Vocal Cord Augmentation with Cultured Autologous Fibroblasts

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Bovine collagen is an acceptable agent for vocal cord medialization; however, it produces only a temporary effect. As a foreign protein bovine collagen is susceptible to host collagenase and can induce immune response. Autologous collagen has become recently available, but it is less effective as a medialization agent. The study examines human skin fibroblasts growing in culture. Human skin bioptates were taken from the retroauricular area. Fibroblasts in culture were tested for scar contractility and ability to produce type I collagen (by flow cytometry with labeled antibodies). After five passages in culture the cells produced normal type I collagen, exhibited normal contractility, and did not induce no tumors in nude mice.

Key Words: *autologous fibroblasts; type I collagen; contraction; oncogenicity; vocal cord medialization*

Bovine collagen has been successfully used for vocal fold medialization since 1984 [5]. It is particularly helpful in treating vocal fold scarring, atrophy, focal defects, and as an injectable medialization agent for vocal fold paralysis and paresis. However, bovine collagen produces only a short-term effect due to destruction by collagenase produced by host fibroblasts and can cause hypersensitivity.

In 1986 more stable and less antigenic glutaraldehyde-crosslinked collagen became available [3]. These agents are now widely used for the treatment of vocal fold defects, atrophy, and medialization [4,11,12].

Injectable autologous collagen was first applied in 1995 [6]. This technique preserves tissue architecture including intermolecular crosslinking. These natural collagen fibers are highly resistant to proteases compared to reconstituted collagen with cleaved telopeptides. Telopeptide integrity is essential for quarter stagger formation of collagen fibers and for crosslinking between adjacent collagen molecules.

Autologous collagen best simulates host collagen, which ensures perfect host tolerance.

The use of injectable autologous human fibroblasts would be expected to result in permanent correction based on local collagen production. Here we studied cultured human fibroblasts for their abilities to produce collagen, form scars, and induce tumors in experimental animals.

MATERIALS AND METHODS

Skin punch biopsy specimens (4 mm) were taken from the retroauricular area of two patients and placed into sterile tubes with 10 ml Eagle's minimal essential medium containing fetal bovine serum, sodium pyruvate, and antibiotic and antimicotic drugs. The specimens were transferred to 60-mm dishes and incubated in a CO_2-incubator (5% CO_2). After 2 weeks the cells were harvested by trypsinization and transferred to 25-cm^5 flasks. After 5 weeks the yield was about 4×10^7 cells.

For evaluation of collagen production, 5×10^5 fibroblasts (passage 6) were washed with phosphate

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buffered saline containing 0.1% sodium azide (Sigma) and 2% fetal bovine serum and stained using mouse monoclonal antibodies against human type I collagen (clone MAB 1340, Chemicon) and FITC-labeled goat polyclonal antibodies against mouse IgG/IgM (36484D-MO38768, BP Phar). The samples incubated with FITC-labeled goat antimouse antibodies only and unstained cells were used as the control. The cells were incubated with antibodies in 50 µl medium for 30 min on ice [4]. No less than 10^5 cells were analyzed on a Becton Dickenson flow cytometer.

Fibroblast contraction potency was evaluated as described previously [15]. For preparing 0.2% atelocollagen gel, 0.3% pepsin-processed type I atelocollagen (pH 7.3) (Sigma) was mixed with 6-fold Minimum Essential Medium and 10% embryo calf serum (4:1:1). Fibroblasts (passage 6, 10^5 cells/ml) dispersed with 0.05% trypsin and 0.02% EDTA (Gibco) in PBS were mixed 1:1 with 0.2% collagen gel (CG) and the mixture was transferred to 35-mm dishes (3 ml per dish). The dishes were incubated at 37°C and 5% CO_2. The diameter of CG was measured every 4 h for 24 h. Cultured fibroblasts from hypertrophic scar (after passage 6) were used as the control.

For evaluation of oncogenicity of injectable fibroblasts, the cells (passage 7) were injected subcutaneously in a dose of 4×10^7 to two 1.5-month BALB/c nude mice.

RESULTS

Autologous human fibroblasts are a potentially exciting natural alternative to bovine fibroblasts or other foreign material is currently used for vocal fold medialization. The use of autogenous product eliminates the risk of immune reactions in the host and ensures better take as the material self-derived. However, some drawbacks of autologous fibroblasts are apparent. Clinical improvement is often less pronounced compared to the effect of bovine collagen, especially at the early stages after implantation. Furthermore, there is a possibility of transformation of patient's normal fibroblasts into pathological fibroblasts (myofibroblasts) responsible for fibrosis and scar contracture [1,2]. CG contraction is due to specific interaction between fibroblasts and collagen fibrils [8,9,14]: fibroblasts rearrange extracellular collagen fibrils. Previous studies showed that abnormal fibroblasts derived from hypertrophic scar exhibit far greater contraction potency than normal cells. In our experiments, fibroblasts after 6 passages in culture had normal contraction potency *in vitro*. They contracted CG and simultaneously some fluid was expelled from the gel. After 24 h the diameters of collagen gels were 27.9±0.8 and 28.1±0.5 mm for patients 1 and 2 respectively, while hypertro-

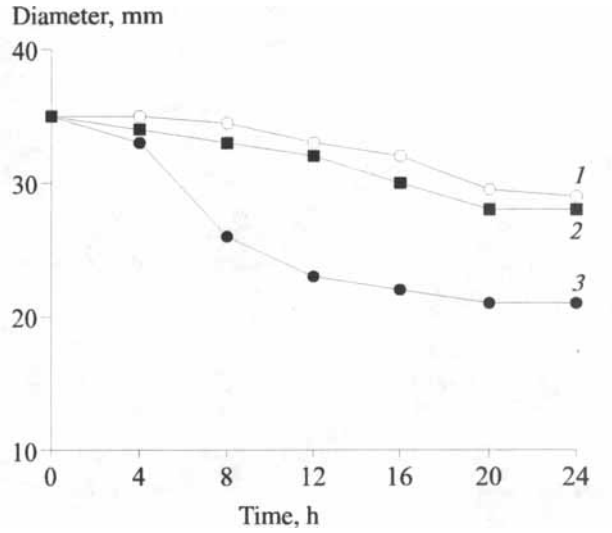

Fig. 1. Contraction of collagen gel after 24-h incubation with fibroblasts isolated from normal skin (patients 1 and 2) and hypertrophic scar (3). Differences are significant ($p<0.05$) in each time point.

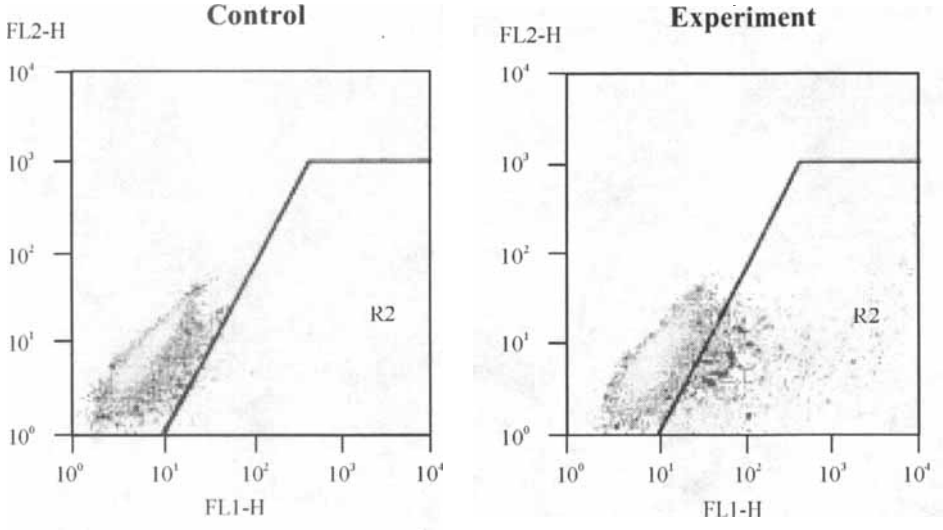

Fig. 2. Expression of type I collagen in fibroblasts after 6 passages in culture.

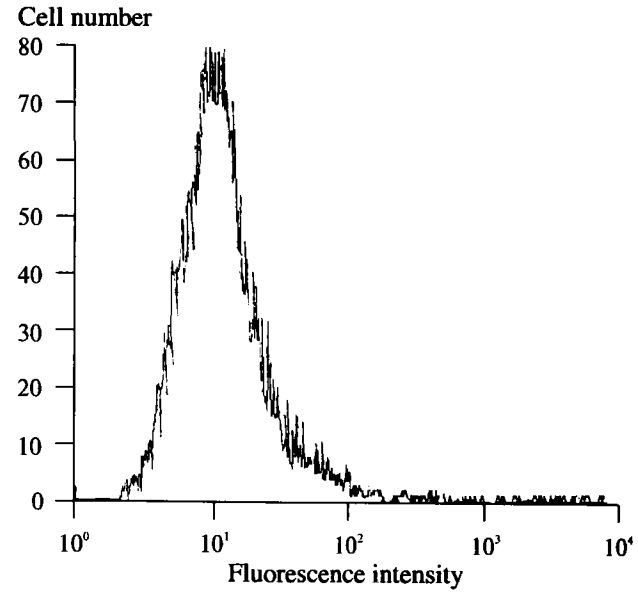

Fig. 3. Fluorescence of cultured fibroblasts (passage 6).

phied scar fibroblasts (passage 6) contracted CG to 21.4±0.6 mm. Moreover, the dynamics of gel contraction by fibroblasts from normal skin was characterized by prolonged lag-period from the start of incubation: 6 *vs.* 2 h for fibroblasts from hypertrophied scar (Fig. 1).

Previous studies showed that fibroblasts lose their ability to synthesize matrix proteins after serial passages *in vitro* [7]. Our flow cytometry study demonstrated that even after 6 passages in culture fibroblasts actively produced type I collagen: fluorescence intensity was 10.2 and 10.03. *vs.* 0.33 and 0.17 arb. units in negative control, respectively (Figs. 2 and 3).

Subcutaneous injection of cultured fibroblasts (passage 7, 4×10^7 cells per mouse) induced no tumors in nude mice within 2 months after transplantation.

Our findings suggest that skin fibroblasts retain normal morphology in culture and produce matrix proteins. Our next goal is to obtain immortal fibroblasts supplies using cell culture technique and to apply these cells as a vocal fold medialization agent.

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