Differential Collagen I Gene Expression in Fetal Fibroblasts

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• ***Purpose:*** **Fetal wound healing is characterized by the regeneration of normal dermis and the absence of scar. Transforming growth factor beta-1 (TGF-β1) is a ubiquitous cytokine with potent fibrogenic effects in both postnatal and fetal wounds. Supplementing fetal wounds with TGF-β1 results in increased fibrosis consisting primarily of collagen I. We hypothesized that the lack of scar formation in fetal wounds may be caused by differential collagen I gene (COL1A1) expression. The authors examined basal collagen Ia gene expression in human fetal, newborn, and adult dermal fibroblasts after stimulation with exogenous TGF-β1.** ***Methods:*** **Subconfluent human dermal fibroblasts from fetal, newborn, and adult cell lines were incubated for 24 hours, then stimulated by incubation for 4 hours with 1 ng/mL of human recombinant TGF-β1, or with media alone for basal collagen gene expression, and then placed in guanidium isothyocyanate buffer. To quantitate COL1A1 gene expression, total cellular RNA was extracted and subjected to northern and slot blot hybridization analysis with Dig-labeled COL1A1 probes. The membrane was exposed to x-ray film for 15 minutes and developed.** ***Results:*** **Scant COL1A1 gene transcript was detected in control fetal fibroblasts. Brief stimulation with of TGF-β1 upregulated the COL1A1 gene transcription in fetal fibroblasts. Gene expression for COL1A1 in both postnatal cell lines appeared similar in treated and untreated cells. Housekeeping control (GAPDH) confirmed no difference in total amount of RNA at the start or end of the experiment.** ***Conclusion:*** **COL1A1 gene expression is notably absent in unstimulated fetal fibroblasts, but is upregulated by TGF-β1. In contrast, postnatal fibroblasts demonstrate significant constitutive COL1A1 gene expression at baseline and unchanged after TGF-β1 stimulation. This differential regulation may contribute to the ability of fetal wounds to regenerate without scar and explain the effect of exogenous TGF-β1 to increase fibroplasia in fetal dermal incisional wounds.**

INDEX WORDS: Fibroblast, fetal wound healing, reverse transcriptase polymerase chain reaction, collagen.

FETAL WOUND HEALING is characterized by an accelerated rate of healing with a more rapid and organized deposition of collagen and extracellular matrix resulting in regeneration of the normal dermal architecture.[1-5] Recent evidence suggests that the ability of fetal skin to heal scarlessly is intrinsic to the fetal fibroblast.[6] Although both adult and fetal fibroblasts migrate into the wound, proliferate, and produce collagen, adult fibroblasts produce large amounts of collagen aligned in parallel bundles perpendicular to the epidermis in a pattern we recognize as scar.[3] In contrast, fetal fibroblasts produce collagen that is aligned in a normal reticular pattern indistinguishable from adjacent unwounded skin.[2]

We have demonstrated previously that there is a fetal fibroblast phenotype distinguished from postnatal fibroblasts by the production of large hyaluronic acid-dependent pericellular matrices,[7] accelerated rates of proliferation (Crombleholme, unpublished observations), distinct chemotactic response to transforming growth factor-beta 1 (TGF-β1), TGF-β2, and platelet-derived growth factor (PDGF).[8-10] In addition, fetal fibroblasts have been demonstrated to have increased hyaluronic acid production,[11] synthesis of particular fibronectin variants and matrix macromolecules,[12,13] and the expression of fetal-specific antigen determinants on their cell surface.[14] It is unknown if this fetal fibroblast phenotype includes differential transcriptional response to signals released in the wound.

TGF-β has ubiquitous expression and diverse biologic activities influencing cell-cell and cell-extracellular matrix interactions particularly during tissue repair.[15,16] TGF-β is known to be released in both adult and fetal wounds,[17] cause accumulation of fibroblasts in wounds,[15] and to stimulate the production of collagen.[16,18-20] TGF-β has also been implicated as a mediator of tissue fibrosis.[21] We have demonstrated previously that there is a lack of upregulation of TGF-β1 gene expression in fetal wound clefts in marked contrast to the upregulation of TGF-β1 gene expression observed in adult wound clefts.[22] Exogenous TGF-β1 applied to fetal skin wounds results in marked fibroplasia and scar formation consisting primarily of collagen type I.[16,18] As a result of these findings, we postulated that the lack of scar formation in fetal wounds may in part be caused by a differential collagen I (COL1A1) gene expression in fetal fibroblasts. We used the reverse transcriptase polymerase chain reaction (RT-PCR) in human dermal fetal, neonatal, and adult dermal

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fibroblasts to determine COL1A1 gene expression under basal and stimulated conditions when exposed to TGF-β1.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Normal human dermal fibroblasts obtained from a 12-week-gestation fetus, newborn foreskin, and a 44-year-old adult (WS1, CCD-43SK, and CCD-866SK, respectively ATCC Rockville, MD) were established in culture in Eagle's minimum essential medium (MEM) with nonessential amino acids and Earle's basic salt solution (BSS) and 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc, Logan, UT). Fibroblast cultures were maintained in 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. In all experiments fibroblasts were used between the 4th and 15th passage to ensure constant phenotype.[23]

Fibroblast cell lines were released from culture with calcium and magnesium-free phosphate-buffered saline (PBS) and 0.1% trypsin EDTA, washed and resuspended in MEM with 10% FBS and counted by hemocytometer to assess viability. Subcultures of fetal, newborn, and adult fibroblast cell lines were placed at a density of 5×10^5 in a T-25 flask and incubated for 24 hours.

TGF-β1 Stimulation of Fibroblast Cell Lines

Each fibroblast cell line was incubated with either media alone or media with human recombinant TGF-β1 (Sigma Pharmaceuticals, Inc, St. Louis, MO) for a 4-hour period at a concentration of 1 ng/mL. The concentration of TGF-β1 chosen was based on results of previous experiments that demonstrated maximal chemotactic effect of TGF-β1 on newborn and adult fibroblasts under the same culture conditions used in the present experiment.[8] In addition, the brief 4-hour period of TGF-β1 stimulation was chosen to eliminate fibroblast proliferation as a factor contributing to total RNA extracted. Each flask was incubated for 4 hours. All growth factors were reconstituted using MEM. Each was 97% pure as assessed by NH_2-terminal sequence analysis and silver staining of SDS-PAGE gels (Sigma Pharmaceuticals, Inc, St Louis, MO). Media were removed, and the cells were washed three times with calcium and magnesium-free PBS, placed in guanidium isothiocyanate buffer for RNA extraction.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from other cellular proteins and nucleic acids by polytron homogenization in guanidium isothiocyanate buffer followed by ultracentrifugation at 12,000*g* for 20 minutes. Total RNA was isolated from the supernatant on a cesium trifluoride gradient. Reverse transcription was performed using a Poly-T primer and the PCR was used with specific oligonucleotide primers for COL1A1 and glyceraldehyde phosphate dehydrogenase (GAPDH) as a control. Oligonucleotide primers were chosen from the following base pair locations: 3439-58 and 3793-74 for COL1A1 and 386-403 and 561-580 for GAPDH. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide to visualize DNA under ultraviolet (UV) light.

Northern and Slot Blot Analysis

Northern blot was performed by transferring the RNA to a nylon membrane using slot blot analysis, followed by UV crosslinking. Prehybridization was carried out at 42°C for 4 hours, then Dig-labeled collagen Ia oligonucleotide probe were added at 53°C (Boehringer Mannheim, Indianapolis, IN). The mixture was allowed to hybridize overnight at 42°C. The membrane was then washed and blocking solution added for 1 hour. Anti-Dig Fab fragment was mixed in and the solution was added to the membrane at room temperature for 30 minutes. After washing with buffer, lumi-phos 530 53°C (Boehringer Mannheim) was added for an incubation of 15 minutes at 37°C. The membrane was then exposed to x-ray film for 15 minutes and developed and photographed. The process was duplicated using GAPDH with the only exception being hybridization was carried out overnight at 53°C.

RESULTS

COL1A1 gene expression was lower in fetal fibroblasts than in adult or newborn fibroblasts under unstimulated conditions (Fig 1). Only a faint band could be detected for control unstimulated fetal fibroblasts. A distinct band was noted for COL1A1 mRNA in both adult and newborn fibroblasts under basal conditions. After 4 hours of TGF-β1 stimulation, COL1A1 gene expression significantly increased in fetal fibroblasts, but did not change in adults or newborn fibroblasts. Hybridization with a probe for GAPDH confirmed equal amount of RNA on the gel for each sample tested.

DISCUSSION

Human fetal dermal fibroblasts have only low constitutive expression of the COL1A1 gene. However, a brief duration of stimulation with TGF-β1 rapidly results in upregulation of the fetal COL1A1 gene expression. No such upregulation of the COL1A1 gene was observed in either neonatal or adult fibroblasts. These results suggest differences between fetal and postnatal fibroblasts in control of the COL1A1 gene at a transcriptional level. These findings are consistent with those of Lorenz et al,[24] who noted both collagen I and III gene induction after a 24-hour incubation of fetal fibroblasts with TGF-β1 and TGF-β2. Interestingly, in Lorenz's study the adult fibroblasts responded as well, possibly related to the longer duration of stimulation, fivefold higher concentration of

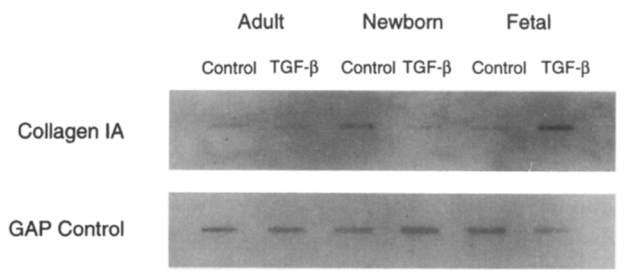

Fig 1. Slot blot analysis of total RNA extracted from human fetal, newborn, and adult dermal fibroblasts after incubation with TGF-β1 (1 ng/mL) or media alone for 4 hours. Hybridization was performed with Dig-labeled oligonucleotide probes to COL1A1 and GAPDH and exposed to anti-Dig Fab fragment and lumi-phos 530 and exposed to x-ray film. COL1A1 gene expression was increased in fetal fibroblasts after exposure to TGF-β1 but newborn and adult fibroblasts were unaffected. Housekeeping control (GAPDH) confirmed equal amount of RNA for each sample tested.

TGF-β used, or synergistic interaction between TGF-β1 and TGF-β2. Induction of the fetal COL1A1 gene by TGF-β1 may be responsible for the ability of exogenous TGF-β1 to convert fetal wounds to a scar-forming phenotype.

Nath et al,[25] demonstrated by in situ hybridization in fetal rabbit wounds that fetal wounds accumulate type I collagen by increasing the number of fibroblasts within the wounds and not by up regulation of gene transcription. In contrast, they found adult wounds accumulate type I collagen by both fibroblast migration and up regulation of gene transcription. Nath et al,[25] speculated that the fetus does not rely on gene induction to deposit collagen because a stimulus for supramaximal collagen production, such as TGF-β1, is absent or inactive in fetal wounds.

Immunohistochemical and in situ hybridization studies in early human fetal skin wound models have found these wounds to be deficient in TGF-β.[18,26,27] It is known however, that at least a brief exposure to TGF-β1 occurs in fetal wounds when it is released from platelets in formation of the initial hemostatic wound plug.[15] Our results suggest that the molecular machinery is available to induce COL1A1 gene expression if the appropriate stimulus, ie, TGF-β1, is present. Van Obberghen-Schilling et al,[28] have shown that TGF-β1 positively regulates its own expression causing up regulation of TGF-β1 gene expression. However, in previous studies in a model of fetal and adult rabbit skin transplanted into nude mice, we found that incisional wounds in fetal skin do not demonstrate up regulation of TGF-β1 by cells lining the wound cleft.[22] In contrast, there was a marked up regulation of TGF-β1 gene expression in adult wound clefts. These results suggested that even though released by platelets into the fetal wound TGF-β1 does not cause induction of TGF-β1 gene expression in cells lining fetal wounds.[22] The continued presence of TGF-β1 is essential for sustaining the fibrotic response that this cytokine elicits. Roberts et al,[29] demonstrated that subcutaneous injection of TGF-β1 in 1-day-old mice induced striking granulation tissue formation surrounded by a collagenous network. But this effect was reversible once exogenous TGF-β1 had been metabolized. A sustained TGF-β stimulus for the induction of COL1A1 gene expression and excess collagen deposition is absent in fetal wounds.

These results support the view that the ability of fetal skin to heal without scar is intrinsic to the fetal fibroblast. However, given the complexity of collagen biosynthesis, the organized collagen deposition that occurs in fetal wound healing is unlikely to be caused by only differences in the fetal fibroblasts' response to signals released in the wound and different COL1A1 gene transcription. The regeneration of normal dermal architecture in fetal wound healing may reflect numerous subtle differences in fetal fibroblast collagen production from transcriptional factors, stability of mRNA, as well as factors affecting translation, collagen type and net accumulation, collagen fibrillogenesis and crosslinking, and complex interactions with the extracellular matrix.

Although layers of control in fibroblast function may be responsible for the fetal fibroblast phenotype, a better understanding of the mechanisms that control it may provide insight into the biology of scarless wound repair.

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Discussion

O.O. Olutoye (Richmond, VA): This is another study that has contributed to defining phenotypic differences between fetal and adult fibroblasts. This study suggests that the collagen type 1 gene is expressed in low levels in the fetal fibroblast but its expression can be induced by the application of exogenous TGF-β. Phenotypic differences between fetal and postnatal fibroblasts, especially with regards to collagen metabolism, may help explain differences in the wound healing response. I have a few questions.

These studies were carried out using only one cell line each of fetal, newborn, and neonatal fibroblasts. Have these observations held true when performed on other fetal fibroblast cell lines at low passage?

The amount of TGF-β released by platelets at the wound site differ between the fetus and adult. In your experiment only one dose of TGF-β was used. Have you used different concentrations of TGF-β in these experiments, and is there a threshold concentration after which the collagen gene is induced in fetal fibroblasts?

Dr Bullard, in her study presented a few minutes ago, demonstrated that TGF-β decreased collagenase in fetal skin. Did you look for any changes in collagenase mRNA in your study?

Finally, can you share your thoughts with us about why you think fibroblasts in a rapidly growing fetus, busy laying down extracellular matrix, would express little collagen mRNA in vitro?

E.K. Gallivan (response): We have now established a library of 32 different fetal fibroblast cell lines in primary cell culture and are now extending our studies using this library.

In answer to your second question we did use only one dose. Previous work, looking at the effects of TGF-β1, 2, and 3 on the adult, newborn, and fetal fibroblast chemotaxis has demonstrated that we get a peak biologic effect in vitro achieved at the concentration of 1 ng/mL. We did not replicate these dose response studies looking at the effect on collagen gene expression, but your point is very well taken. Not only may the adult fibroblast require a higher level of TGF-β to respond, but a longer exposure to TGF-β may be necessary to up regulate the TGF-β1 gene expression.

In answer to your third question regarding Dr Bullock's excellent study with collagenase, we have not done that work in our lab. But as mentioned, we are trying to extend our studies with the 32 cell lines we have established.

My thoughts on why I think fibroblasts express little collagen in vitro—we speculate that the difference is one of degree. The response of adult tissue to wounding happens rather rapidly. In 3 to 4 days there is an explosion of collagen deposition leading to scar formation; whereas, the fetus has a more measured response commensurate over the growth, and the long duration of gestation. And when disturbed by wounding we think that perhaps the lack of TGF-β in the fetal wound means that there is a lack of the machinery being turned on.