Background: Fetal wounds can heal without any histological evidence of scarring. Fetal wounds lack the inflammatory infiltrate characteristic of adult wounds, and the fetal environment is not necessary for scarless healing to occur. Recent evidence suggests that fibroblasts are the main effector of scarless healing in fetal tissue. What has not been shown is what profile of growth factors the fibroblast uses to influence wound repair.

Objective: To determine the expression of growth factors (transforming growth factors \(\beta_1\), \(\beta_2\), and \(\beta_3\); acidic and basic fibroblast growth factors; keratinocyte growth factor; and platelet-derived growth factor AA, BB, and AB) of fetal and adult fibroblasts in vitro.

Design: Adult and fetal fibroblasts were grown in culture, and messenger RNA was extracted by standard techniques. Northern hybridization was used to identify messenger RNA transcripts for the aforementioned growth factors. Densitometry was used to compare growth factor messenger RNA expression with that of a ubiquitously expressed control, glyceraldehyde phosphate dehydrogenase.

Results: The data suggest that fetal and adult fibroblasts express acidic and basic fibroblast growth factor and transforming growth factor \(\beta_1\). Adult fibroblasts show twice the relative expression of these growth factors compared with fetal fibroblasts.

Conclusions: The adult fibroblasts demonstrate a relative excess production of cytokines compared with fetal fibroblasts. This is thought to contribute to suboptimal wound healing in adult wounds compared with the scarless healing of fetal wounds.

MATERIALS AND METHODS

CELL CULTURE

Cell lines for human adult and fetal fibroblasts were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Camden, NJ. All cell lines were genetically normal, and all were obtained from skin biopsies specimens. Cell line GM0023 was taken from a 31-year-old woman, GM0024 from a 31-year-old man, GM0011 from a male fetus of 8 weeks’ gestation, and GM0468 from a male fetus of 18 weeks’ gestation.

Cells were grown in culture with standard sterile techniques with the use of Eagle minimum essential medium supplemented with fetal bovine serum (20%), penicillin G sodium (100 µg/m), and streptomycin sulfate (100 µg/mL). Cells were grown to 80% confluence, and early passages (between 9 and 14) were used for messenger RNA (mRNA) extraction. In preparation for mRNA extraction, cells were trypsinized, washed with phosphate-buffered saline, and frozen immediately at −80°C. A total of 10^7 cells was used for each cell line to isolate mRNA.

mRNA ISOLATION AND BLOT PREPARATION

Poly-A RNA from the different fibroblasts was prepared by means of a protein denaturing kit (RNA-easy Kit; QIAGEN, Chatsworth, Calif). The RNA was then denatured in 50% formamide, separated on 1.2% agarose gels, stained with ethidium bromide, and transferred onto Nytran (Schleicher & Schuell, Keene, NH) membranes. The RNA was then immobilized onto the membranes at 80°C under vacuum for 2 hours.

PROBE PREPARATION

Probes were obtained through the generosity of several donors; Sandra O. Gollnick, PhD (Roswell Park Cancer Institute, Buffalo, NY), provided TGF-β1, TGF-β2, TGF-β3, and with permission from Bristol-Myers Squibb (Cranberry, NJ); Charles E. Murry, PhD (University of Washington, Seattle, Wash), provided PDGF-A and PDGF-B; and Judith Abraham, PhD (Scios Inc, Sunnyvale, Calif), provided acidic- and basic-FGF. Plasmid DNAs were used for transfection into competent Escherichia coli (Epicurien Cells; Stratagene, San Diego, Calif), positive clones were identified, and probe DNA was isolated by standard protocols. Appropriate restriction enzymes were used to isolate probes from plasmids, and fragments were separated on 1% low-melting agarose gels in Tris acetate buffer, pH 8.0. The probe fragment was cut out of the gel, and a 100-ng equivalent was labeled by means of a random primer kit (Pharmacia-Biotech, Piscataway, NJ) and α-deoxycytosine triphosphate. The probe for KGF was an oligonucleotide designed from the KGF complementary DNA sequence. It was labeled at the 5’ end by a kinase reaction with γ-labeled phosphorus 32 adenosine triphosphate. Multiple tissue Northern blots (adult human tissues) (Clontech, Palo Alto, Calif) were used to identify transcript size of the different probes. The probe for the mRNA sequence for glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a control probe in Northern blot analysis. The probe was produced as a polymerase chain reaction product with the use of primer sequences from Apostolakos et al.

HYBRIDIZATION AND AUTORADIOGRAPHY

Blots were prehybridized with salmon sperm DNA (200 µg/mL) in Church hybridization buffer (0.5-mol/L sodium phosphate, pH 7.2, 7% sodium dodecyl sulfate, 1-mmol/L EDTA) at 63°C for 3 hours, then hybridized with the appropriate probes overnight at 65°C in the same buffer for 12 to 16 hours. Blots were then washed with Church wash buffer (40-mmol/L sodium phosphate, pH 7.0, and 1% sodium dodecyl sulfate), once at room temperature and twice at 65°C for 20 minutes each. Autoradiography was performed for 1 to 2 days at ~70°C.

Autoradiographs were scanned into a computer, and densitometry was performed with the National Institutes of Health Image Analyzer program (National Institutes of Health, Bethesda, Md) with the use of GAPDH expression as a reference.

RESULTS

The 9 growth factors evaluated were found to be expressed in normal tissues (Table). The size of the transcripts correlated to those reported by other investigators. All fibroblast cell lines expressed mRNA for the control probe GAPDH (Figure 1).

Of the 9 growth factors, 0 were not expressed in any of the fibroblast cell lines. These factors were TGF-β2, TGF-β3, KGF, PDGF-AA, PDGF-BB, and PDGF-AB. The remaining growth factors (TGF-β1, aFGF, and bFGF) were expressed in varying degrees in the fibroblast cell lines (Figure 1). These results were confirmed by repeating the Northern blotting for each probe. Because of the large number of cells required to harvest enough mRNA for a single specimen (10^7 cells), the number of specimens available for Northern blot evaluation was limited, and thus no statistical evaluation of the differences in expression of these growth factors could be performed.
Our data show that adult fibroblasts express TGF-β1 in levels of 55% to 80% relative to the GAPDH standard. Acidic FGF is expressed in levels of 45% to 85% of GAPDH, while bFGF is expressed in 15% to 55%. In contrast, fetal fibroblasts expressed TGF-β1 in levels of 25% to 60% relative to GAPDH, aFGF in 0% to 24%, and bFGF in 0% to 27% (Figure 2).

Previous experiments have shown differences between the fetal and adult wound environments in terms of the compositions of cellular and extracellular matrixes. The fetal environment has been identified as a possible contributor to the differences in fetal and adult wound repair. Fetal wounds are bathed in sterile amniotic fluid that is rich in extracellular matrix molecules, such as hyaluronic acid and fibronectin. This may contribute to the fetal extracellular matrix being more fluid and therefore more permissive to cellular migration and orderly collagen deposition, resulting in restoration of normal cellular architecture. However, several investigators have shown that the fetal wound is capable of scarless repair independent of this environment. For example, Ferguson and Howath wounded 2-day-old pouch opossums (Monodelphis domestica) and found that, despite being removed from the amniotic fluid environment, their wounds healed without scarring. They continued to demonstrate scarless healing for 4 weeks after birth in the non-sterile environment of the mother’s pouch, after which time they healed with extensive scarring. Other investig-
Growth factors play a major role in wound healing. They attract inflammatory cells into the wound, stimulate angiogenesis, and regulate the production of extracellular matrix proteins. What remains to be determined is how the differences between fetal and adult wounds are regulated by growth factors and, ultimately, how we may intervene in adult wounds to influence the wound repair outcome. To this end, Whitby and Ferguson used immunohistochemistry to demonstrate differences in the growth factor profile between fetal and adult wounds. Platelet-derived growth factor, a chemoattractant of inflammatory cells, was identified early in both types of wounds. It then disappeared 24 to 48 hours earlier in fetal wounds than in adult wounds. Fibroblast growth factor, a stimulator of angiogenesis, was found in adult wounds but was notably absent in fetal wounds. Transforming growth factor β, a major stimulant of fibrogenesis and wound contraction, was also seen only in adult but not fetal wounds. In contrast, we demonstrated expression of all 3 of these growth factors in one of our fetal cell lines. This may be because of the greater sensitivity of Northern blotting compared with immunostaining; however, mRNA expression does not ensure production of proteins and, thus, Western blot studies are under way in our laboratory to determine the expression status of these proteins.

The 3 main sources of growth factors in a wound are platelets, which are deposited in the wound from the blood; tissue macrophages, which migrate into the wound from the blood; and fibroblasts, which are native to the wounded tissue. Lorenz et al grafted human fetal skin onto adult athymic mice, thus perfusing fetal tissue with adult blood. This eliminated platelets and macrophages as potential sources of fettallike growth factors in wounds created on the grafted fetal tissue. Grafts that were placed in a subcutaneous pocket healed without scarring despite being perfused with adult blood. Immunohistochemistry for collagen types I and III of both human and murine origin demonstrated that the subcutaneous grafts healed with human collagen in a pattern indistinguishable from the surrounding unwounded tissue. They concluded that fetal fibroblasts native to the grafted tissue were the critical effector cells responsible for scarless repair.

If the fetal fibroblast is indeed the major cellular effector of scarless wound repair, it is likely that the fibroblasts relay signals in a paracrine and autocrine fashion to direct the type of repair that occurs. Growth factors are the most likely messengers of these signals, and, therefore, knowledge of the differences in the expression of growth factors between fetal and adult fibroblasts would be invaluable in determining how the fetal fibroblast directs the wound repair process to occur without scarring. Importantly, no previous data exist that document the differences in growth factor expression between fetal and adult fibroblasts. Our data demonstrate that 6 growth factors were not expressed by either the adult or fetal fibroblasts when grown in culture. These factors were TGF-β isoforms 2 and 3, KGF, and PDGF isoforms AA, BB, and AB. For the remaining growth factors evaluated, in general, adult fibroblasts demonstrated twice the relative expression of the fetal fibroblasts. This is consistent with Adzick and Lorenz’ theory of “cytokine poisoning” in which excess cytokines and inflammatory infiltrate are present in adult wounds and serve to speed healing at the expense of suboptimal wound repair. One of the fetal cell lines, GM0468, demonstrated expression levels similar to those of the adult cell lines, while the other fetal cell line, GM0011, expressed only TGF-β1. It is interesting that cell line GM0468, taken from an 18-week-gestation fetus, produced adult proportions of growth factors, while cell line GM0011, taken from an 8-week-gestation fetus, produced much less TGF-β1 and neither aFGF nor bFGF. This observation also supports the theory that as the fetus ages, more growth factors are produced, eventually leading to cytokine excess and suboptimal wound repair.

Ultimately, the goal of fetal wound healing research is to manipulate the adult wound to heal in a fashion similar to that of scarless fetal repair. Growth factors play an important role in both adult and fetal wound healing, and it is possible that by altering the growth factor profile of a wound, either by adding growth factors or by blocking their actions, we may be able to manipulate the wound healing process. For example, Werner et al demonstrated that expression of KGF, a member of the FGF family responsible for rapid reepithelialization in normal adult wound healing, is deficient in diabetic wound healing. They used this evidence to explain the beneficial effects of exogenous FGF in the treatment of diabetic wounds with impaired healing. Cullen et al. showed that the addition of TGF-β1 or PDGF-AB to fetal and neonatal fibroblast cell cultures is capable of modulating the secretion of proteases important in cell migration during wound healing and thus affecting repair outcome. Shah et al. used TGF-β blocking antibodies to markedly decrease TGF-β levels in adult wounds and thus decrease scarring while maintaining normal tensile strength and achieving near-normal dermal architecture. Other investigators have demonstrated that neutralizing both TGF-β1 and TGF-β2 has a greater, synergistic antiscarring effect compared with neutralizing either isoform alone, and that the addition of TGF-β3 isoform to a wound down-regulates levels of isoforms 1 and 2 and also results in an antiscarring effect. Similarly, expression of growth factors in our experiment may have been influenced by fetal bovine serum, which itself contains growth factors and was used to perform the fibroblast cell culture. To examine the role of serum and to confirm our preliminary data, we are currently performing studies in the presence and absence of serum in a larger series of fibroblasts.

Our results suggest that fibroblasts contribute to the cytokine excess found in the adult wound. However, confirmation of these results from a larger sample population would be useful. Also, it should be stated that direct measurement of growth factors in fetal and adult wounds, or the creation of a complete growth factor...
file,” would require measurement of secreted growth factors found in the extracellular matrix. The technique used herein indirectly measures the growth factor production of fibroblasts by measuring mRNA, which is presumed to represent the extracellular secretion of growth factors.11 We plan to confirm this by analyzing the cell supernates collected from the fibroblasts in our ongoing studies for the secretion of matrix proteins. In addition, growth factor expression in wound fibroblasts may differ significantly from that in fibroblasts grown in cell culture. A wound model, such as that published by Tuan et al,23 would allow further study. They used fibrin gels stabilized on plastic culture plates to demonstrate how fibroblasts can actively reorganize the matrix into a collagen-containing, contracted, scarlike tissue. Study of the growth factor expression of these “active” fibroblasts would seem the next logical step in determining how fetal fibroblasts can effect scarless wound healing.

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REFERENCES