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Prostaglandin E2 differentially regulates contraction and structural reorganization of anchored collagen gels by human adult and fetal dermal fibroblasts

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Abstract

Contraction and remodeling of granulation tissue by fibroblasts is a crucial component of dermal wound healing. Postnatal wounds heal with imperfect repair and scar formation, whereas tissue repair in fetal wounds is regenerative. Prostaglandin E2 (PGE2) modulates the behavior of fibroblasts in the wound bed. This study was designed to investigate the mechanism by which PGE2 regulates an in vitro model of granulation tissue, anchored collagen gels, by human adult and fetal dermal fibroblasts. We hypothesized that PGE2 differentially regulates contraction and remodeling of anchored collagen gels by these fibroblast phenotypes. These results indicate that once tension was generated, fetal fibroblasts exerted lower contractile forces resulting in less collagen contraction. This coincided with less prominent stress fibers, yet fetal fibroblasts were able to substantially remodel the collagen architecture. This mechanism was differentially modulated by PGE2 and was mimicked with a PGE2 receptor agonist, indicating a cyclic adenosine monophosphate (cAMP)-dependent mechanism through the EP2 receptor. However, direct up-regulation of cAMP led to decreases in contraction and remodeling by both fibroblast phenotypes indicating an altered signaling pathway. Therefore, targeting cAMP via the EP2 receptor could potentially decrease adult fibroblast contractile forces to the levels of the fetal fibroblast phenotype in order to decrease dermal scarring.

In the adult dermis, fibroblast contraction and remodeling of granulation tissue is an important step in the final outcome of scar tissue formation. This imperfect dermal repair is characterized by disorganized collagen that does not completely recapitulate the original structure and function of the dermal tissue.¹ The degree of scarring has been linked to early

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stages of wound healing in which inflammatory mediators guide fibroblast activity in the wound bed. In contrast, fetal wound healing is characterized by an altered inflammatory response and minimal contraction leading to complete regeneration of dermal wounds.² Crucial to this regenerative wound healing is the intrinsic phenotype of the fetal fibroblast, considered to be the key effector of scarless fetal wound healing.³ The fetal fibroblast phenotype, with its unique response to inflammatory mediators, contributes to dynamic interactions integral to the scarless outcome.

Contraction and remodeling by fibroblasts is dictated by actin cytoskeletal forces generated inside the cells known as traction forces. Early in adult wound healing, closure of the wound is thought to be initiated by fibroblast migration at the wound edges.⁴ As the wound begins to close due to migratory forces, resistance from the newly formed tissue causes the differentiation of fibroblasts into myofibroblasts.^{5,6} The myofibroblast phenotype is characterized by large traction forces known as contractile forces, which are due to up-regulation of α -smooth muscle (SM) actin.⁵ In contrast, myofibroblasts are either not detected or are only transiently expressed in fetal wounds of certain sizes.^{7,8} This implies that fetal fibroblasts do not differentiate into myofibroblasts and that migratory forces are sufficient for appropriate collagen organization; however, little is known about their contraction and remodeling abilities and the influence of inflammatory mediators on these activities.

Prostaglandin E2 (PGE2) is an important inflammatory product of the arachidonic acid cascade catalyzed by cyclooxygenase-2, which is involved in inflammation and fibrotic responses during tissue repair.^{9,10} The effects of PGE2 are transduced through four E-prostanoid (EP) receptors by means of calcium- and cyclic adenosine monophosphate (cAMP)-dependent signaling cascades.^{11–14} In particular, the EP2 and EP4 receptor pathways modulate migration and chemotaxis by up-regulation of cAMP,¹⁵ and its subsequent effects on cell morphology due to cytoskeletal rearrangement lead to decreases in free floating collagen gel contraction.^{16–18} PGE2 also inhibits the differentiation of fibroblasts into the highly contractile myofibroblast phenotype via the EP2 receptor by inhibiting α -SM actin expression,¹⁹ suggesting a role in modulating cellular force generation. In addition, we have shown a differential cAMP/protein kinase A (PKA)-dependent mechanism for human fetal and adult dermal fibroblast migration²⁰ and free floating collagen gel contraction²¹ via the EP2/EP4 and EP2 pathways, respectively. Therefore, changes in the actin cytoskeleton and resulting force generation due to PGE2 signaling pathways may contribute to differences in fetal and adult contraction and remodeling.

In vitro models of contraction and remodeling that experience mechanical loading, such as attached or anchored collagen gels, have been shown to approximate granulation tissue due to biological and mechanical similarities.²² Fibroblasts in contracting anchored collagen gels proliferate, exhibit α -SM actin, and remain polarized in a spindle-shaped, bipolar morphology indicative of a myofibroblast phenotype.^{23,24} The contractile forces generated by α -SM actin stress fibers are exerted through focal adhesions, similar to fibronexus junctions found in contractile fibroblasts in granulation tissue.²⁵ Anchored collagen gel contraction results in not only compaction of the collagen matrix as in free floating collagen gels but also remodeling of gel fibrils resulting in fibril reorganization and alignment as well as cellular alignment.^{23,24} Therefore, this in vitro model is useful for studying changes in fibroblast phenotype and the resulting forces generated for contraction and remodeling.

Our group was the first to perform a direct comparison of free floating collagen gel contraction by human adult and fetal dermal fibroblasts, and we observed a fetal phenotype with faster migration (i.e., greater contraction) and a differential response to PGE2 via

cAMP up-regulation through the EP2 receptor pathway.²¹ Although this model is considered representative of contraction and remodeling, the contraction is a result of migratory forces akin to invasion of fibroblasts into the wound bed, and not the contractile forces exerted by myofibroblasts during granulation tissue remodeling.²⁴ The current study extends the findings of our previous work by utilizing an anchored collagen gel model as an in vitro analog of granulation tissue to evaluate the effects of PGE2 and the involvement of the EP2 receptor pathway related to adult and fetal fibroblast collagen gel contraction and physical remodeling of the fibril architecture in a mechanically loaded environment.

MATERIALS AND METHODS

Chemicals

Pharmacological agents used in these experiments were PGE2 (Cayman Chemical, Ann Arbor, MI), butaprost (the free acid form), an EP2 receptor agonist (Cayman Chemical), and forskolin, a direct activator of adenylate cyclase and subsequent cAMP levels (Calbiochem, San Diego, CA).

Cell culture

Strains of human dermal fibroblasts were previously generated in our laboratory, which were produced during standard surgical procedures, with institutional review and approval for work with human-derived materials. Two human adult dermal fibroblast cell strains (derived from 20- and 66-year-old patients) and two human fetal dermal fibroblast cell strains (of 16 and 18 weeks gestation) were obtained from frozen stocks. Frozen cells were thawed and cultured in Dulbecco's modified eagle medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen) and 10 U/mL penicillin, 10 U/mL streptomycin sulfate, and 0.025 µg/mL of Amphotericin B (Antibiotic-Antimycotic; Life Technologies, Rockville, MD) in a humidified atmosphere containing 5% CO₂ at 37 °C. Culture medium was changed twice per week and cells were subcultured as they became confluent. All cells were used before passage 10 (approximately 15 total population doublings). Near confluent cultures were washed twice with 1× Hank's balanced salt solution (Invitrogen), trypsinized (Invitrogen), and resuspended in medium. Cells were counted with a hemacytometer then centrifuged and resuspended in the appropriate volume for use in the experiments.

Anchored collagen gels

The anchored collagen gel assay is a modification of a previous technique.²⁶ All work was conducted using sterile technique. The type I collagen source, Vitrogen (Angiotech Biomaterials, Palo Alto, CA), was prepared using the manufacturer's protocol of an 8 : 1 : 1 ratio of Vitrogen, 10 × PBS (Sigma, St. Louis, MO), and 0.1 M NaOH (Sigma) while adjusting the final pH with a small amount of 0.1 M HCl (Sigma), to a final collagen concentration of 2.4 mg/mL, pH 7.4. Collagen gels with a volume of 150 µL were cast in 22×10 mm silicone molds placed on 22×40 mm glass coverslips (Chase Scientific Glass, Norcross, GA) in 60×15 mm Petri dishes (Cardinal Health, Warrendale, PA). The silicone molds were created using a commercially available 601 A/B mixture (Wacker Silicone, Lansing, MI). Inside the molds on the glass coverslips, 5×10 mm steel mesh pieces (Small Parts Inc., Miami Lakes, FL) were glued using a silicone adhesive (Henkel Consumer Adhesives, Avon, OH) at opposite ends of the longest dimension of the mold to provide anchoring for the collagen gels. The Petri dishes were placed in a 37 °C water bath for 1 hour for collagen gelation. Fibroblasts were seeded on to the collagen gels inside the molds in a volume of 400 µL containing 5.0×10⁴ cells in medium supplemented with 10% fetal bovine serum. The dishes were incubated for 30 minutes to ensure fibroblast adhesion to the collagen gels. The molds were removed with tweezers, and 7 mL of medium supplemented

with 2% fetal bovine serum (control) with and without pharmacological agents was then added. Pharmacological agents were added to this medium at the following concentrations (exceeding equilibrium values for the EP-specific ligands¹²): 10 μ M PGE2, 10 μ M butaprost (an EP2-specific PGE2 agonist), and 25 μ M forskolin (an adenylate cyclase activator). The culture dishes were incubated for another 30 minutes to allow for equilibration of the medium then, if required, the collagen gels were loosened from the glass coverslips by gentle pipetting or mechanical release with a metal hook. Collagen gels without cells were also created by following this procedure but omitting the fibroblasts in the 400 μ L applied to the anchored collagen gels.

Initial experiments utilized a volume of 75 μ L of collagen solution. One of these experiments, presented here, was carried out to 6 days with 10% fetal bovine serum to determine the time period for maximum contraction. From these early experiments, we determined that a 24-hour-period was sufficient for observing major changes in collagen contraction and remodeling. Also, we found that switching to a volume of 150 μ L of collagen solution provided more stability between the anchors. Therefore, collagen gels were imaged at 0, 2, 4, and 24 hours with a digital camera (Diagnostic Instruments, Sterling Heights, MI) connected to a dissecting microscope (Olympus, Center Valley, PA). Changes in collagen gel area were measured using Metamorph software (Universal Imaging, Downingtown, PA). Percent contraction was calculated as the change in area from the initial area at time zero. The collagen gels were imaged with a grid underneath for size calibration.

Cell morphology and actin cytoskeleton imaging

Cell morphology was imaged using $\times 100$ phase contrast microscopy (Nikon, Melville, NY) at each time point. For fluorescence imaging, collagen gels were fixed with 10% formalin (Sigma) and blocked with 10% horse serum (Vector, Burlingame, CA) in PBS for 1 hour. Cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 20 minutes. F-actin and α -SM actin were visualized with Texas Red-X phalloidin (1 : 200; Invitrogen) and FITC monoclonal anti- α -SM actin (1 : 300; Sigma), respectively, after 1 hour of incubation. The collagen gels were imaged using an Olympus FluoView FV1000 confocal microscope (Olympus). Images of the fibroblasts were captured at $\times 600$ in the center area of the anchored collagen gels.

Small angle light scattering (SALS)

SALS is a well-developed technique that has been described previously.²⁷ Briefly, a continuous, unpolarized laser light from a 4 mW HeNe (Uniphase, Manteca, CA) with a wavelength of 632.8 nm was passed through a collagen gel with a spatial resolution of 200 μ m. The fixed collagen gel was placed in a custom-built glass holder with PBS and moved on two axes with a precision linear travel system composed of computer controlled stepper motors (Arrick Robotics, Hurst, TX). The internal planar fibril architecture of the specimen causes a scattering of the laser light that is collected on a projection screen at each position within the collagen gel. SALS video images of the laser light scattering is collected, digitized to a resolution of 640 $\times$ 480 eight-bit pixels, and analyzed with custom software. The resulting angular distribution of scattered light intensity, $I(\Phi)$, is interpreted by the software as a distribution of fibril angles corresponding to the tissue sample at each position. This analysis gives the preferred fibril direction, represented as a vector, which is the centroid, Φ_c , of $I(\Phi)$ and an orientation index, OI, which is defined as the angle that contains 50% of the total fibrils, represented by a specific color. Randomly oriented fibril networks result in high OI values while highly oriented fibril networks result in low OI values. The resulting angular distribution of scattered light can be used to quantify the fibril architecture in terms of the orientation and distribution of collagen fibrils at each position within the collagen gels.

The anchored collagen gels were fixed at 24 hours and scanned with SALS. The center region of the anchored collagen gels, which undergo the largest amount of contraction, were analyzed for collagen fibril orientation by using the OI values. In order to accurately quantify each region for each collagen gel, these center regions were analyzed by using square areas consisting of 12×12 vectors (corresponding to square areas of OI values) yielding a total of 144 values per collagen gel.

Statistics

Percent contraction values are expressed as mean $\pm$ standard deviation (SD). The OI values for the center region were averaged for one representative value for each area for each collagen gel and then averaged for each experimental group and expressed as mean $\pm$ SD. Comparisons were made based on evaluation of the data for normality and equal variance. Data passing the normality and equal variance tests were analyzed by either a Student's *t* test or a one-way ANOVA followed by the Holm–Sidak Method to detect statistically significant differences. Data failing the normality and equal variance tests were analyzed by the Mann–Whitney Rank Sum Test. All statistical analyses were performed using SigmaStat 3.0 (Systat Software, Point Richmond, CA). A value of $p < 0.05$ was considered statistically significant, and $p < 0.01$ was considered highly significant.

Collagen gel contraction and remodeling data generated using multiple strains of either human dermal adult or fetal fibroblasts were pooled together. Our previous work has shown very consistent results among individual human adult and fetal dermal fibroblast cell strains with respect to migration and contraction forces.^{20,21} In this work, we also observed similar consistency in our anchored collagen gel contraction data for the fibroblast cell strains that were used in those previous studies. Therefore, the number of cell strains used in each experiment was minimized ($n=2$) since we once again found fairly uniform results (i.e., small standard errors) for each fibroblast phenotype with respect to degree of contraction of anchored gels. Given the consistency in the data, subsequent experiments utilized one representative cell strain for each phenotype per condition tested (butaprost and forskolin).

RESULTS

Cellular morphology changes from stellate to spindle-shaped over time under basal conditions

Experiments were conducted with two different human dermal adult and two different human dermal fetal fibroblast cell strains. Collagen gels were seeded with 5.0×10^4 cells in medium supplemented with 2% fetal bovine serum and allowed to contract over 24 hours. Over time, the anchored collagen gels decreased in volume due to compaction of the lattice, and fibril alignment was evident due to cellular alignment between the anchors (Figure 1). Early on, fibroblasts appeared stellate and spread with multiple protrusions in no preferred direction. However, as the anchored collagen gels began to contract and the fibrils began to align, the fibroblasts became spindle-shaped and polarized between the anchors. This altered morphology and alignment was more pronounced in the center and corners of the collagen gels as opposed to the midportions along each anchored end.

Contraction is eventually greater with adult fibroblasts but remodeling is similar under basal conditions

Under basal conditions, fetal fibroblasts exhibited a dynamic response in their ability to contract anchored collagen gels as time progressed (Figure 2A). At 2 hours, fetal fibroblasts exhibited a greater contraction of anchored collagen gels compared with their adult counterparts, 12.5 vs. 9%, respectively ($p < 0.01$). However, at 4 hours, there was no significant difference in the degree of contraction between adult (18.3%) and fetal (15.6%)

fibroblasts. Then at 24 hours, collagen gel contraction was significantly higher for adult vs. fetal fibroblasts, 32.8 vs. 24.4%, respectively ($p < 0.01$). Over a 6-day period in a separate experiment, this increased collagen gel contraction ability by adult fibroblasts vs. their fetal counterparts was significant up to 3 days (Figure 2B, $p < 0.01$). Overall, except at early time points, fetal fibroblast contraction of anchored collagen gels was significantly lower under basal conditions compared with adult fibroblasts. At 24 hours, collagen gel reorganization by both adult and fetal fibroblasts was significantly greater (i.e., lower OI values) than collagen gels without cells (Figure 2C). The OI values of 40.7° and 38.6° in the center region for adult and fetal fibroblasts, respectively, were not significantly different from each other but were far less than the collagen gels without cells at 57.1° ($p < 0.01$), indicating that significant remodeling and alignment of collagen fibrils was occurring and measurable in our system.

Our explanation for the observed dynamic variation of fetal fibroblasts in this model is that under initial conditions and up to 2 hours, the anchored collagen gels are under very little tension, so the environment is similar to free floating collagen gels, in which fetal fibroblasts exert greater traction forces and induce more contraction.²¹ Between 2 and 4 hours, the anchored gels undergo sufficient initial contraction (about 15%) to develop tension, after which time, adult fibroblasts develop more actin stress fibers, relative to their fetal counterparts (Figure 3), exhibiting a myofibroblast phenotype, which results in greater contraction under tension at 24 hours. Although both adult and fetal fibroblasts began orienting and aligning between the anchor points and exhibited a spindle-shaped, polarized morphology by 4 hours, the differences in contraction and presence of more prominent stress fibers in adult fibroblasts suggests alterations in the regulation of the fetal fibroblast cytoskeleton, which were maintained at 24 hours as well (data not shown), even though there were no significant differences measured in collagen gel remodeling.

Collagen gel contraction and remodeling is differentially inhibited by PGE2

Compared with untreated controls, 10 μ M PGE2 significantly inhibited adult fibroblast contraction at 2, 4, and 24 hours by 46, 46, and 29%, respectively (Figure 4A, $p < 0.01$). Collagen gel contraction by fetal fibroblasts was not inhibited by PGE2 at any of the observed time points (Figure 4B). By 24 hours, collagen reorganization by adult fibroblasts in the center region of the collagen gels was significantly inhibited by PGE2 by 9.4° (Figure 4C, $p < 0.05$). Fetal fibroblasts were not inhibited in their ability to reorganize the center region of the collagen gels in response to PGE2. Throughout the structure of the collagen gels, the lack of reorganization and alignment of collagen fibrils was quite evident with PGE2 for adult fibroblasts, particularly in the center region, as was the degree of remodeling by cells in general (Figure 5). At 4 hours, adult fibroblast spreading was significantly inhibited by PGE2 with destabilization of the actin cytoskeleton, whereas fetal fibroblasts retained their spindle-shaped, polarized morphology on the anchored collagen gels and maintained actin filaments (Figure 6). These early differences in cell spreading suggest distinct differences in the regulation of the actin cytoskeleton of adult and fetal fibroblasts that contribute to their differential ability to contract the anchored collagen gels as well as their ultimate reorganization and alignment of collagen fibrils.

Collagen gel contraction and remodeling is differentially inhibited by the EP2 receptor agonist butaprost

In our previous work, we had tested multiple EP receptor agonists to determine the signaling pathway responsible for changes in morphology and concomitant contraction. These results indicated that morphological changes in the actin cytoskeleton of fibroblasts on free floating collagen gels is a result of the EP2 receptor signaling pathway.²¹ Therefore, only the EP2 receptor agonist butaprost was tested in this study. Butaprost exerted the same effects as

PGE2 by decreasing adult fibroblast collagen gel contraction by 46, 52, and 30% at 2, 4, and 24 hours, respectively (Figure 7A, $p < 0.01$). However, fetal fibroblast collagen gel contraction was not affected by butaprost (Figure 7B). In addition, this EP2 receptor agonist mimicked the effects of PGE2 by decreasing collagen fibril orientation by 19.2° in the center region of the adult fibroblast gels but not the fetal fibroblast gels (Figure 7C, $p < 0.01$). At 4 hours, the adult fibroblast morphology with the EP2 receptor agonist resembled the cellular shapes seen with PGE2 (results similar to Figure 6). Adult fibroblast spreading was clearly inhibited, but fetal fibroblasts maintained a spread morphology that allowed for continued contraction and remodeling similar to basal levels. As previously mentioned, this result indicates differential regulation of the actin cytoskeleton through the EP2 receptor for adult vs. fetal fibroblasts.

Collagen gel contraction and remodeling is inhibited by forskolin

Forskolin bypasses receptor pathways by penetrating the cell membrane and causing direct activation of adenylate cyclase, the enzyme responsible for the production of cAMP, the second messenger for the EP2 receptor. Therefore, forskolin was utilized to confirm the inhibitory effects of PGE2 and the EP2 receptor agonist on anchored collagen gel contraction and remodeling to confirm the regulatory role of cAMP in this model system. Adult fibroblast collagen gel contraction was significantly inhibited by forskolin, 38 and 43% at 2 and 4 hours, respectively (Figure 8A, $p < 0.05$). Anchored collagen gel contraction was also significantly inhibited for fetal fibroblasts by 48 and 26% at 2 and 4 hours, respectively (Figure 8B, $p < 0.05$). Reorganization and alignment of collagen fibrils was significantly reduced by forskolin for both adult and fetal fibroblasts by 9.3° and 5.3° , respectively, in the center region of the collagen gels (Figure 8C, $p < 0.05$). For both the adult and fetal fibroblasts, cellular spreading was hindered with forskolin at 4 hours in a similar manner as seen with the adult fibroblasts with PGE2 and the EP2 receptor agonist (results similar to Figure 6).

DISCUSSION

We have effectively utilized an anchored collagen gel model tethered at two ends that allows for compaction as well as fibril reorganization to investigate the differential effects of PGE2 and the EP2 receptor pathway on human adult and fetal dermal fibroblasts. Similar models have been used to evaluate tension and orientation,²⁸ force transduction,²⁹ mechanical anisotropy,²⁶ and collagen fibril alignment.³⁰ Although many variables are associated with these contraction techniques that make them sometimes difficult to reproduce, they do share many common features such as the morphological responses by fibroblasts. Our results show that as the anchored collagen gels contracted, fibroblast morphology changed from a stellate to a spindle, polarized shape that has been described in the literature as a shift to the myofibroblast phenotype.^{23,24} Morphological changes such as these are characteristic of collagen gels that become mechanically loaded with cells aligning along the lines of tension.^{23,24} This alignment has been observed in regions that experience the most tension and therefore undergo the most contraction and remodeling leading to fibril reorganization and alignment—the center and corners. This phenomenon has been previously documented and is due to the nature of the anchoring, which creates different zones of tension within the collagen gels.³⁰ Interestingly, fibroblasts in regions near the mid-portions along the anchored ends maintained a stellate morphology and random orientation for a longer period and demonstrated less remodeling, indicating less tension in these areas.

These changes in morphology and mechanical loading coincided with the shift in the ability of human fetal dermal fibroblasts to contract anchored collagen gels under basal conditions. At 2 hours, the fetal fibroblasts exhibited greater contraction, but this ability was lost over time. Initially, the collagen gels were quite loose and thus did not exhibit significant tension.

Early anchored collagen gel contraction, therefore, may approximate free floating collagen gel contraction. Our previous work with free floating collagen gels showed that fetal fibroblasts maintained a significantly greater ability for contraction than adult fibroblasts over the entire time period.²¹ However, free floating collagen gels contract quickly without any mechanical loading or ordered, physical remodeling of the collagen fibrils (i.e., contraction purely by compaction of the collagen matrix). Fibroblasts remained in a stellate morphology indicative of a migratory phenotype vs. a spindle-shaped, contractile, myofibroblast phenotype. This stellate morphology was seen at the early timepoints on the anchored collagen gels. Therefore, early contraction of anchored collagen gels may also be a result of migratory forces and would account for the increased contraction by fetal fibroblasts early on.

As time progressed, the anchored collagen gels became noticeably taut between the anchors. At 4 hours, a significant degree of cellular alignment was seen as well as the polarized cell morphology for both fibroblast phenotypes. These changes are a result of physical remodeling of the matrix and fibril alignment leading to tension between the anchors. Spindle-shaped, polarized cell morphology, and the presence of stress fibers have been verified in the literature as indications of the myofibroblast phenotype which is known to exert large contractile forces.^{23,24} Staining of the actin cytoskeleton confirmed the existence of stress fibers at 4 hours, but fetal fibroblasts exhibited less prominent stress fibers than adult fibroblasts. However, no statistically significant differences were seen at 4 hours for gel contraction, indicating a larger increase in the rate of adult fibroblast contraction. By 24 hours, adult fibroblasts had contracted the anchored collagen gels to a significantly greater extent than fetal fibroblasts. The differences in the relative stress fiber expression between adult and fetal fibroblasts also persisted at 24 hours (data not shown). This trend in contraction continued for up to 3 days after which time no statistically significant differences were detected between fetal and adult fibroblasts. However, beyond 24 hours, collagen gel contraction is much slower since contraction tends to plateau once a mechanical equilibrium is reached (i.e., contraction eventually reaches a maximal equilibrium value). These data suggest that differences may exist in contractile force production between adult and fetal fibroblasts due to altered cytoskeletal dynamics.

Despite these differences in stress fibers and contraction, differences were not apparent in collagen organization and alignment in the center region at 24 hours. Once neighboring fibrils begin to compact and align based on geometry, spacing between groups must eventually compact as well. Ultimately, aligned fibrils may become packed together and only spacing between bundles begins to shift. Small shifts in collagen fibrils lead to large changes in macroscopic geometry due to the interconnectivity of the collagen architecture.³¹ Therefore, there may be a threshold level at which collagen fibril alignment is indistinguishable due to the mechanical nature of the tethering of the anchored collagen gel model, especially since we found differences in macroscopic contraction by adult and fetal fibroblasts that correlated to differences in actin stress fibers. However, SALS was sensitive enough to demonstrate significant remodeling by both fibroblast phenotypes when compared with collagen gels without cells as well as distinguishing between control and treated groups. Therefore, SALS was able to detect appreciable changes in reorganization and alignment of the collagen gel structure making it a useful technique for assessing changes in collagen fibril remodeling.

Although we did not measure cellular proliferation, which is up-regulated in anchored collagen gels,⁴ we did not expect significant changes to occur in contraction based on changes in cell number within the first 24 hours using 2% FBS. In fact, we have observed slightly higher rates of cell proliferation in culture for fetal fibroblasts; therefore, it is likely that increases in adult fibroblast contraction were due to differences in contractile force

generation. These results suggest that adult fibroblasts have an inherent ability to exert larger contractile forces than fetal fibroblasts due to differential regulation of the actin cytoskeleton and the presence of more prominent stress fibers. It is also possible that fetal fibroblasts do not fully differentiate into a myofibroblast phenotype and consequently cannot exert the larger contractile forces that lead to excessive contraction and remodeling of granulation tissue resulting in scar tissue in adult wounds. This hypothesis is supported by the fact that the myofibroblast phenotype is either transiently expressed or not detected at all in fetal wounds.^{7,8}

PGE2 has been identified as an important mediator of dermal wound healing^{9,10} with specific effects on fibroblast behavior.^{15,32,33} Of particular interest is the work by Kolodnick et al.¹⁹ showing that PGE2 can inhibit the differentiation of fibroblasts into myofibroblasts via the EP2 receptor pathway by up-regulation of cAMP. In addition, inhibitors such as glucocorticoids have been shown to increase fibroblast-mediated collagen gel contraction by inhibiting PGE2 production^{34,35} indicating the importance of this pathway to the wound healing process. We have also identified the influence of this pathway on human adult and fetal dermal fibroblasts in regulating migration²⁰ and free floating collagen gel contraction,²¹ results that paralleled each other since free floating collagen gel contraction is a result of migratory forces. In particular, PGE2 had a differential effect in both cases, and fetal fibroblasts exhibited a more stable morphology and cytoskeleton that led to higher rates of both migration and free floating collagen gel contraction when compared with adult fibroblasts. The effects of PGE2 on contraction and physical remodeling of an anchored collagen gel, an in vitro analog of granulation tissue, are unknown. Interestingly, the effects of PGE2 were similar in this study as well. PGE2 significantly inhibited anchored collagen gel contraction and remodeling by adult fibroblasts but had no effect on fetal fibroblasts. The changes in cellular morphology for the adult fibroblasts were quite evident and indicated dramatic changes in the cytoskeleton and loss of stress fibers. In contrast, fetal fibroblasts maintained their actin cytoskeleton and stress fibers. This result suggests that fetal fibroblasts have a more stable and active cytoskeleton that retains the ability to generate forces for collagen contraction and remodeling in the presence of PGE2, but the nature and mechanism of these forces is still in question. This observation could also suggest the lack of a true myofibroblast phenotype in fetal fibroblast cultures. This response to PGE2 is conserved regardless of the mechanical nature of the underlying substrates and subsequent changes in phenotype (i.e., free floating vs. anchored collagen gels); however, the amount of contraction is significantly different when compared with adult fibroblasts.

Since we had previously identified the importance of the EP2 receptor signaling pathway in regulating free floating collagen gel contraction,²¹ we utilized the EP2 receptor agonist butaprost in this study to determine whether this pathway was responsible for the effects observed with PGE2. Butaprost significantly inhibited anchored collagen gel contraction and remodeling by adult fibroblasts in a manner that closely mimicked the effects of PGE2, including changes in cellular morphology. In contrast, fetal fibroblasts were not affected in their ability to contract or remodel the anchored collagen gels. The EP2 receptor pathway involves up-regulation of cAMP and has been shown to destabilize actin filaments and inhibit free floating collagen gel contraction.^{36–38} A similar effect was observed in the present study for adult fibroblasts on anchored collagen gels suggesting an alteration in contractile force production. However, this cAMP-dependent mechanism does not appear to affect the activity of fetal fibroblasts, since they once again retained their ability to contract the anchored collagen gels, implying a stable and functional cytoskeleton in the presence of butaprost. This cAMP-dependent mechanism was confirmed with forskolin, a direct activator of adenylate cyclase and subsequent cAMP production. Both adult and fetal fibroblast contraction and remodeling was significantly inhibited indicating that the effects of cAMP are conserved. We have shown previously that these human dermal adult and fetal

fibroblasts express equivalent levels of all four EP receptors.²⁰ In addition, basal cAMP levels for these adult and fetal fibroblasts are similar but only adult fibroblast cAMP levels increase in response to exogenous PGE2 or butaprost.²⁰ Therefore, it is likely that some other step in the EP2 signaling pathway leading to cAMP up-regulation is responsible for the differential effects seen with PGE2 and butaprost, or that the EP2 receptor is less responsive in fetal fibroblasts.

Taken collectively, these studies suggest that fetal fibroblasts exert less contractile force than their adult counterparts in an anchored collagen gel model but still may retain the ability to adequately remodel the collagen architecture. These results are in contrast to our previous work in which fetal fibroblasts maintained a greater ability to contract free floating collagen gels, which exemplifies an important distinction between the two collagen gel in vitro models of contraction, as described in the introduction. Therefore, the use of these two models suggest that fetal fibroblasts exert substantial migratory forces but not contractile forces when compared with their adult counterparts, and this is supported by the appearance of less prominent stress fibers. In addition, these reduced contractile forces may be sufficient for localized extracellular matrix remodeling, even in areas experiencing some level of tension. Recently, Tomasek et al.³⁹ suggested that an intermediate phenotype exists, the proto-myofibroblast, which exerts contractile forces utilizing stress fibers not containing α -SM actin. The proto-myofibroblast phenotype can be induced with mechanical tension and exhibits cytoplasmic actin stress fibers and fibronexus adhesion complexes. They also reported that bulk force measurements for proto-myofibroblasts on anchored collagen gels approach those of fully differentiated myofibroblasts. This observation suggests that stress fibers in general, regardless of their actin monomer composition, can exert different levels of contractile forces to cause anchored collagen gel contraction. Therefore, contraction of in vitro models of granulation tissue undergoing tension are a result of a myofibroblast-like phenotype capable of exerting contractile forces and do represent a shift from the “normal” fibroblast phenotype. Overall, we have shown that fetal fibroblasts exhibit less prominent stress fibers, regardless of their origin, resulting in less contraction under tension. Therefore, fetal fibroblasts may stay in a proto-myofibroblast phenotype in which stress fibers are evident and similar force levels can be achieved; however, these stress fibers may be composed of more cytoplasmic actins than α -SM actin. In either case, fetal fibroblasts maintained a propensity for collagen gel contraction and remodeling despite the presence of PGE2 in both free floating and anchored collagen gel assays.

An implication from this study is that smaller traction forces, whether they are migratory forces, diminished contractile forces, or somewhere in between, may be sufficient for extracellular matrix remodeling in fetal wound healing. In fact, these diminished forces could explain the lack of excessive contraction and remodeling of the wound bed in fetal wounds and lack of scar tissue formation. In the presence of PGE2, fetal fibroblasts retained their ability to actively contract and remodel the anchored collagen gels; however, inhibition caused by forskolin implies that a cAMP-dependent mechanism for regulating the cytoskeleton is still conserved. This mechanism has been described for inhibited free floating collagen gel contraction in which up-regulation of cAMP activates PKA which phosphorylates the myosin light chain kinase leading to destabilization of actin-myosin complexes.³⁶ It is likely that a similar pathway involving cytoskeletal alterations is involved in inhibition of anchored collagen gel contraction; however, the alteration in the fetal fibroblast signaling pathway responsible for the differential effects in contraction and remodeling remains unknown.

Overall, the effects of PGE2, and specifically the EP2 receptor signaling pathway, suggest a regulatory mechanism for modulating cellular force generation in vivo, particularly in the case of adult wound healing. Specifically, targeting the cAMP regulatory mechanism

through the EP2 receptor may provide a therapeutic target for decreasing adult fibroblast contractile forces to a “fetal level” by reducing stress fiber expression and thereby reducing the amount of contraction and remodeling ultimately resulting in less scar contracture. The structural and functional benefits to patients from such an alteration of the natural course of postnatal wound healing would represent a significant advance in the management of this vexing clinical challenge. In order to further explore these differences in adult and fetal wound healing, we will focus our future work on changes in actin expression and specific cytoskeletal proteins involved in force production in order to further characterize these differences in contraction and remodeling.

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Figure 1.

An example of the time course of anchored collagen gel contraction (top panel) and morphologic changes in the center from stellate to polarized (bottom panel) by human adult dermal fibroblasts in control conditions at 0, 2, 4, and 24 hours ($\times 100$ phase contrast). Images (top panel) were analyzed for changes in area and expressed as percentage contraction based on the collagen gel sizes at $t=0$. Scale bar represents 5 mm.

Figure 2.

Human fetal dermal fibroblast contraction of anchored collagen gels is diminished (A) over the first 24 hours (B) and is less than their adult counterparts at later time points. (C) Both human adult and fetal dermal fibroblasts cause significant reorganization and alignment of collagen fibrils compared with control gels without cells. All anchored collagen gel assays were performed using 5.0×10^4 cells in control conditions (DMEM and 2 or 10% FBS). $n=9$ and 17 for the adult and fetal fibroblast contraction over 24 hours, respectively, and $n=14-17$ and $n=6-11$ for the adult and fetal fibroblast contraction over 6 days, respectively. For the OI analyses, $n=8$, 4, and 13 for no cells, adult fibroblasts, and fetal fibroblasts, respectively. Values represent mean $\pm$ SD, and * $p < 0.01$ for adult vs. fetal fibroblast contraction and OI comparisons.

Figure 3.

Human adult and fetal dermal fibroblasts exhibit a spindle-shaped, polarized morphology and alignment between the anchors (top and bottom) in the center of the anchored collagen gels; however, adult fibroblasts exhibit more prominent stress fibers than fetal fibroblasts. Stress fibers were observed at $\times 600$ with confocal microscopy at 4 hours in control conditions using Texas Red-X phalloidin (1 : 200) and FITC monoclonal anti- α -SM actin (1 : 300) for F-actin and α -SM actin visualization, respectively.

Figure 4.

PGE2 inhibits the contraction of anchored collagen gels by (A) human adult dermal fibroblasts but not by (B) human fetal dermal fibroblasts at 2, 4, and 24 hours. (C) At 24 hours, PGE2 inhibits the organization of collagen fibrils in the center region of anchored collagen gels by human adult dermal fibroblasts but not by human fetal dermal fibroblasts. Anchored collagen gels were assessed in control conditions with and without 10 μ M PGE2. $n=9$ and 11 for the adult control and PGE2 contraction assays, respectively, and $n=17$ and 9 for the fetal control and PGE2 contraction assays, respectively. $n=4$ and 11 for the adult control and PGE2 OI assays, respectively, and $n=13$ and 8 for the fetal control and PGE2 OI assays, respectively. Values represent mean $\pm$ SD, and * $p < 0.01$ for control vs. PGE2 contraction comparisons and $p < 0.05$ for control vs. PGE2 OI comparisons.

Figure 5.

(A) An example of a collagen gel without cells showing little organization or alignment. Examples of (B) reorganization and alignment of a collagen gel in control conditions, and (C) the effects of PGE2 on human adult dermal fibroblast organization of an anchored collagen gel at 24 hours. Red values indicate low OI values (i.e., high orientation) and blue areas indicate high OI values (i.e., low orientation). The vectors represent the preferred fibril direction in each position. The control case shows a high degree of reorganization of the collagen gel when compared with the collagen gel without cells. In contrast, the PGE2 inhibited the degree of collagen fibril orientation and thus overall reorganization of the collagen gel when compared with the untreated control.

Figure 6.

The spindle-shaped, polarized morphology was inhibited by PGE2 for human adult dermal fibroblasts and coincided with a loss of stress fibers, whereas human fetal dermal fibroblasts maintained a spindle-shaped, polarized morphology in the presence of PGE2 and retained stress fibers in the center of the collagen gels. Stress fibers were observed at $\times 600$ with confocal microscopy at 4 hours with 10 μM PGE2 in the center of the anchored collagen gels using Texas Red-X phalloidin (1 : 200) and FITC monoclonal anti- α -SM actin (1 : 300) for F-actin and α -SM actin visualization, respectively.

Figure 7.

The EP2 receptor agonist butaprost inhibits the contraction of anchored collagen gels by (A) human adult dermal fibroblasts but not by (B) human fetal dermal fibroblasts at 2, 4, and 24 hours. (C) At 24 hours, the EP2 receptor agonist butaprost inhibits the organization of collagen fibrils in the center region of anchored collagen gels by human adult dermal fibroblasts but not by human fetal dermal fibroblasts. Anchored collagen gel assays were assessed in control conditions with and without 10 μ M butaprost. $n=9$ and 8 for the adult control and EP2 receptor agonist contraction assays, respectively, and $n=17$ and 9 for the fetal control and EP2 receptor agonist contraction assays, respectively. $n=4$ and 7 for the adult control and EP2 receptor agonist OI assays, respectively, and $n=13$ and 6 for the fetal control and EP2 receptor agonist OI assays, respectively. Values represent mean $\pm$ SD, and * $p < 0.01$ for control vs. EP2 receptor agonist contraction comparisons and $p < 0.05$ for control vs. EP2 receptor agonist OI comparisons.

Figure 8.

The contraction of anchored collagen gels by (A) human adult dermal fibroblasts and (B) human fetal dermal fibroblasts is inhibited by forskolin at 2 and 4 hours. (C) At 24 hours, forskolin inhibited the organization of collagen fibrils in the center region of anchored collagen gels by both human dermal adult and fetal fibroblasts. Anchored collagen gel assays were assessed in control conditions with and without 25 μ M forskolin. $n=9$ and 5 for the adult control and forskolin contraction assays, respectively, and $n=17$ and 5 for the fetal control and forskolin contraction assays, respectively. $n=4$ and 4 for the adult control and forskolin OI assays, respectively, $n=13$ and 5 for the fetal control and forskolin OI assays, respectively. Values represent mean $\pm$ SD, and * $p < 0.05$ for control vs. forskolin comparisons.