Objective: Because subglottic stenosis (SGS) represents one of the most challenging pathologies confronting the pediatric otolaryngologist, our laboratory is investigating the role fibroblasts play in mucosal scar formation in the course of SGS development. Our objective is to establish cell transplantation into the subglottic mucosal wound bed as a viable tool for examining the cellular processes that underlie the development of SGS.

Design: A series of 2 animal experiments, with animals assigned to a control, vehicle-only, or cell-treated group.

Setting: John G. Rangos Sr Research Center, Children’s Hospital of Pittsburgh, Pittsburgh, Pa.

Subjects: Twenty-six New Zealand white rabbits. This animal model has been well established in the study of SGS formation.

Interventions: Fluorescently labeled exogenous fibroblasts were transplanted into the injured subglottis of the rabbits.

Results: Exogenous fibroblasts derived from fetal and adult dermis and subglottic mucosa were successfully transplanted into the injured subglottic mucosa of adult rabbits. Transplanted fibroblasts survived into the latter stages of wound healing (at 14 and 21 days) and appeared to be associated with a mild inflammatory cell influx and active remodeling of the mucosal wound bed.

Conclusion: Cell transplantation is a viable tool for the study of fibroblast activity in the mucosal wound bed.


SUBGLOTTIC STENOSIS (SGS) in children, especially newborns, has become increasingly more prevalent during the past quarter century owing to widespread adoption of prolonged endotracheal intubation for respiratory support.¹-¹² Current therapeutic approaches to SGS are largely surgical in nature, addressing the gross pathology associated with airway narrowing.⁹-¹² Unfortunately, surgical approaches are associated with postoperative alterations in airway morphology and function. After surgical intervention, laryngeal anatomy and function are altered in as many as 78% and 44% of cases, respectively.⁹ Although other techniques may improve on this outcome, normal vocalization is recovered in only 25% to 33% of patients undergoing reconstruction and decannulation.¹⁰-¹² We hypothesized that a minimally invasive approach to correction of SGS may meet with improved success, especially for secondary outcome measures such as proper vocalization. An understanding of the cellular and molecular processes underlying the formation of SGS is of paramount importance for the development of such novel therapeutic strategies. Our laboratory is investigating SGS formation through the prism of mucosal wound healing and scar formation.

Scarring is a consequence of the imperfect wound healing response to injury, which eventuates in repair rather than regeneration of the connective tissue by the resident fibroblasts. Postnatal wound healing is characterized by hemostasis, inflammation, fibroplasia, cellular proliferation, and remodeling.¹³ Repair ensues with the deposition of fibrin and fibronectin into the wound bed and replacement of these by collagen secreted by fibroblasts under the influence of soluble mediators released from a variety of cells present in the local wound environment. During an extended period of weeks to months, the collagen fibrils are remodeled (synthesized and degraded) into the ultimate repaired connective tissue or scar. Scar formation, also called fibrosis, represents the end stage of postnatal wound healing and may be associated with an irretrievable loss of specialized function. In airway mucosa, the fibrotic healing response results in a thick-
enled mucosal lamina propria and a narrowed lumen. This result is often compounded by replacement of the cartilaginous framework with scar tissue.

In contrast to postnatal wound healing, fetal airway mucosal healing occurs without scar formation. In the middle to late stages of gestation, fetal wounds heal rapidly and are essentially indistinguishable from unwounded tissue, clinically and histologically. In addition to fetal airway mucosa, this phenomenon has been described in other tissue types, including skin; however, it is not universal to all fetal tissues. Fetal fibroblasts have been shown to be at least partially responsible for this regenerative healing phenotype.

The wound-healing properties of fetal tissue and the fetal fibroblast hold promise for future clinical applications in at least 2 ways. First, by delineating the differences between fetal and postpartum healing, we can gain insight into ways of manipulating wound healing to effect a more favorable anatomic and functional result. Second, determining whether fetal cells retain their scarless healing response outside the uterus may enable their use in various reconstructive and tissue-engineering efforts.

One approach is to transplant exogenous fetal fibroblasts into an adult wound to determine any subsequent impact on the course of wound healing. A previous study has demonstrated that transplanted fetal fibroblasts can survive in an adult dermal wound, remain active, and potentially participate in the wound-healing process. Herein, we extend our focus to the analysis of mucosal wound healing. We used a previously described SGS animal model to determine the viability of fibroblast transplantation into a mucosal wound bed and to characterize the subsequent fate and impact of these transplanted cells. Previous work in our laboratory has established that SGS can be consistently induced in the New Zealand white rabbit model using a variety of techniques. For this study, scraping of the posterior mucosa with a scalpel was used to create a full-thickness injury. Chemical and thermal cautery were deemed inappropriate methods of inducing the mucosal injury owing to their possible negative impact on the survivability of transplanted cells. Although scraping of the posterior mucosa does not mimic the injury encountered during human SGS development (ie, intubation), it is a validated method of inducing chronic SGS in an animal model.

We selected the following 2 outcome measures for this study: (1) engraftment, survival, and localization of transplanted cells in the wound bed and (2) inflammatory response to transplanted fibroblasts. These 2 outcome measures will allow us to determine the viability of this approach for the study of fibroblast activity in the mucosal wound bed. Given that allogeneic fibroblasts are generally thought to elicit an immune/inflammatory response in immunocompetent adult animals, we deemed it important to ascertain whether the transplantation of exogenous fibroblasts into the subglottic mucosal wound bed results in excessive inflammatory cell infiltration. To further address this issue, we chose subglottic mucosa and skin tissue types as sources of fetal and adult fibroblasts. Skin was chosen as a companion to subglottic mucosa because (1) fetal and adult dermal repair exhibit properties similar to mucosal healing; (2) skin fibroblasts are most readily available as donor cells; and (3) tissue mismatch may exacerbate the immune/inflammatory response to transplanted fibroblasts.

Exogenous fibroblasts were transplanted immediately after creation of a mucosal injury to maximize engraftment into the wound bed and to allow for maximal interaction between transplanted fibroblasts and immune/inflammatory cells. Finally, 2 time points, 14 and 21 days, were chosen for killing the animals and tissue analysis on the basis of our previous experience with this experimental model to capture inflammatory and fibrotic events during SGS formation.

This is a pilot study, designed to test the feasibility of fibroblast transplantation into a subglottic wound bed. Because of experimental constraints, the analysis is primarily qualitative and descriptive; nevertheless, it will establish proof of principle and a novel experimental approach for the study of fibroblast activity during SGS formation.

METHODS

CELLS

All cells were obtained from existing frozen stock. Adult rabbit fibroblasts were derived from animals older than 3 months, whereas fetal fibroblasts were obtained from 21-day-old fetuses (full-term age, 31 days) as follows. Fetal rabbits were removed from the uterine sac, and the airway was incised using an anterior approach (via the cricoid cartilage and cricothyroid membrane). The subglottic mucosa was removed with a scalpel and used for subsequent fibroblast outgrowth. Dorsal skin (5 × 5 mm) was excised without underlying fascia and muscle and used for subsequent fibroblast outgrowth. Adult tissue was harvested in a similar manner and underwent additional decontamination with an antibiotic-antimycotic solution (10× concentration). Skin and mucosal tissues were minced into 1-mm pieces, and each piece was placed in a sterile culture dish. Explants were allowed to adhere for 5 minutes, after which fibroblast growth media (Dulbecco’s Modified Eagle Medium; Invitrogen Corp, Carlsbad, Calif) supplemented with 10% fetal bovine serum and the antibiotic-antimycotic solution (Invitrogen Corp) was added. Explants were left undisturbed for 7 to 10 days after setup and then analyzed for fibroblast outgrowth. Adherent fibroblasts (fetal, adult, dermal, and mucosal) were subcultured and used in subsequent experiments. All cells were used before passage 5 to minimize possible phenotypic alterations during in vitro culturing. Cells were labeled with a fluorescent dye (CM-Dil; Molecular Probes, Eugene, Ore) according to established protocols.

ANIMALS

For this study, the New Zealand white rabbit was selected. This animal model has been previously shown to be applicable for the study of SGS and mucosal airway wound healing. Three experiments were compiled for this study, for a total of 26 animals.

AIRWAY WOUNDING

All animal experiments were conducted under approved protocols compliant with committee regulations for institutional animal care and use. Animals were prepared for surgery as follows. The neck fur was clipped and the skin was prepared with...
In this study, a total of 5 animals died prematurely (in these cases, airways were obstructed by a clot of what appeared to consist of fibrinous and mucous exudate), allowing us to analyze cell survival and histological findings for the 21 remaining animals. None of the premature deaths were associated with excessive SGS, but rather were deemed surgical complications and were not extensively studied. We consider this data set to represent a pilot study to analyze the viability of transplanted fetal and adult fibroblasts in an injured subglottis and the degree of host response to these cells as illustrated by the inflammatory infiltrate. The 2 outcome measures chosen for this study were (1) cell survival and distribution in the mucosal wound bed and (2) inflammatory response to transplanted fibroblasts. The degree of tissue remodeling and granulation tissue formation was also analyzed, as illustrated by the general anatomy and specific presence of the master wound-healing regulator TGF-β1.

Data indicate that fetal and adult skin fibroblasts were capable of engrafting into adult rabbit subglottic wounds. Both cell types could be found in the granulation tissue subsequent to mucosal wounding, as late as 14 days after injury. Despite equivalent numbers of transplanted fibroblasts, there were more fetal than adult skin fibroblasts present in the mucosal granulation tissue at 14 days after injury (Figure 1). It is unclear whether this is owing to improved graft take, increased escape from immune detection, or higher rates of survival and proliferation.

Analysis of the host response to cell transplantation in the subglottis was conducted using cell markers CD45 (pan–T/B-cell marker) and ED1 (macrophage marker). Data suggest that transplanted adult dermal cells localize to the

**Figure 1.** Transplanted cell presence in the subglottic region. Fibroblast count was determined and semiquantitatively analyzed using detection of fluorescence in fresh frozen sections. Data are presented as means, with error bars representing standard error of the mean.

**MICROSCOPIC ANALYSIS**

Tissue specimens were embedded in optimal cutting temperature embedding compound (OCT; Miles Inc, Elkhart, Ind) and cryosectioned into approximately 10-µm-thick sections, at −23°C. Cell presence in the wound bed was ascertained as previously reported. Specifically, cryosections were imaged using a microscope equipped with a tetramethylrhodamine-isothiocyanate–labeled filter (Nikon Inc, Melville, NY) and coupled to an imaging software package (Metamorph; Universal Imaging Corporation, Downingtown, Pa), and the total amount of fluorescence present in the wound bed was measured. Representative slides were processed for immunohistochemical characterization of the connective tissue components using standard techniques. Hematoxylin-eosin staining was used to ascertain the overall histological appearance of the injured mucosa. The degree of inflammation in the wound bed was characterized using pan–B/T-cell marker monoclonal mouse anti-CD45 (Sigma-Aldrich Corp, St Louis, Mo) and macrophage marker monoclonal mouse anti-ED1 (Biosource International, Camarillo, Calif). Wound remodeling was assayed using a monoclonal anti–transforming growth factor-β1 (TGF-β1) antibody (R&D Systems, Minneapolis, Minn). In all cases, the secondary antibody was a biotin-conjugated donkey anti–mouse antibody (Jackson ImmunoResearch, West Grove, Pa), followed by a conjugate consisting of horseradish peroxidase and streptavidin and 3-amino,9-ethyl-carbazole chromogen (ScyTek Laboratories Inc, Logan, Utah).

**RESULTS**

The remaining animals were killed at 21 days after injury, and the airways were removed and cryosectioned.

**TISSUE ANALYSIS**

Animals were killed by means of intracardiac administration of pentobarbital sodium (50 mg/kg) after heavy sedation via administration of ketamine hydrochloride (35 mg/kg) and xylazine hydrochloride (5 mg/kg). The neck skin and fascia were incised, and the airway was removed as a whole-tissue sample from the larynx down to the first 2 rings of the trachea. The tissue was wrapped in sterile gauze, snap frozen in liquid nitrogen, and stored at −80°C for further analysis.
wound bed and appear to be associated with a slight increase in CD45-positive cells in the wound bed (Figure 2) compared with control wounds. Cells positive for CD45 were present in control wounds at 14 days after injury, and transplanted fibroblasts do not completely colocalize with these putative inflammatory cells. Nevertheless, given the relevance of an immune response to exogenous cells in the wound bed, we thought it necessary to begin to address this issue. The CD45-positive cells localized primarily to the laminar portion of the injured mucosa in control and fibroblast-treated wounds (Figure 2 and Figure 3). The ED1-positive cells were generally localized in the injured and uninjured mucosal epithelium.

We focused additional analysis on determining whether those inflammatory cells in the granulation tissue colocalized with the fluorescently labeled fibroblasts. Figure 3 is a composite of representative micrographs of fetal skin fibroblasts transplanted into an injured adult subglottis and addresses this issue. Transplanted fibroblasts were evident in the mucosal wound bed surrounded by extracellular matrix (illustrated by hematoxylin-eosin staining in Figure 3B) and appeared to partially colocalize with CD45-but not ED1-positive cells. Activity of transplanted fibroblasts in the mucosal wound bed cannot be ascertained using available experimental approaches. In lieu of direct evidence of exogenous fibroblast activity, TGF-β1 was used as a surrogate to indicate wound bed activity. In wounds containing adult (Figure 2) or fetal (Figure 3) skin fibroblasts, TGF-β1 expression in the wound bed appeared to be concentrated in the area surrounding the transplanted fibroblasts. This is in contrast to the normal wound margins, where TGF-β1 appears to be localized primarily in the epithelial layer. In summary, despite tissue-type mismatch, both fetal and adult skin fibroblasts were able to engraft and survive in the mucosal wound bed through the first 14 days of SGS formation.
The initial experiment using fetal and adult skin fibroblasts was followed by a second experiment using subglottis-derived fetal and adult fibroblasts to address the same issues. This experiment was performed primarily to determine whether there are tissue-specific differences in transplanted exogenous fibroblasts. Because dermal and mucosal fibroblasts may not share all characteristics, we wanted to test the possibility that transplanted mucosal fibroblasts elicit a more subdued inflammatory response in the context of subglottic mucosal healing. Both fetal and adult subglottic fibroblasts were found to graft and survive in the mucosal wound bed (Figure 4). Fetal subglottic fibroblasts seemed to exhibit increased survival at the experiment end point (Figure 5), which in this case was extended to 21 days, to begin to address the later and chronic phases of subglottic healing. In general, inflammation, exemplified by the CD45- and ED1-positive cell influx, and remodeling, exemplified by TGF-β1 staining, paralleled our findings using dermal fibroblasts (data not shown).

**COMMENT**

Subglottic stenosis is a narrowing of the airway lumen that is caused by mucosal scarring, a process that is at least partially dependent on fibroblast infiltration, proliferation, and subsequent deposition of the replacement extracellular matrix. Subglottic stenosis formation follows the steps encompassed during wound healing in other tissue, but in the airway excessive scarring can impair normal speech and respiration. Our focus is to understand the cellular processes underlying scar formation as part of SGS by focusing on fibroblast activity in the wound bed. Specifically, we used the natural phenotype variant represented by fetal wound healing. Our long-term goal is to determine the degree to which the fibroblast phenotype is responsible for the final outcome of wound healing. By replacing endogenous fibroblasts with exogenous fibroblasts of adult and fetal origin, we can determine whether or not the natural history of SGS and the final outcome of wound healing can be changed. This initial study was designed to address the following 4 preliminary issues associated with fibroblast transplantation into an injured subglottis: (1) successful delivery of cells into the wound bed, (2) grafting and survival of cells in the wound bed, (3) immune and inflammatory host responses to exogenous cells, and (4) active participation of transplanted cells in the wound-healing process.

This pilot study extends previous fibroblast transplantation studies in rabbit skin wounds by our group and confirms the feasibility of transplanting fetal and adult airway and dermal fibroblasts into subglottic mucosal wounds. The purpose behind the use of dermal-derived fibroblasts was to ascertain whether tissue mismatch would result in (1) failure to graft in the airway mucosal wound bed or (2) excessive inflammation. Our data suggest that skin-derived fibroblasts of fetal and adult origin can engraft into a mucosal wound bed and survive as long as 14 days after injury. Engraftment of all exogenous fibroblasts (fetal, adult, dermal, and mucosal) was accompanied by an apparent inflammatory infiltrate composed primarily by CD45-positive cells. This result is potentially important because inflammation has generally been associated with negative wound-healing outcomes. More specifically, excessive inflammation has been linked to more scarring. Although our analytical methods are not quantitative, there was no detectable differ-
ence in the degree of inflammatory infiltrate accompanying any particular fibroblast phenotype.

The underlying goal of our studies is to use this approach to begin to understand the behavior of fetal and adult mucosal fibroblasts in a subglottic wound bed. As such, it is important to ascertain whether exogenous fibroblasts survive for extended periods in the wound environment and whether they retain cellular activity. Our data indicate that exogenous fibroblasts can survive as long as 21 days after airway injury. Transplanted fetal fibroblasts of dermal and mucosal origin were found in larger numbers at the time of death than were adult counterparts. This finding was a semiquantitative assessment based on the amount of fluorescence present within the mucosal wound bed. Increased fetal fibroblast presence in the wound bed can result from (1) increased engrafting, (2) decreased immune rejection, or (3) more robust cell survival or proliferation mechanisms. The precise contribution of each of these phenomena is unclear at this point and will be addressed in subsequent experiments.

Histological analysis showed that transplanted cells were present in the mucosal granulation tissue and were surrounded by reconstituted extracellular matrix components. Individual cell activity in the wound bed is difficult to ascertain. In this pilot study, we used immunohistochemical staining for TGF-β1, a master regulator of wound healing, to determine the degree of tissue remodeling at the time of death. In general, there was an increase in concentration of TGF-β1 associated with the transplantation of exogenous fibroblasts into the mucosal wound bed. Subsequent studies will extend this line of analysis by using more extensive and quantitative techniques aimed at describing exogenous cell activity during wound healing.

A follow-up study is planned that will expand on this pilot data. More specifically, it will address the reliability of our cell delivery technique, the degree of inflammation associated with cell transplantation, and the impact on extracellular matrix deposition and organization of transplanted fetal fibroblasts. We believe this avenue of analysis has the potential to answer some of the questions regarding the role of fibroblasts in SGS formation and may lead to modalities of directing fibroblast activity toward decreased scar formation and more regenerative healing.

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Figure 4. Photomicrographs demonstrate the survival of transplanted fetal (A; arrowhead; CM-Dil–positive cells) and adult (B; arrowhead; CM-Dil–positive cells) subglottic fibroblasts into the latter phases of subglottic stenosis formation (21 days after injury). CRT indicates cartilage; GRN, granulation tissue; and LP, lamina propria (original magnification ×100).

Figure 5. Bar graph depicts the survival of adult and fetal subglottic fibroblasts transplanted into the injured subglottis at 21 days after injury. The presence of transplanted cells was determined and semiquantitatively analyzed using detection of fluorescence in fresh frozen sections. Data are presented as means, with error bars representing standard error of the mean.
Correspondence: Patricia A. Hebda, PhD, Department of Pediatric Otolaryngology, Rangos Research Center, Children’s Hospital of Pittsburgh, 3460 Fifth Ave, Pittsburgh, PA 15213 (hebda@pitt.edu).

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