduction in HYAL activity was accompanied by increased HA turnover. The results are consistent with the decrease in HYAL activity, which would be consistent with increased HA turnover, leading to reduced HA turnover.

Fig. 8. Assay of cell-free HYAL activity. The CM from confluent cultures of fibroblasts (●) or myofibroblasts (○) was passed over DEAE-Sephacel, concentrated in 0.1 M sodium formate buffer, pH 3.7, to correct for cell number, and incubated with [3H]HA for 72 h. The HA size distribution was then examined by size exclusion chromatography on a Sephacryl S-500 column and compared with the original [3H]HA preparation, incubated in the absence of the CM concentrate (□).
accretion were the product rather than the cause of the differentiation. This was confirmed by using 4-MU to inhibit HAS activity. No resultant change in myofibroblast morphology was seen.

The accumulation of HA may have been the consequence of either an increase in HA synthesis or a decrease in its degradation. Examining potential differences in HA-degradative capacity between the cells, by pulse-chase analysis and by adding radiolabeled high molecular weight HA to the cells in culture, confirmed that fibroblasts degraded HA more readily than myofibroblasts. The principal enzymes involved in the turnover of HA are the HYAL group of enzymes (10, 23, 24). There are several HYAL genes but only three (HYAL 1, HYAL 2, and HYAL 3) encode proteins that are broadly expressed in somatic tissues. HYAL 1 is ubiquitously expressed and is found in urine and circulating in plasma (25, 26). It is also a lysosomal enzyme, which cleaves HA to small disaccharides. In contrast, and circulating in plasma (25, 26). It is also a lysosomal enzyme, which cleaves HA to small disaccharides. In contrast, HYAL 2 is anchored to the plasma membrane by a glycosylphosphatidylinositol anchor and cleaves HA at a much slower rate to a limit product of −50 disaccharides (−20 kDa) (27). Enzyme activity for HYAL 3 has not been described; nevertheless, at least in chondrocytes, it is up-regulated by inflammatory cytokines in parallel with HYAL 2 (28). It has been demonstrated previously that stimulation of HA synthesis may be associated with its increased catabolism (29, 30) and that the rate-limiting step in HA degradation in chondrocytes and keratinocytes is its internalization attached to its receptor CD44 (31, 32). There was no difference, however, in the expression of CD44 between the two cell types in the current study (not shown), and coincident with a lower expression of HAS 2 there was a decrease in the expression of HYAL 2. Combined with the Western blot data, showing significantly more HYAL 1 and HYAL 2 in the culture medium of myofibroblasts, this suggests that a release of HYAL enzymes from myofibroblasts to the medium may be another mechanism with the potential to contribute to the reduced HA degradation seen in these cells.

The expression of HYAL 1 and HYAL 2 mRNA by fibroblasts derived from periodontal ligament has been described previously (33). HYAL activity has also been identified in the CM of these cells. Furthermore, HYAL 1 is released by other fibroblast lines (34), whereas expression of HYAL 2 in Xenopus laevis results in HYAL 2 release into the extracellular environment as well as its expression on the cell surface and intracellularly (35). Although it is possible to release HYAL 2 from the cell surface by exogenous proteinase treatment or phospholipase C hydrolysis of its glycosylphosphatidylinositol link (36), the endogenous mechanisms of release of the HYAL enzymes from mammalian cells are not clear.

HYAL 2 has been proposed as a coreceptor for HA at the cell surface along with CD44 (10, 37). Its suggested role is to begin the degradation of HA to smaller chains (−20 kDa) (27) as the endosome forms. These chains are then degraded further by the HYAL enzymes and other glycosidases in the lysosome (10, 38, 39). Fig. 2 clearly shows intracellular low molecular weight breakdown products of HA and that there are no such products on the surface of either cell type. There are, however, similar HA fragments in the medium of both cell types. There are two potential sources for HA fragments in the extracellular milieu. They may have leaked from the endosome as degradation began but before complete closure of the organelle. Alternatively, they may have been generated at the cell surface by cleavage of HA tethered to CD44. Cell surface HYAL activity has been suggested (10), and Xenopus HYAL 2 activity at neutral pH has been described (40). Recently, however, a novel mechanism for creating acidic microenvironments for HA degradation has been described by Bourguignon et al. (41). The authors propose a mechanism of HA degradation at the cell surface based on the formation of acidic microenvironments under the control of the Na+/H+ exchanger, NHE1, as the result of HA binding to CD44. Our findings also emphasize the importance of cell-associated but non-soluble HA-degradative activity. In addition, confocal microscopy of the cells demonstrated that the expression of both HYAL 1 and HYAL 2 was lower in myofibroblasts and with a more diffuse cellular distribution. The change in this localization was unexpected but together with the published data of Bourguignon et al. may provide a mechanism explaining why removing HYAL from its normal location markedly alters the turnover of HA. In addition, because changes in CD44 function may be induced by a variety of pathological events it will be important in future work to evaluate the extent to which differences in CD44 function affect the formation and localization of the HYAL-containing microdomains.

The evidence, therefore, fits a model in which there is cell-associated HYAL activity that is redistributed and shed from myofibroblasts when they have differentiated. This reduces their capacity for HA degradation and consequently allows HA to accumulate. To the best of our knowledge, this level of control of HA accumulation has not been suggested previously, and the control mechanisms behind the shedding or secretion of the enzymes are currently under investigation.

When present as a high molecular weight polymer, HA is anti-inflammatory and antiangiogenic. Its persistence at sites of wounding or tissue injury, however, is associated with progressive fibrosis and scarring in a number of organs including the lung and the kidney (5, 11, 42, 43). The data presented suggest that as a result of the differentiation of resident fibroblasts to a myofibroblastic phenotype, HA accumulates and may be a major contributor to the maintenance of the cells in that phenotype. The result would be the excessive production of a fibrotic matrix leading to irreversible scarring. Discerning a possible role for the released HYALs is more speculative. They may, however, serve two functions. They may act as binding proteins, attaching to matrix- or cell-associated HA and being internalized with that HA. Alternatively, once sequestered to the extracellular environment they may be activated in newly generated acidic microenvironments at sites of inflammatory cell activation. This would release HA fragments, with immunomodulatory, proinflammatory, and angiogenic properties (11, 44, 45). Furthermore, tetrasaccharide fragments are antiapoptotic (46) and would therefore promote cell accumulation in areas in which HA is being degraded, allowing for the establishment of a potent profibrotic feedback mechanism.

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