Interstitial fluid flow induces myofibroblast differentiation and collagen alignment in vitro

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Summary

The differentiation of fibroblasts to contractile myofibroblasts, which is characterized by de novo expression of α-smooth muscle actin (α-SMA), is crucial for wound healing and a hallmark of tissue scarring and fibrosis. These processes often follow inflammatory events, particularly in soft tissues such as skin, lung and liver. Although inflammatory cells and damaged epithelium can release transforming growth factor β_1 (TGF-β_1), which largely mediates myofibroblast differentiation, the biophysical environment of inflammation and tissue regeneration, namely increased interstitial flow owing to vessel hyperpermeability and/or angiogenesis, may also play a role. We demonstrate that low levels of interstitial (3D) flow induce fibroblast-to-myofibroblast differentiation as well as collagen alignment and fibroblast proliferation, all in the absence of exogenous mediators. These effects were associated with TGF-β_1 induction, and could be eliminated with TGF-β_1 blocking antibodies. Furthermore, $\alpha_1\beta_1$ integrin was seen to play an important role in the specific response to flow, as its inhibition prevented fibroblast differentiation and subsequent collagen alignment but did not block their ability to contract the gel in a separate floating gel assay. This study suggests that the biophysical environment that often precedes fibrosis, such as swelling, increased microvascular permeability and increased lymphatic drainage – all which involve interstitial fluid flow – may itself play an important role in fibrogenesis.

Key words: Fibrosis, α-Smooth muscle actin, Transforming growth factor β, β_1 Integrin, Shear stress

Introduction

Myofibroblasts play a key role in both physiological wound healing and pathological fibrocontractive conditions such as various forms of fibrosis and desmoplasia (Gabbiani, 2003; Serini and Gabbiani, 1999). During early wound healing, growth factors released by inflammatory cells stimulate fibroblasts to migrate into the provisional clot matrix, where they proliferate and reconstitute a collagen-rich extracellular matrix (ECM) (Martin, 1997). The gradual increase in ECM stiffness by fibroblast tractional forces is mandatory for their further evolution into myofibroblasts (Hinz and Gabbiani, 2003b), which actively close the wound by contraction. Once epithelium has covered the wound, myofibroblasts normally disappear by apoptosis and the granulation tissue eventually evolves into a scar containing few cells (Desmouliere et al., 1995). Under pathological conditions of fibrosis, however, the myofibroblasts do not undergo apoptosis but instead proliferate and overproduce ECM. Fibrosis is the pathologic hallmark of many common fibrocontractive diseases, including pulmonary fibrosis (Thannickal et al., 2004), hepatic cirrhosis and chronic glomerulonephritis (Desmouliere et al., 2003), systemic sclerosis (scleroderma) (Varga and Jimenez, 1995) and desmoplastic stromal response (Desmouliere et al., 2004; Mueller and Fusenig, 2004; Walker, 2001).

Myofibroblasts are generally characterized by expression of α-smooth muscle actin (α-SMA) protein, the actin isoform typical of smooth muscle cells, conferring a high contractile activity to these cells (Hinz et al., 2001a), although α-SMA is not required for collagen gel contraction in vitro (Grinnell, 1994; Hinz and Gabbiani, 2003a; Vanni et al., 2003). The primary inducer of fibroblast-to-myofibroblast differentiation is transforming growth factor β1 (TGF-β_1) (Desmouliere et al., 1993; Ronnov-Jessen and Petersen, 1993), acting either via paracrine release by inflammatory, epithelial or tumor cells (Werner and Grose, 2003) or via autocrine regulation (Kim et al., 1990). Mechanical factors that either provide resistance to matrix contraction or exert tensional forces on the fibroblast cytoskeleton can also modulate fibroblast differentiation. For example, when wound granulation tissue fibroblasts were subjected to mechanical tension in vivo by immobilizing the edges of full-thickness wounds, α-SMA expression was upregulated; tension release by frame removal led to stress fiber disassembly and downregulation of α-SMA expression (Hinz et al., 2001b). In vitro, fibroblasts cultured in three-dimensional (3D) collagen gels exhibit increasing levels of α-SMA expression with increasing matrix stiffness and/or externally applied stretch (Grinnell et al., 2003) but do not differentiate in free-floating gels (Arora et al., 1999). Thus, mechanical forces are strongly implicated in myofibroblast differentiation.

Here, we demonstrate that low levels of interstitial flow (i.e. fluid flow through a 3D matrix) can itself induce collagen alignment and fibroblast-to-myofibroblast transition via autocrine upregulation of TGF-β_1. We previously reported that human dermal fibroblasts align under interstitial flow in 3D collagen gel cultures, perpendicular to the direction of flow (Ng and Swartz, 2003). As aligned fibroblasts and matrix fibers are often seen in wound and fibrotic tissues (Darby et al., 1990; Hinz et al., 2001b), we proposed that interstitial flow could itself contribute to fibrosis even in the absence of inflammatory cells as observed in idiopathic pulmonary fibrosis (Pardo and Selman, 2002; Thannickal et al., 2004). Interstitial flow is present in soft tissues as an important component of the microcirculation between blood and lymphatic vessels, and interstitial flow is increased during events such as inflammation and wound healing where an influx of inflammatory cells and active angiogenesis both contribute to increased fluid flux into the surrounding tissues. The levels of flow we imposed reflect probable pathological values, as they were three to ten times higher than those reported for normal tissue (Chary and Jain, 1989). In the context of desmoplastic stroma, the high interstitial pressure of tumors may lead to an increased outflow of tumor interstitial fluid into the stromal tissues surrounding the tumors (Heldin et al., 2004; Jain, 2001; Swabb et al., 1974). Thus, our findings suggest that the biomechanical environment associated with inflammation (which is accompanied by cytokines), vascularized tumors, remodeling blood vessels or increased lymphatic flow (which is not necessarily associated with cytokines), can itself stimulate myofibroblast differentiation.

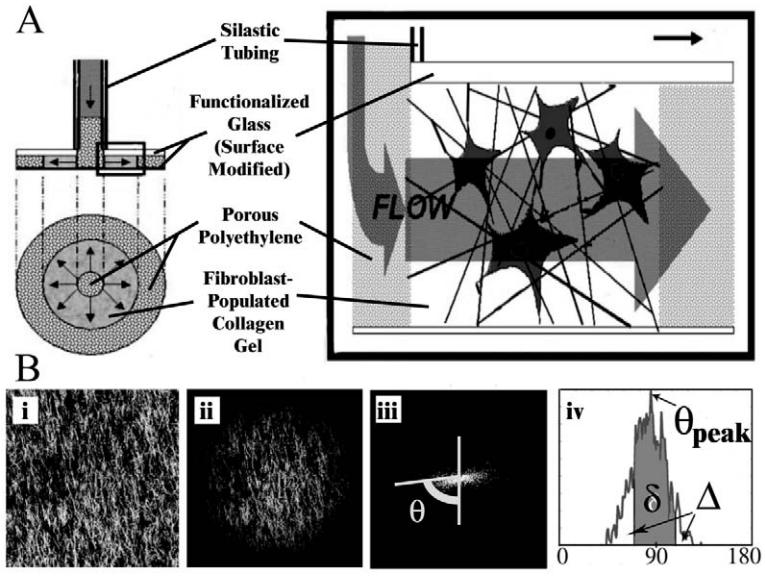


Fig. 1. Experimental set-up and alignment determination. (A) Design features of radial interstitial flow tissue culture chamber. The chamber is made of porous polyethylene and surface-modified glass materials to anchor the ECM and allow direct visualization. (B) Algorithm for image quantification of alignment and orientation. The confocal image (i) is modified (ii) to remove edge effects. (iii) A FFT transformation is performed to obtain a power spectrum from which (iv) an intensity frequency histogram is plotted and an alignment index ($=(\delta/(\Delta+\delta))/(\delta/(\Delta+\delta))_{ideal}$) and the peak angle ($\theta_{peak}$) extracted.

Materials and Methods

Culture of human dermal fibroblasts

CCD1079sk neonatal human dermal fibroblasts (HDF, American Type Culture Collection, Manassa, VA) were expanded in α-MEM supplemented with 10% fetal bovine serum (GIBCO BRL, Grand Island, NY) and 1% penicillin/streptomycin (Sigma, St Louis, MO) and used in passages 7-9.

Preparation of fibroblast-populated matrices and application of interstitial flow

Collagen gels (2 mg/ml), seeded with 5×10^5 fibroblasts/ml, were cast in an interstitial flow chamber (Fig. 1A) as previously described (Ng and Swartz, 2003). The set-up was immersed in media overnight for cell attachment at 37°C, 5% CO_2 in an incubator. For the induction of flow, the chamber was connected to a reservoir of growth medium via a peristaltic pump and a pressure manometer. The flow was delivered at 0.012 ml/minute, leading to a weighted average velocity of 6.3 μm/second. Medium surrounded the chamber and could diffuse through the outer and inner PE rings. Two static controls were used, one mechanically constrained and the other floating. In the first, the gel-filled chamber was set up as before but with no connection to the flow delivery apparatus. The mechanically relaxed static control consisted of the cell-populated gel seeded into an eight-well Lab-Tek coverslip chamber system (Nalge Nunc, Naperville, IL) and allowed to contract freely throughout the experiment. All cultures were maintained in a humidified 37°C, 5% CO_2 incubator.

Immunofluorescence staining

The entire gel was fixed by immersion in 2% paraformaldehyde in PBS for 30 minutes and permeabilized in 0.5% Triton X-100. To detect f-actin and α-SMA, it was immersed overnight at 4°C in 150 nM Alexa 488-conjugated Phalloidin (Molecular Probes, Eugene, OR) and 5 μg/ml monoclonal Cy3-conjugated mouse anti-human α-SMA antibody (clone 1A4, Sigma). In some cases, the gels were also incubated in 500 nM TOTO-3 (Molecular Probes) for nuclear counterstaining. To detect proliferation, gels were incubated with 0.8 μg/ml monoclonal mouse anti-human Ki-67 (clone MIB-1, DakoCytomation, Carpinteria, CA) and then 10 μg/ml Alexa 546-conjugated rabbit anti-mouse IgG (Molecular Probes), followed by counterstaining with Phalloidin and TOTO-3. To visualize TGF-β_1 protein expression, gels were incubated with 20 μg/ml rabbit anti-human TGF-β_1 (Promega), followed by incubation with 2.5 μg/ml Alexa 647-conjugated goat anti-rabbit IgG (Molecular Probes).

Confocal fluorescence and reflectance microscopy

Images were taken using laser-scanning confocal microscopy (Leica LCS SP2 laser microscope system, Mannheim, Germany). Confocal reflectance contrast microscopy was performed to visualize collagen fibers using a 40× (1.25 NA) oil objective lens with modifications based on a previous protocol (Brightman et al., 2000; Friedl et al., 1997). To detect Phalloidin and reflectance simultaneously, samples were excited with a 488 nm Ar laser and both the respective emission signal and reflected light passed through an RT 30/70 beam splitter and collected in two separate channels. Two other channels were used to detect emission signals from the α-SMA and TOTO-3 stains, which were excited by He-Ne lasers (543 nm and 633 nm respectively). Samples were vertically scanned from the bottom coverslip with a total depth of 20-100 μm and a pinhole diameter of 40-70 μm. The sequential images were collected at a step depth of 0.3-2.0 μm and reconstructed

using Leica LCS (Leica) or Volocity (Improvision, Lexington, MA) software.

Bioneutralization studies

For bioneutralization studies, antibodies with known function-blocking activity were added to the cell suspension and incubated for 30 minutes at 37°C prior to seeding in the collagen matrices. Concentrations were chosen in accordance with previously demonstrated blocking concentrations. They were also maintained in the culture medium throughout the experiment at a lower concentration as indicated by pre-incubation and experiment: mouse anti-human $\alpha_1\beta_1$ integrin (clone SR84, BD Biosciences Pharmingen, 10 and 2 μg/ml) (Rettig et al., 1984; Setty et al., 1998), mouse anti-human $\alpha_2\beta_1$ (clone BHA2.1; Chemicon; 20 and 10 μg/ml) (Li et al., 2003) and rabbit anti-human TGF-β_1 (Promega; 0.8 μg/ml) (Zatelli et al., 2000).

Image analysis quantification

Fibroblast proliferation, density, spreading and expression of α-SMA and TGF-β_1 were quantified using ImageJ (NIH, Bethesda, MD). All cells in each image (typically 50-200) were evaluated, using three images per experiment, with three to five experiments per condition. Particle counting was used to determine the number of proliferating (Ki67+) cells normalized to the total cell number (TOTO-3+). α-SMA and TGF-β_1 were quantified by calculating the projected areas of their signals and normalizing those to the f-actin signals. To quantify the projected areas, each image was first converted into a binary image using the threshold function with fixed limits determined from sample images; these were then despeckled, and the total area of signal (α-SMA or TGF-β_1) was divided by the total cell area (i.e. the total area of f-actin signal). Cell density (number of cells/mm^3) was quantified directly from cell counts (TOTO-3+) whereas cell spreading was expressed as the fraction of projected cell area (f-actin signal) per total projected image area.

Fast Fourier image analysis and quantification of cell and collagen fiber orientation and alignment

Fast Fourier transform (FFT) analysis, an indirect method previously applied to quantify collagen fiber alignment in SEM and histological images of ligaments, sclerodermal lesions and scar tissues (Chaudhuri et al., 1987; Nishimura and Ansell, 2002; Pourdeyhimi et al., 1997; van Zuijlen et al., 2002), was used here to evaluate the orientation distribution of structures in confocal images. We developed a MATLAB program to perform the analysis (Fig. 1B). First, an image was imported as a matrix array and Welch windowing was applied to reduce edge effects caused by discontinuities in the imperfect periodic images. The FFT algorithm then transformed the windowed image into a power spectrum, which was highly contrasted before the intensity frequencies were summed to determine the orientation intensity distribution histogram.

From each orientation histogram, the peak angle, or angle of highest frequency, was determined. However, although this indicates the angle at which the most cells or fibers are aligned, it does not reflect how many objects (cells or fibers) are aligned at this angle; if the objects were perfectly randomly oriented, the peak angle would be arbitrary and irrelevant. Thus, we also defined an alignment index to reveal the fraction of cells or fibers that were aligned within 20° of the peak angle and this was normalized to the fraction of randomly oriented fibers that would lie within this range (i.e. 40°/180°=0.22). A randomly aligned matrix would have an alignment index of 1; the higher the value, the higher the fraction of cells or fibers aligned near the peak angle.

Statistical analysis of parametric and non-parametric data

Normally distributed data were represented by bar graphs showing the mean and s.d., and unpaired Student's or Welch's *t*-tests were used to compare mean differences between data with unequal or equal variances, respectively (equality of the variances were assessed using an *F*-test). Comparisons of three groups or more were performed using ANOVA with Dunnett's post-test. For non-normally distributed data, which were represented by medians and 95% confidence intervals using box plots, the nonparametric Mann-Whitney test was used to compare median differences whereas the Krushal-Wallis test with Dunn's post test was used to compare three groups or more.

Results

Interstitial flow induces cell and matrix alignment

To investigate the effects of interstitial flow on fibroblast and matrix organization, fibroblast-populated collagen gels were subjected to an average interstitial flow velocity of 6.3 μm/second (leading to an approximate shear stress of 0.1-0.3 dyn/cm^2; see Discussion for details). Fibroblasts aligned perpendicular to the direction of flow within 2 days (Fig. 2A), correlating with the alignment of the surrounding collagen fibers in the same direction (Fig. 2B). Analysis of FFT intensity frequency histograms confirmed these qualitative observations of cell and matrix alignment: the alignment index, a measure of the fraction of cells or ECM fibers that are aligned to within 20° of the peak angle, showed significant differences between both cells and collagen fibers under flow compared to those in static conditions (Fig. 2C). Furthermore, under interstitial flow, the peak angle was oriented at 83±18° for cells under flow but were randomly aligned (peak angle 58±46°) in static conditions (Fig. 2D); similar trends were seen with fiber orientation, with peak angles of 90±10°, and 84±52° for flow and static conditions, respectively (Fig. 2D).

Interstitial flow promotes myofibroblast differentiation and proliferation

To investigate whether the aligned cells were proliferating and differentiating into myofibroblasts, we immunostained fibroblast-populated collagen gels with Ki67 (a proliferation marker) and α-SMA and quantified confocal images as described in Materials and Methods. Interstitial flow induced α-SMA expression in about 5% of fibroblasts after 2 days and 97% after 5 days (Fig. 3A,B). In contrast, virtually all fibroblasts in static conditions remained undifferentiated after 2 days and only 14% were α-SMA-positive after 5 days. In addition, interstitial flow increased the proliferating cell fraction compared to both static controls (Fig. 3C). Thus, it can be concluded that interstitial flow (order of microns per second) enhances myofibroblast differentiation and proliferation in collagen matrices.

TGF-β_1 mediates flow-induced myofibroblast differentiation

As TGF-β_1 is the major known inducer of myofibroblast differentiation, we examined whether TGF-β_1 was involved in the interstitial flow response. First, we observed by immunostaining that interstitial flow strongly induced TGF-β_1 protein expression by the fibroblasts, whereas none could be detected under static conditions (Fig. 4A,B). This was consistent with the expression of α-SMA shown in Fig. 3B. When an anti-TGF-β_1 blocking antibody was introduced into

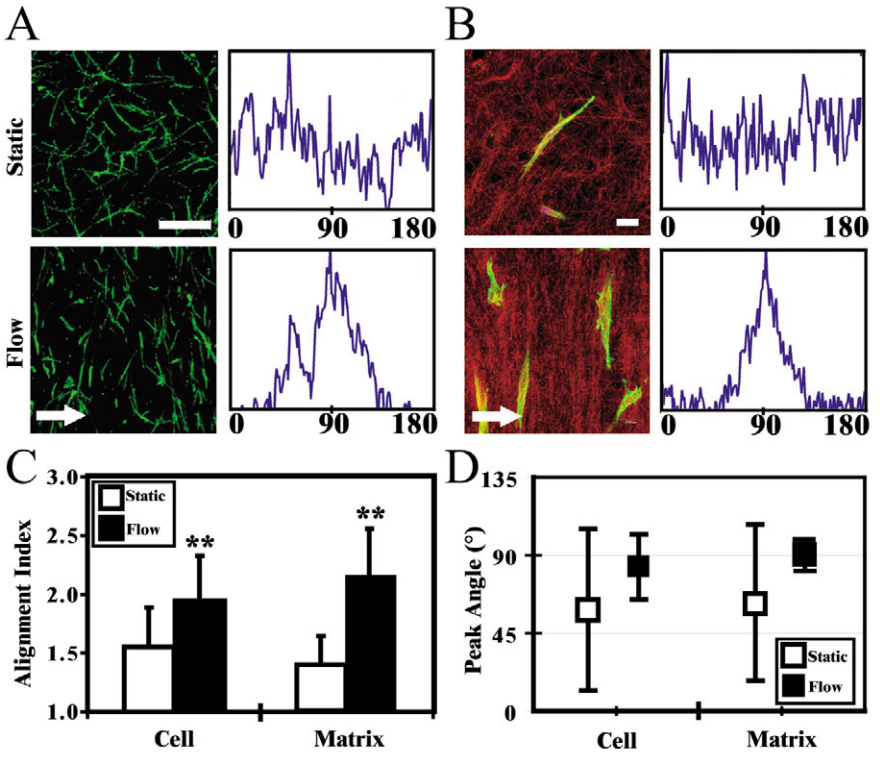
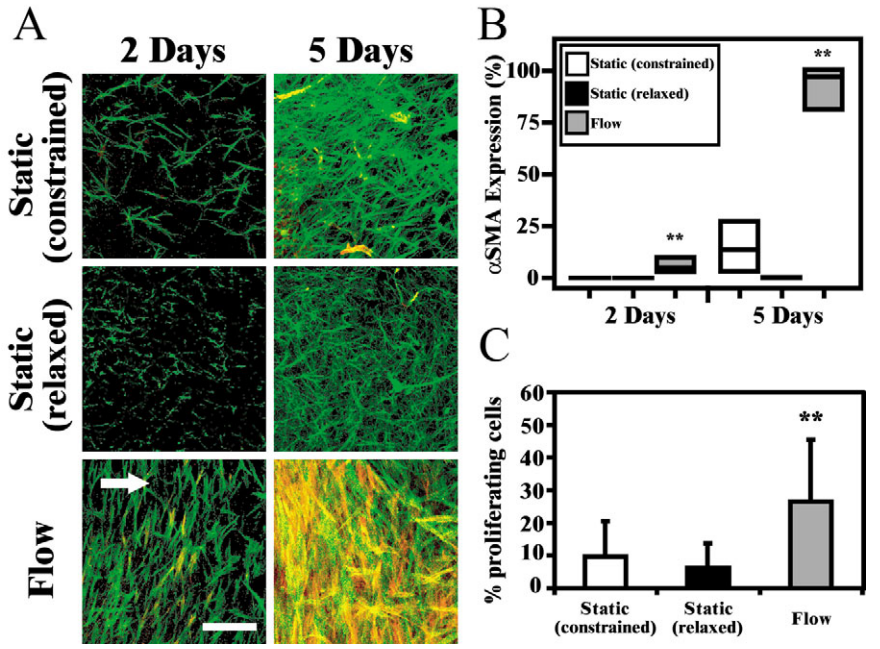


Fig. 2. Alignment of human dermal fibroblasts in a collagen matrix subjected to radial interstitial flow. Confocal images of cells (A) and matrix fibers (B) with their corresponding FFT analyzed intensity frequency histograms. f-actin is labelled green with the confocal reflection in red; arrow indicates flow direction. These observations were quantified by alignment index (C) and peak angle (D) for cell and matrix alignment, respectively. Unpaired *t*-tests were used for statistical analysis of the means; significant differences (**P<0.01) were observed in alignment index in both cells and matrix under flow conditions compared to that measured under static conditions using Mann-Whitney test. Bar, 200 μm (A); 20 μm (B).



the flow media (Fig. 4A), α-SMA expression was eliminated (Fig. 4C), suggesting that the mechanism by which interstitial flow induces α-SMA expression is mediated through the upregulation of TGF-β_1.

Furthermore, TGF-β_1 neutralization abolished flow-enhanced cell density and spreading without affecting cell alignment. Without the blocking antibody, cell density and spreading (i.e. projected cell area per cell) were both increased by interstitial flow after 2 days, but with TGF-β_1 neutralization, no increase in density or spreading was seen (Fig. 4D,E). Interestingly, blocking TGF-β_1 only slightly affected the flow-induced cell alignment: the alignment index was not changed (Fig. 4F) but there was a greater distribution in peak angle (Fig. 4G). In contrast, collagen fiber alignment was reduced (Fig. 4F), indicating that α-SMA is required for the cells to align the matrix but not to align themselves. Taken together, these results indicate that interstitial flow causes an upregulation of TGF-β_1 expression, which induces α-SMA expression, which in turn causes matrix alignment.

Interstitial flow effects are mediated through $\alpha_1\beta_1$ integrin

Matrix remodeling depends on the transmission of intracellular contractile forces to the ECM at sites of integrin-type cell-matrix adhesions. Fibroblasts are known to mechanically interact with collagen fibers primarily through β_1 integrins, particularly $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Heino, 2000). To investigate whether ligation and signaling through these β_1 integrins were important in mediating the fibroblast differentiation response to

Fig. 3. Interstitial flow induces α-SMA expression in fibroblasts. (A) Confocal images of cells at 2 and 5 days showing α-SMA expression under mechanically constrained static and interstitial flow conditions (green, f-actin; red, α-SMA; arrow indicates flow direction). (B) Significantly higher levels of α-SMA expression are seen in fibroblasts undergoing interstitial flow at both time points (**P<0.01 using Dunn's test). Box plot shows 95% confidence intervals with midline showing the median. (C) The percentage of proliferating (Ki67+) cells after 2 days was higher under interstitial flow conditions than either constrained or relaxed static controls (bar represents the mean value and error bars, s.d.; **P<0.01 using Dunnett's test). Bar, 200 μm.

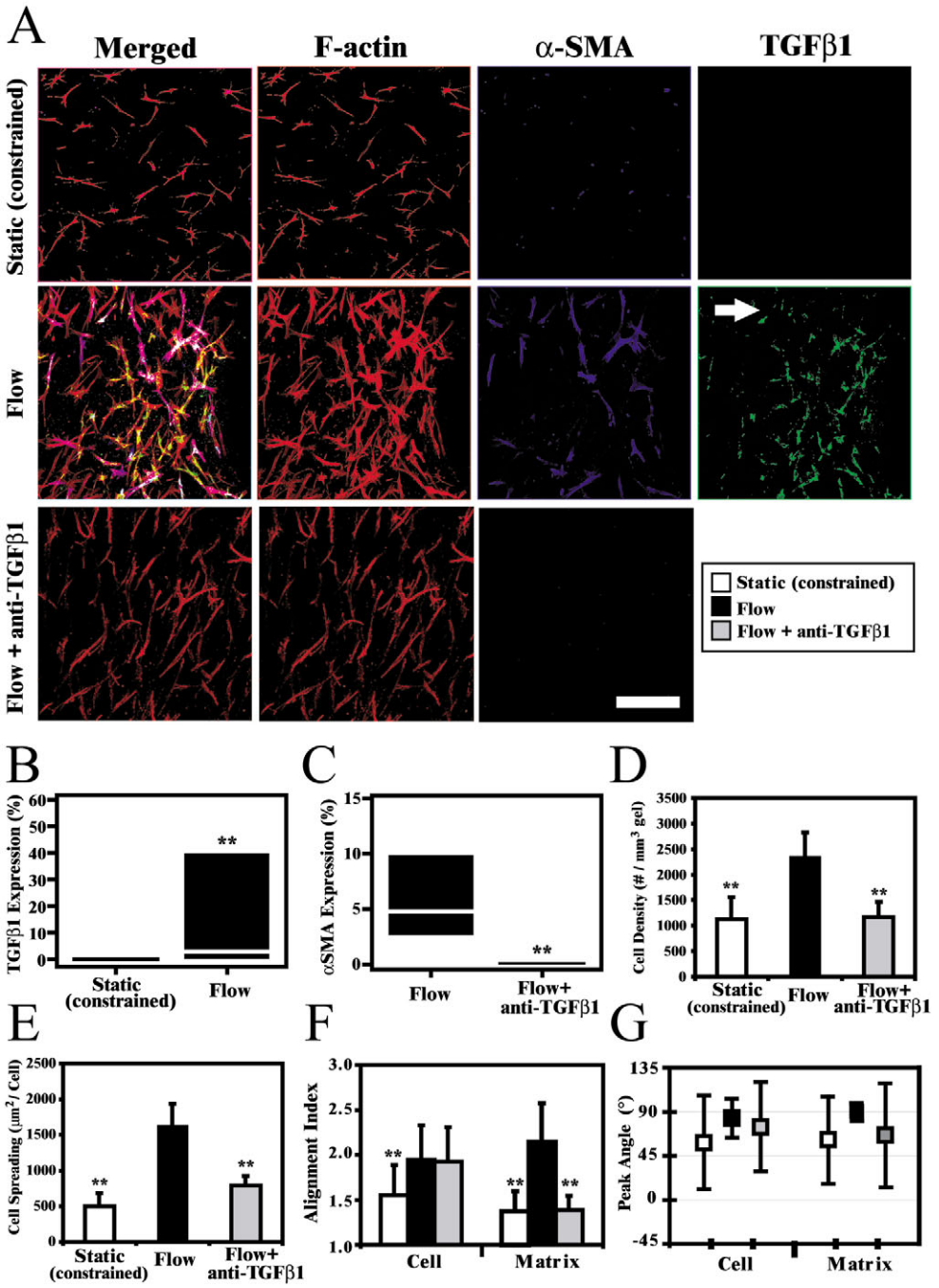


Fig. 4. TGF-β_1 mediates flow-induced myofibroblast differentiation. (A) Confocal images showing TGF-β_1 and α-SMA expression in mechanically constrained static (top) and flow conditions, without (middle) and with (bottom) TGF-β_1 neutralizing antibody (red, f-actin; green, TGF-β_1; blue, α-SMA; arrow indicates flow direction). (B) TGF-β_1 expression quantification (**P<0.01 using Dunn's test) in fibroblasts under constrained static and interstitial flow conditions for 2 days. Box plot shows 95% confidence intervals with midline showing the median. (C) α-SMA expression quantification in normal compared to bioneutralized flow conditions (**P<0.01 using Mann-Whitney test). (D,E) Quantification of cell density in terms of number of cells per mm^3 and cell spreading in terms of projected cell area (μm^2/cell) (**P<0.01 using Dunnett's test). (F) Alignment and (G) orientation of the cells and matrix fibers under each condition (*P<0.05; **P<0.01 using Dunnett's test). Bar, 200 μm.

interstitial flow, blocking antibodies were used to specifically target $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. We found that although both reversed the effects of flow on α-SMA expression, cell density and cell spreading, $\alpha_1\beta_1$ integrin blocking completely neutralized α-SMA expression (Fig. 5A-D). The differences in α-SMA expression between blocking of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ were significant (P=0.0002 using Mann-Whitney test), indicating that $\alpha_1\beta_1$ was a more potent regulator of α-SMA expression than $\alpha_2\beta_1$. Furthermore, blocking of $\alpha_1\beta_1$ integrin had a smaller effect than $\alpha_2\beta_1$ blocking on cell density and spreading (Fig. 5C,D). Importantly, specific blocking of $\alpha_1\beta_1$ had little effect on the ability of the fibroblasts to attach to the gel (Fig. 5A) and contract it (Fig. 5G), in contrast to blocking of $\alpha_2\beta_1$ integrins, which caused significant cell detachment and prevented matrix contraction (Fig. 5A,G). Finally, blocking $\alpha_1\beta_1$ prevented both cell and matrix alignment (Fig. 5E,F); the lack of change in cell alignment during $\alpha_2\beta_1$ blocking was probably due to the fact that very few cells remained attached to the matrix when $\alpha_2\beta_1$ is blocked. These results suggest that ligation of $\alpha_1\beta_1$ integrins is necessary for interstitial flow-induced myofibroblast differentiation and subsequent cell proliferation, cell alignment and matrix alignment.

Discussion

Much information has recently emerged about the cellular and

biochemical events that underlie tissue remodeling towards a fibrotic phenotype. For example, the development of fibrosis follows a similar pathway to normal wound healing except in most cases there is a chronic progression of the disease which is characterized by: (1) a continuous insult or stimulus (chemical or mechanical); (2) excessive synthesis of collagen and other ECM components; and (3) a decrease in resolution owing to a downregulation of matrix-degrading enzymes (Mutsaers et al., 1997). In addition, in many tissues fibrosis is typically preceded by chronic inflammation, which provides a source of inflammatory cell-derived cytokines such as TGF-β_1 that are crucial mediators of fibrogenesis. However, fibrosis can also follow non-inflammatory events such as swelling in lymphedema (Campisi and Boccardo, 2002) and in idiopathic pulmonary fibrosis (Pardo and Selman, 2002); the mechanisms of myofibroblast differentiation and subsequent progression to fibrosis are still not clear in such pathologies, and the role of interstitial flow that typically accompanies such processes has not previously been addressed.

Our results here suggest that interstitial flow alone may be sufficient to induce and sustain fibrosis, even in the absence of TGF-β_1 secretion by other cells such as inflammatory, epithelial or tumor cells, and correlates with key features of the progression of an inflammatory state to a fibrotic pathology. We show that fibroblasts in 3D collagen cultures undergoing somewhat superphysiological levels of interstitial flow (6 μm/second, or roughly 1-3 dyn/cm^2 shear stress) exhibit features commonly observed in tissues of fibrotic phenotype such as scar tissue and the desmoplastic stroma around tumors: the cells proliferate, differentiate into myofibroblasts, and align parallel to each other. Our observation of cell and fiber alignment here is consistent with in vivo observations during wound healing where collagen fibers and fibroblasts become aligned within the wound bed (Ehrlich and Krummel, 1996; Tomasek et al., 2002); furthermore, perpendicular cell alignment to fluid flow has previously been seen in 2D shear studies on smooth muscle cells (Lee et al., 2002). The flow-induced increase in cell proliferation also mimics the pathological condition: whereas in normal wound repair, myofibroblasts eventually undergo apoptosis while the granulation tissue evolves into a scar with a sparse cell population (Tomasek et al., 2002); in fibrotic conditions, they continue to proliferate and overproduce ECM, leading to elevated fibroblast density (Ehrlich and Krummel, 1996).

To investigate the mechanism underlying this interstitial flow-induced myofibroblast differentiation, we examined

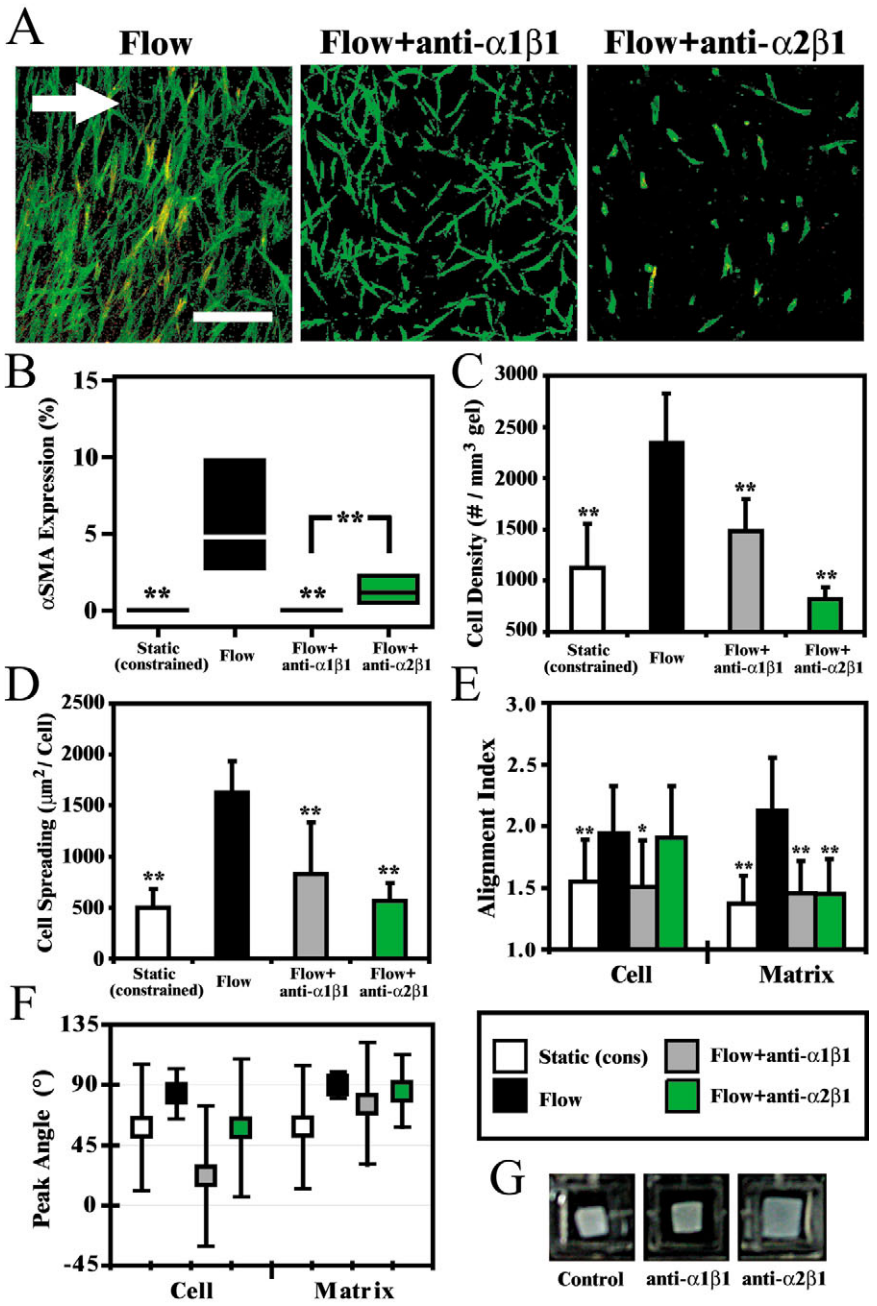


Fig. 5. Interstitial flow effects are mediated through $\alpha_1\beta_1$ integrins. (A) Confocal images of cells under normal, $\alpha_1\beta_1$- and $\alpha_2\beta_1$-neutralized flow conditions for 2 days (green, f-actin; red, α-SMA; arrow indicates flow direction). (B) Bar graphs indicating α-SMA expression levels for the respective normal and bioneutralized conditions (**P<0.01 using Dunn's test); box plot shows 95% confidence intervals with midline showing the median. (C,D) Effects of integrin blocking on cell spreading and density (**P<0.01 using Dunnett's test). (E,F) Effects of integrin blocking on the alignment and orientation of cell and collagen fibers under the various experimental conditions (*P<0.05; **P<0.01 using Dunnett's test). Error bars indicate s.d. (G) Contraction of free-floating gels by fibroblasts after no treatment (control) or treatment with either of the integrin-blocking antibodies as indicated; only neutralizing $\alpha_2\beta_1$ integrin prevents contraction. Bar, 200 μm.

the role of TGF-β_1, a potent and well-known inducer of α-SMA expression (Arora et al., 1999; Dugina et al., 2001; Kunz-Schughart et al., 2003; Vaughan et al., 2000) as well as a stimulus of collagen production (Roberts et al., 1986) and inhibitor of collagen proteolysis (Mutsaers et al., 1997). First, we saw that interstitial flow triggered the autocrine production of TGF-β_1 in fibroblasts, correlating with the increased α-SMA expression, and that blocking TGF-β_1 with antibodies completely prevented the flow-induced α-SMA expression. Furthermore, this TGF-β_1 induction is probably responsible for the flow-enhanced cell proliferation, as TGF-β_1 has been shown to prevent apoptosis (Phan, 2002; Zhang and Phan, 1999) and induce cell proliferation, at least in vascular smooth muscle cells (although it also has been found to inhibit proliferation under certain conditions) (Gibbons, 1994).

To further explore how flow triggers TGF-β_1 signaling, we examined the roles of β_1 integrins. These vital cell matrix adhesion molecules play crucial roles in transducing ECM strain to the cell (Chiquet et al., 2003; Eckes et al., 1999) and are known to be important in tissue repair and fibrosis by regulating cell proliferation, survival, differentiation, migration, matrix deposition and wound contraction (Danen and Sonnenberg, 2003; Eckes et al., 1999; Mutsaers et al., 1997). Although several different integrins can mediate collagen matrix remodeling, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{11}\beta_1$, and $\alpha_v\beta_3$ (Tamariz and Grinnell, 2002), we focused on $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins because β_1 integrins have been associated with tensional force generation in fibroblasts within a collagen type I matrix (Jenkins et al., 1999; Schiro et al., 1991) and because $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins are largely responsible for regulating ECM remodeling in fibroblast-populated collagen gels (Langholz et al., 1995). The results from our bioneutralization experiments suggest that β_1 integrins do indeed play a specific role in mechanical force-regulated formation of α-SMA, particularly $\alpha_1\beta_1$, as blocking antibodies against $\alpha_1\beta_1$ completely inhibit the flow-induced expression of α-SMA without interfering with the ability of the fibroblasts to contract the gel. Thus, although our gel contraction data is consistent with earlier reports that $\alpha_2\beta_1$ integrin is specifically required for gel contraction (Jenkins et al., 1999; Schiro et al., 1991), it also demonstrates that the $\alpha_1\beta_1$ integrin is specifically involved in flow-induced differentiation but not required for ECM adhesion or contraction by the fibroblasts. This indicates that the $\alpha_1\beta_1$ integrin plays a specialized function specific to fibroblast differentiation and is not surprising as $\alpha_1\beta_1$ is abundant in smooth muscle cells (Heino, 2000) and is thought to be the sole integrin utilized by contracting myofibroblasts in wound healing in vivo (Racine-Samson et al., 1997).

Thus, these studies suggest a mechanism of flow-induced myofibroblast differentiation and matrix remodeling as illustrated in Fig. 6. As for the fundamental mechanotransduction mechanism triggering these responses, it is still unclear exactly how the cell might sense such low levels of interstitial flow. It has been established that mechanical forces like stretch can drive fibroblast differentiation toward a myofibroblast phenotype (Arora and McCulloch, 1994; Hinz et al., 2001a; Hinz and Gabbiani, 2003b), although the mechanism remains unclear; furthermore, 2D shear stress can induce autocrine TGF-β_1 expression in vascular smooth muscle cells both in vitro on confluent cell monolayers (Ueba et al., 1997) or in vivo after experimental artery injury (Song et al., 2000). Here, the levels of interstitial flow imposed are extremely small: based on a measured average hydraulic conductivity of 1×10^{-9} cm^2 at the beginning of the experiment that decreased to 2×10^{-10} cm^2 after 5 days of interstitial flow owing to matrix remodeling (Ng and Swartz, 2003), we estimated the average fluid shear stress on the cells to vary between 0.15 and 0.33 dyn/cm^2 (Wang and Tarbell, 1995). It is not known whether such small shear stresses can be sensed by the cells, although 2D stresses as low as 0.1 dyn/cm^2 imposed on endothelial cell monolayers can elicit gene upregulation (Barakat and Lieu, 2003). Little to no stretch would be expected to be imposed on the cell as the ECM is anchored in all directions, although non-affine deformation behavior in a collagen matrix can lead to nonuniformly distributed strain; however, this would tend to decrease, rather than increase, imposed strain on the cell (Pedersen and Swartz, 2005). Other possible mechanisms may include small changes in the local extracellular biochemical environments: for example, changes in extracellular distribution and transport of cell-secreted cytokines (Swartz, 2003).

In conclusion, our data demonstrate the influence of interstitial flow on myofibroblast differentiation, fibroblast proliferation and matrix alignment; all of which are distinct and important characteristics of fibroblasts in fibrotic tissues. Its strong ability to induce myofibroblast differentiation occurs without exogenous inflammatory mediators. This suggests that interstitial flow may help to modulate fibroblast phenotypes and drive the progression of fibrotic diseases, including organ fibrosis (lung, liver, renal or heart), defective wound healing

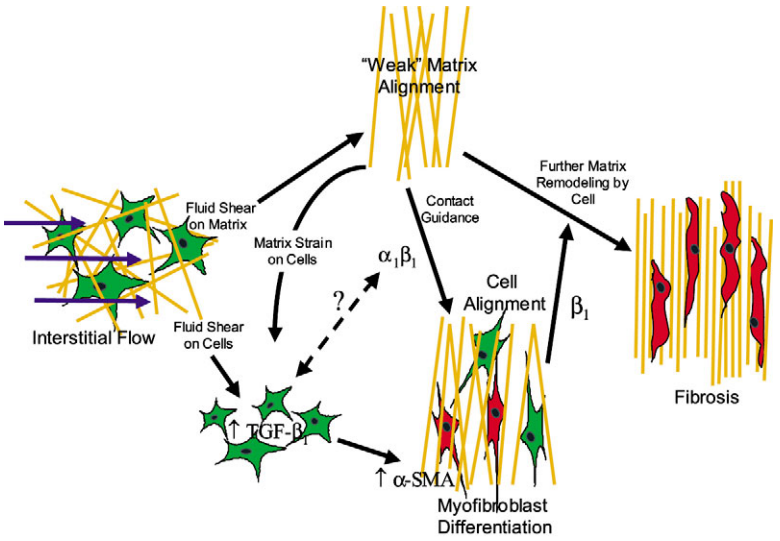


Fig. 6. Proposed mechanism of interstitial flow-driven myofibroblast differentiation and matrix remodeling. First, flow itself can impose shear stress on the cells directly and strain on the cells via stresses on the ECM fibers to which the cells attach. Either of these may trigger TGF-β_1 expression, the latter through $\alpha_1\beta_1$ integrin signaling. TGF-β_1 drives α-SMA expression as the fibroblasts differentiate into myofibroblasts and align the matrix fibers.

like Duputryen's contacture (Tomasek et al., 2002) and connective tissue diseases like artherosclerosis, scleroderma and asthma. Interestingly, we have previously found that the same range of interstitial flow also enhances endothelial morphogenesis (Ng et al., 2004), which is another essential component of the wound healing process (Ehrlich and Krummel, 1996). Taken together, these results suggest that the biophysical environment of tissues undergoing chronic inflammation and/or swelling may significantly affect long-term tissue remodeling towards a fibrotic state. Our results also have a potential use in regenerative medicine and may be useful in the design of therapeutic approaches to prevent fibrotic disease or to promote wound healing.

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