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Preliminary Survival Studies on Autologous Cultured Skin Fibroblasts Transplantation by Injection

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In the correction of aesthetic impairments on the face, dermal, and superficial subcutaneous defects, adequately safe implant material is required. Cultured autologous skin fibroblasts, as a protein repair system, create a living injectable system that has been utilized effectively to treat rhytids, depressed scars, subcutaneous atrophy, acne irregularities, and laser wounds. To evaluate the new method, we have investigated the survival and collagen secretion of autologous transplanted fibroblasts. In this study, rabbit fibroblasts were cultured and expanded. Cells (8×10^7/ml) were injected into the superficial and deep dermal junction of the dorsal ears. Two rabbits were injected independently with labeled [^{3}H]TdR fibroblasts; similarly, eight rabbits were given unlabeled transplanted cells in the right ear and vehicle in the left. Each site was injected three times with the same amount of cells every 2 weeks. The grafts were evaluated for 5 months. After explantation, the samples were collected from the injected sites and stained with autoradiography, H&E, and sirius red, respectively. According to the histological observations, the [^{3}H]TdR-labeled cells survived and large amounts of embryo fibroblasts were found in the experimental subgroup of the labeled cell group. The depth of dermis was significantly different between the experimental subgroup (701.3 ± 31.5 μm) and the control subgroup (638.3 ± 23.9 μm) of the unlabeled group ($p < 0.01$). There was also a significant difference of collagen III between the experimental subgroup (2.63 ± 1.41 cm^2) and the control subgroup (1.05 ± 0.90 cm^2) ($p < 0.05$). There was no significant difference of collagen I between the experimental subgroup (56.25 ± 14.41 cm^2) and the control subgroup (55.41 ± 16.59 cm^2) ($p > 0.05$). The results obtained demonstrate that the distinction of the depth of dermis should be interpreted by the increase of collagen III, instead of collagen I, which is produced by the transplanted fibroblasts. The investigation indicated that transplanted autologous skin fibroblasts could provide a potential and effective approach to treat minor facial tissue deficiencies.

Key words: Fibroblasts; [^{3}H]TdR; Autoradiography; Cell transplantation; Collagen; Implant material

INTRODUCTION

The world of filler substances for soft tissue augmentation has undergone a tremendous revolution over the last several years. Many more implant substances are now available for dermal and superficial subcutaneous defects on the face in clinic trials since bovine collagen became the first material available for injection into rhytides and scars in 1977 (31). These filler substances can be classified into three groups: biological, artificial implant, and active cell transplantation. Biological implants are available as bovine collagen (2,7,13,16,17), Autologen (1,21,28,49), Dermalogen (22,43,48), and hyaluronic acid gel (15,23,38,39,42). Artificial implants, such as Artecoll (12,30,34,47) and Bioplastique (19,20, 44,45) are used in the clinic. Autologous fat cells (9,18, 33) and cultured fibroblasts (4,29,52,54) have also been used as soft tissue augmentation.

Each of these soft tissue augmentation materials has its adverse reactions. Swelling, bruising, erythema, hypersensitivity, and moderate pain are common reactions to the biological and artificial implants. A consequence of their biocompatibility is that they are usually absorbed within 3 to 6 months, or appear many years later in the form of dislocation, granuloma formation, late allergic reaction, or with rough surfaces (48); thus, they cannot be used for permanent soft tissue augmentation, especially on the face (28). Autologous fat cell injection has been reported for many years but, at best, only 50% percent of the implanted fat cells connected to the subcutaneous capillaries of the recipient bed could survive (6,25,27,50). Also transplanted adipocytes cannot fill

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miniwrinkles on the face. It is hoped that these new products for soft tissue augmentation will prove to be safe and effective and will achieve great patient and physician satisfaction.

Using autologous skin fibroblasts as an injectable system to repair dermal and subcutaneous defects such as rhytids and scars opens up a new window for reconstructive surgeons. This system is made available by Isolagen Technologies (Paramus, NJ) and utilizes the patient's own fibroblast protein repair system, which can produce collagen locally and correct contour deformity (52). This system can restore a patient's dermis that has a reduction of fibroblasts and collagen integrity as a result of photodamage, chronological aging, scarring, trauma, acne, or infection. The system can also be stored indefinitely in liquid nitrogen and provide a virtually unlimited source of nonaging fibroblasts. Recent research shows that dermal fibroblasts within restylane may be successfully injected as living grafts for long-term retention (55). Further clinical studies show that autologous fibroblast transplantation could perform augmentation rhinoplasty (26).

Thymidine (TdR) labeled with ^3H, a specific precursor available for studies of DNA synthesis, is incorporated nearly exclusively into DNA (11), but is only available for a short period of time and can therefore be used as a labeling tool for fibroblasts. Consequently, the survival rate of transplanted cells can be monitored (32,37).

The objective of the present work is to investigate whether the transplanted cultured autologous fibroblasts can survive a long time and secrete new collagen.

MATERIALS AND METHODS

Animals and Study Design

Animals. Ten 2-month-old New Zealand white rabbits were obtained from the Experimental Animal Center of the Peking Union Medical College. Animal weights at the initiation of study ranged from 1.4 to 1.6 kg. All animal experiments were performed in compliance with the college research council's guidelines for animal care.

Study Design. The 10 animals were divided into two groups: the [^{3}H]TdR-labeled autologous fibroblasts transplantation group (two animals) and the unlabeled autologous fibroblasts transplantation group (eight animals). In every group, the right ears were injected with the autologous fibroblasts as the experimental subgroup, while the left ears were injected with the phosphate-buffered saline (PBS) without autologous fibroblasts as the control subgroup.

In Vitro Tissue Culture

Primary Cultivation of Autologous Rabbit Dermal Fibroblasts. Primary cultivation of autologous fibroblasts was carried out as described elsewhere (35). Normal skin biopsies were derived from rabbits and then trimmed, cut into small pieces, and digested with dispase (Type II, Roche) at 37°C for 1 h. After digestion, the epidermis was separated from the dermis with forceps. The dermis was cut into small pieces to 1 × 2 mm with a scalpel and was incubated in tissue culture dish at 5% CO_2/95% O_2 and 37°C in Dulbecco's modified Eagles medium (DMEM, GIBCO, USA) supplemented with 10% heat-inactivated (v/v) fetal bovine serum (FBS, Hyclon, USA). Penicillin, streptomycin, and glutamine, were added to the culture dish. The skin segments were left undisturbed for 7 days until the medium was changed. There was considerable outgrowth of fibroblasts by the 10th day before skin segments were removed. When the cultures reached confluence in the dish, the adherent cells were treated with 0.25% trypsin at 37°C for 3–5 min. The detached fibroblasts were collected by centrifugation, resuspended in DMEM medium containing 10% FBS, and cultured in 175-cm^2 tissue culture flasks at 5% CO_2/95% O_2 and 37°C. The fibroblasts were subcultured twice at 5-day intervals. The fourth generation of fibroblasts could be used for the cell transplantation.

Cell Labeling With [^{3}H]TdR. The fibroblasts were labeled with [^{3}H]TdR (Chinese Science College), which is incorporated nearly exclusively into DNA. The whole procedure was accomplished according to the radioactive substance safety regulations. When the fibroblasts became preconfluent, the culture medium was removed by aspiration; 16 μCi [^{3}H]TdR in 4 ml DMEM medium containing 10% FBS was added into one culture flask with [^{3}H]TdR at a final concentration of 4 μCi/ml. After incubation for 12 h at 37°C, they were digested with 0.25% trypsin at 37°C for 3 min. The fibroblasts were harvested by centrifugation (2000 × *g* for 8 min). The cell pellet was resuspended in 8 ml PBS and washed three times to exclude free [^{3}H]TdR that did not enter into the fibroblasts. The cell pellet was resuspended in 500 μl of DMEM medium containing 10% FBS and the cells were counted under phase-contrast microscope.

Cell Counting and Preparation of Cell-Containing Suspension. Before transplantation, all autologous fibroblasts in different tissue flanks belonging to one animal were collected together for cell counting and transplantation into the same animal.

At confluency, the fibroblasts of the forth generation were trypsinized. The cell pellet was suspended in 500 μl of DMEM medium containing 10% FBS. A 10-μl suspension of cells diluted with 0.05% Evan's blue dye in a 1:9 ratio was counted using a hemocytometer under a phase-contrast microscope. According to the result, the cell suspension was centrifuged and resuspended in an

appropriate volume of PBS to yield a final cell concentration of 8×10^7/ml in both the unlabeled cell group and the labeled group.

In Vivo Experiments

In both groups, cell suspension (8×10^7/ml) was transferred into a 1-ml syringe and injected into the superficial and deep dermal junction of the dorsal aspect of the right rabbit ear as the experimental subgroup. The same volume of PBS containing no cells was injected into the left ear of the same animal as the control subgroup. A hole was made to mark the injection site and to localize subsequent biopsy. Each site was injected with 8×10^7 cells or 1 ml PBS over 30 s. Another two injections at the same site were made at 2-week intervals. The animals were sacrificed and the injection sites were explanted 5 months after transplantation.

Autoradiography

Autoradiography was carried out as described elsewhere (14). The explanted material from the [^{3}H]TdR-labeled cell group was rapidly frozen to –40°C and cryostat sections of 8 μm were produced. The frozen sections were thaw mounted on nuclear emulsion IV (China Institute of Atomic Energy) coated slides. The tissue slides were kept in the dark in a storage box at 4°C for 2 weeks. After being developed for 12 min and fixed for 8 min, the tissue sections were stained with methylgreen & pyronin.

Histology

The explanted material from the unlabeled cell group was fixed into 10% buffered formaldehyde, dehydrated in a series of alcohols, and embedded in paraffin. Vertical sections 6 μm thick were prepared and stained with hematoxylin & eosin (H&E) and sirius red. The samples were evaluated independently by three blinded investigators using both light and polarization microscopes.

Dermal Depth. The dermal depth from the epithelium to the cartilage was calculated on 20× magnification under a microscope (UFX-II, Nikon, Japan). There were three different slices used for each location and the data were collected three times on one slice by three different investigators.

Collagen Content. After the sirius-stained slices had been photographed under the polarization microscope (Olympus, Japan), the total absolute area of the red thick type I collagen fibers and the green thin type III collagen on one photo were calculated with MIAS software by the automatic image analyzer (QTM970, Cambridge).

Statistical Analysis

Both the dermal depth and the collagen content of skin were analyzed by SPSS 11.0 software comparing the experimental and the control subgroup in the unlabeled cell group. The significance of differences was evaluated by paired t-test and a value of $p < 0.05$ was considered significant.

RESULTS

Gross Morphology

The general condition of all rabbits in the study through the follow-up period was good without accident, death, or wound infections. The skin on the injected sites appeared normal, without temporary erythema or swelling by 5 days in both groups. Five months after transplantation, the skin overlying the injected sites was soft, harmonious with the surrounding skin, and without prominence or nodules in both of the groups.

Cell Survival of the [^{3}H]TdR-Labeled Cell Transplantation Group

Five months after injection, the transplanted [^{3}H]TdR-labeled fibroblasts could still be detected in the experimental subgroup. Black spots can be observed on the green nucleus of the labeled cells. Some clusters of labeled cells attached to the ear cartilage could be observed clearly (Fig. 1A). There were four distinguishable black dots overlying the nucleus in a typical labeled cell (Fig. 1B). No [^{3}H]TdR-labeled fibroblasts could be found in the control subgroup.

A large amount of extracellular matrix was laid down around the [^{3}H]TdR-labeled cell. No lymphocytes, macrophages, and fibrous membrane circulated clusters of [^{3}H]TdR-labeled cells were apparent. The [^{3}H]TdR-labeled fibroblasts indicated that the transplanted autologous fibroblasts should survive after 5 months.

Histology of the Unlabeled Cell Transplantation Group

H&E Stains. In the unlabeled cell group, lots of embryo fibroblasts were found and converged in the experimental subgroup, with no macrophages and lymphocytes present. The fibroblasts appeared with a spherical nucleus and a limited amount of cytoplasm. Every cell was further surrounded by a thin layer of extracellular matrix with bright red stain, which symbolized more collagen had been produced (Fig. 2A). In the control subgroup, fibroblasts are usually attached to individual collagen bundles and elongated longitudinally while cell densities were lower than those in the experimental subgroup (Fig. 2B).

Sirius Red Stains. Under polarization microscope morphometry, collagen I showed up as red thick type fibers, and collagen III showed up as green thin type fibers in the same area. In the experimental subgroup, collagen III appeared as more discontinuous green fragments, which were arranged irregularly (Fig. 3A). In the

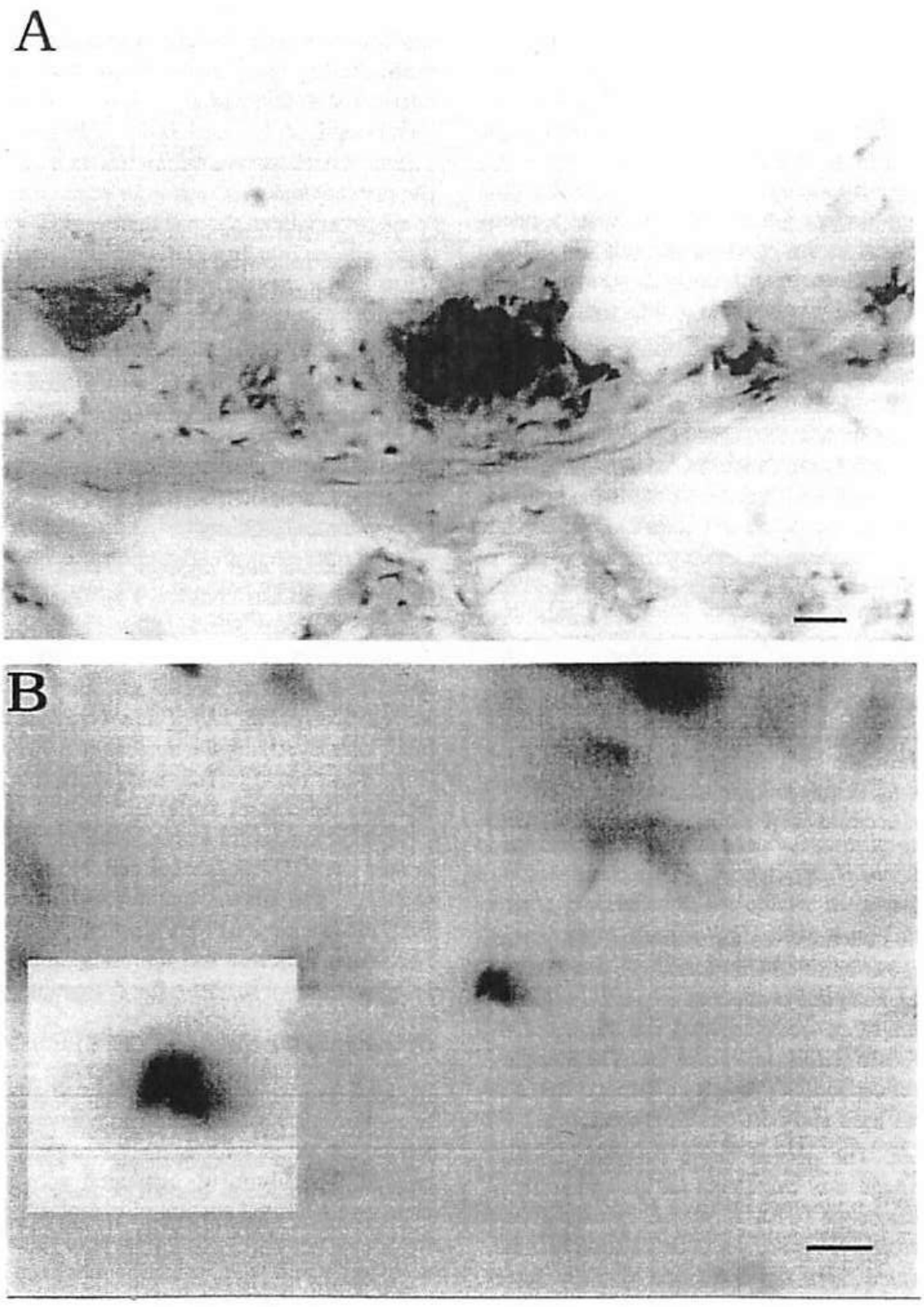

Figure 1. Autoradiography stained with methylgreen & pyronin in the skin 5 months after [^{3}H]TdR-labeled fibroblast transplantation. (A) The [^{3}H]TdR-labeled cells survived near the ear cartilage, distributed around the injected sites (100×). Scale bar: 20 μm. (B) The four black grains on the nucleus shows the labeled fibroblast, which survives for 5 months posttransplantation (500×). Scale bar: 5 μm. The same scale bar represents 2.5 μm in the inset.

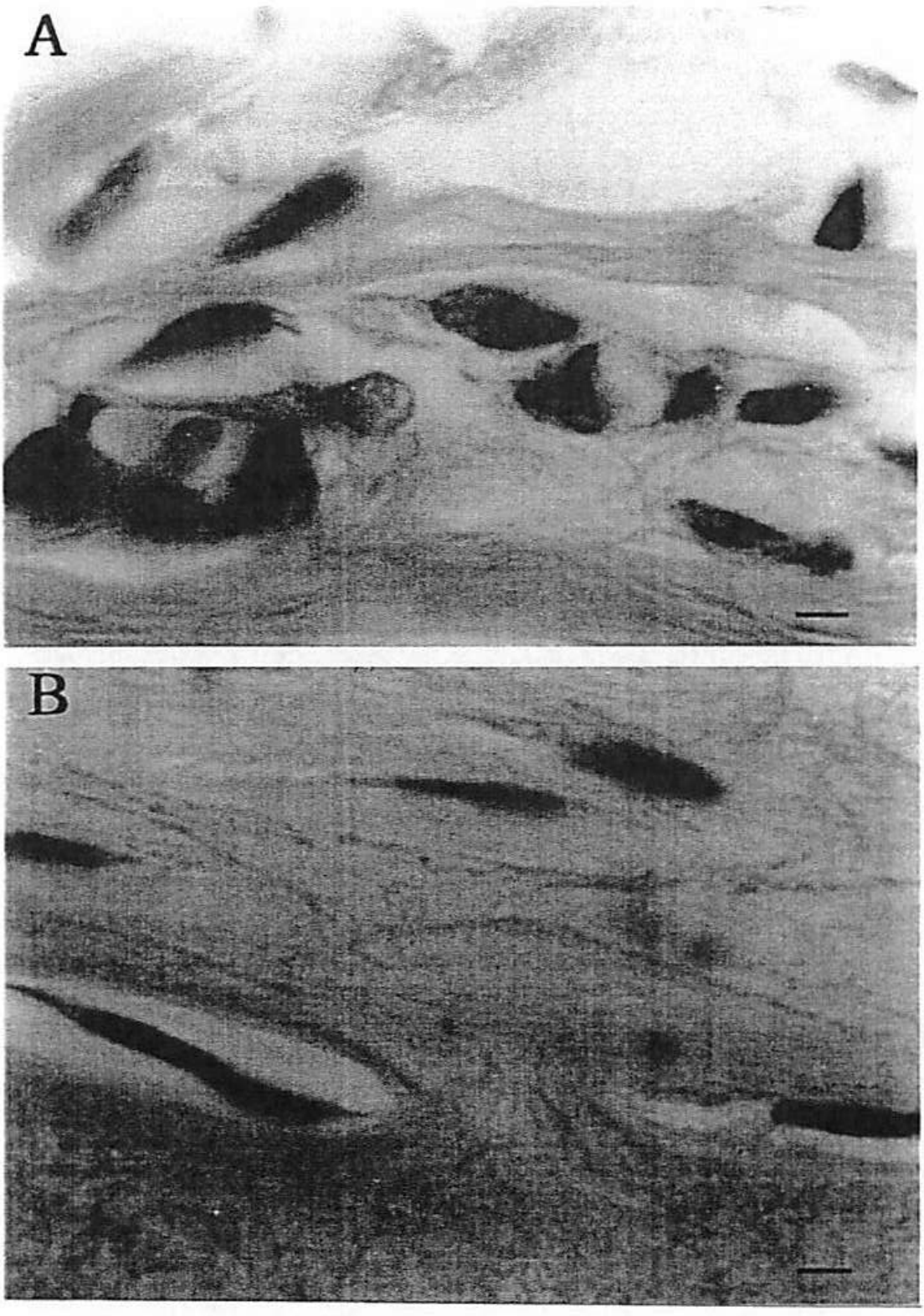

Figure 2. Histological staining with hematoxyline & eosin sections of the experimental subgroup (A) and the control subgroup (B) of the nonlabeled autologous fibroblasts group (200×). (A) A lot of embryo fibroblasts with a spherical nucleus and some amounts of cytoplasm in the skin 5 months after fibroblasts transplantation. The eosin surroundings of the extracellular matrix are more colorful. (B) The normal skin usually shows deployment along bundles of collagen fiber, with elongated nucleus. Scale bar: 10 μm.

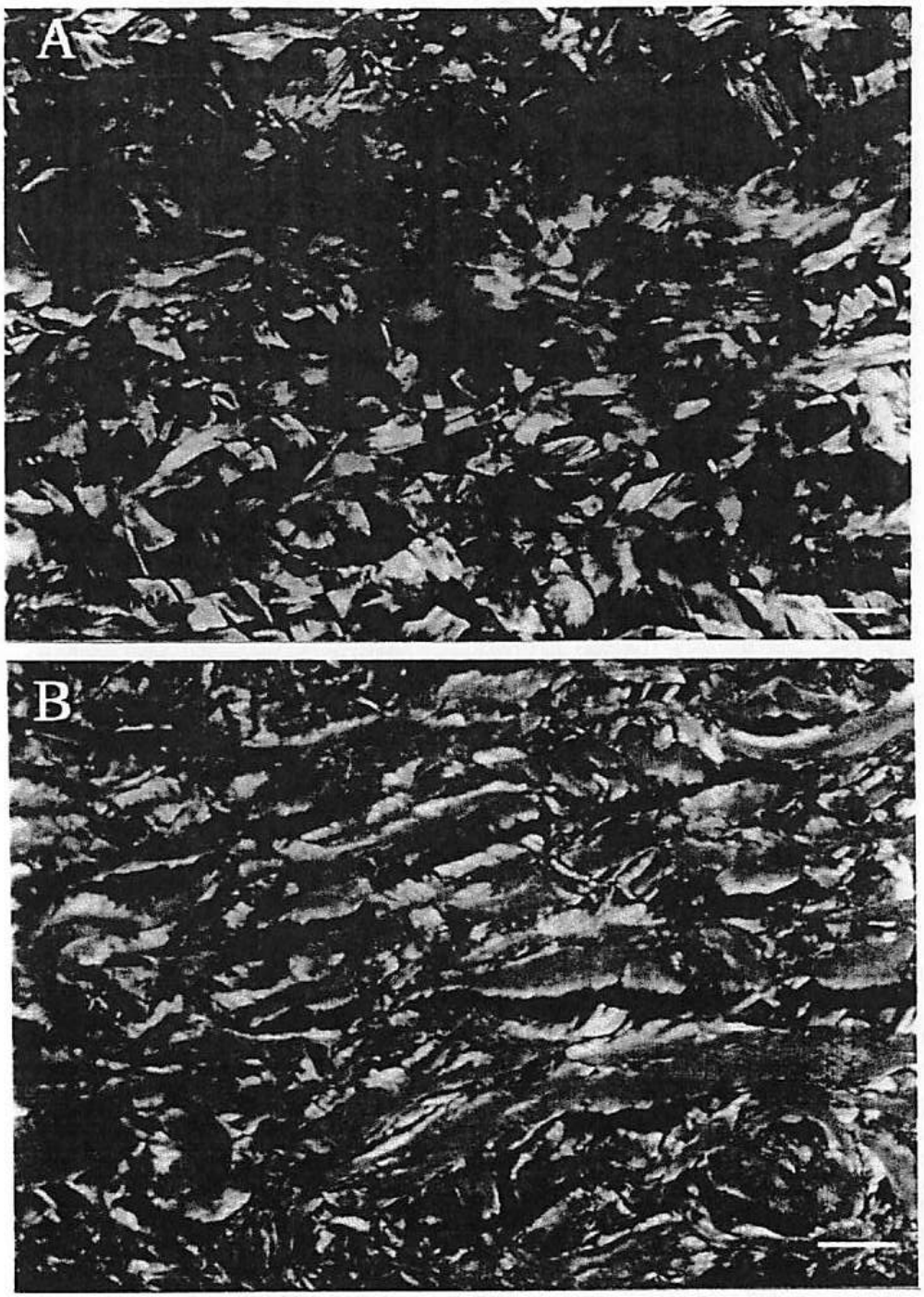

Figure 3. Histological staining with sirius red under the polarization microscope morphometry sections of the experimental subgroup (A) and the control subgroup (B) of the unlabeled autologous fibroblasts group. (A) Collagen III appears as more discontinuous green fragments, which are arranged irregularly in the skin 5 months after fibroblast transplantation (300×). (B) Collagen I appears as largely continuous red streaks with minimal green collagen III in normal skin (300×). Scale bar: 10 μm.

control subgroup, collagen I showed up as continuous red bundles with minimal green collagen III staining, suggesting that collagen I was the main fibers (Fig. 3B).

The Dermal Depth. The dermal depth of the experimental subgroup was 701.3 ± 31.5 μm, in contrast to a dermal depth of 638.3 ± 23.9 μm for the control subgroup ($p < 0.01$, paired t-test).

The Skin Collagen. The absolute area of the collagen I fibers in the experimental subgroup (55.41 ± 16.59 cm^2) was lower than that in the control subgroup (56.25 ± 14.41 cm^2), but this difference did not reach significance ($p > 0.05$, paired t-test). The absolute area of collagen III in the experimental subgroup (2.63 ± 1.41 cm^2) was significantly higher than that in the control subgroup (1.05 ± 0.90 cm^2) ($p < 0.05$, paired t-test) (Table 1).

DISCUSSION

The development and use of materials for soft tissue augmentation have truly exploded over the past few years, along with patient demand and manufacturer interest. An ideal filler substance should attain the following requirements: good biocompatibility, permanence, painlessness, minimal side effects, and easy to use for physicians. For the soft augmentation filler on the face, these criterions are especially important.

Autologous fibroblasts have been broadly investigated to prevent wound contraction and improve wound healing (8,36). Since 1996, Boss has used the autologous fibroblasts (Isolagen, Paramus, NJ) as an injectable system to repair dermal and subcutaneous defects in 1450 patients (4). Histologic analysis in these studies demonstrated that fibroblast injections increase collagen formation, accompanied by a concomitant increase in thickness and density of dermal collagen. The phase III clinical trial suggests that the treatment has safely and effectively produced improvement in rhytids, acne scars, and other dermal defects continuing for at least 12 months after injection (53). Autologous fibroblast transplantation does not require extensive surgical extraction; it produces ongoing improvement in facial contour effects without the hypersensitivity complications and harvesting challenges associated with other treatments, so it should be the ideal soft augmentation filler on the face. This study was undertaken to determine whether transplanted autologous fibroblasts could survive in vivo and secrete new collagen, causing changes in skin depth and collagen in the injected site.

Table 1. The Absolute Area of Collagen I and Collagen III

Group	Collagen I (cm^2)	Collagen III (cm^2)
Control subgroup ($n = 8$)	56.25 ± 14.41	1.05 ± 0.90
Experimental subgroup ($n = 8$)	55.41 ± 16.59*	2.63 ± 1.41†

*$p = 0.91 > 0.05$.
†$p = 0.02 < 0.05$.

At present, most fluorescent dyes used to trace the fate of the transplanted cell, for example, DiI (3,40,41), bisbenzamide (BB) and carboxyfluorescein diacetate (CFDA) (24,46,51), could persist in vivo for up to 60 days. These fluorescent stains could be incorporated into the cytoplasm of cultured cells. If exogenous fibroblasts were labeled by these fluorescent dyes, it is unsuitable for identification of exogenous cells that would survive in vivo for more than 3 months, so that it could not provide the powerful proof that the transplanted autologous fibroblasts could produce more lasting effect in the clinical trials than other filler substances. The method of [^{3}H]TdR-labeled transplanted cells in vitro—tracing in vivo—imaging by autoradiography after explantation was first described in our experiment. The investigation of DNA synthesis with radioactively labeled precursors has developed into a special and independent field of cell kinetics. After addition into the culture flasks, TdR rapidly diffuses from the culture medium and is available to the fibroblasts within 20 min (5). Its addition to a cell culture leads to label all cells in S phase (i.e., they are synthesizing DNA). Researchers have shown that the proportion of endogenous DNA that is replaced by exogenous TdR is very small, only between 0.01% and 0.1%. As the exogenous fibroblasts become relatively inactive, the cells in S phase are minimal, and so the portion of exogenous [^{3}H]TdR that is incorporated into DNA is very small (5). This result implies that the labeled fibroblasts in tissue detected by autoradiography, which had been washed three times by PBS before transplantation, should be the fibroblast that we injected. In the [^{3}H]TdR-labeled autologous fibroblast transplantation group, some clusters of labeled cells attached to the ear cartilage could be observed clearly, and more importantly, there were four distinguishable black dots overlying the nucleus in a typically labeled cell, which shows that transplanted fibroblasts are active, even after 5 months.

As we know, collagen secreted by the transplanted living fibroblasts is indeed our focus. Collagen is the main structural component of skin and is the most abundant extracellular matrix component of skin. Collagen types I and III form the bulk of the dermis, and their ratios in skin changes following birth. Collagen production involves a series of steps that occur within the fibroblast and in the extracelluar space. Type I collagen constitutes approximately 80% of collagen in adult human skin and is the major structural protein in the body. Type III collagen has also been referred to as fetal collagen due to its abundance in fetal tissue. It accounts for about half

of the total collagen in fetal skin. Following birth, production of type I collagen exceeds that of type III collagen, with the resulting ratio of type I to type III collagen being approximately 6:1 in adult human skin. It may therefore play a role in development of dermal collagen.

Sirius red stains show the collagen I and collagen III in one area under the polarization microscope morphometry method. Collagen I appeared as red or yellow thick fibers, and collagen III appeared as green thin fibers (10). Between the experimental subgroup and the control subgroup in the unlabeled autologous fibroblasts transplantation group, collagen I content showed no significant difference, but collagen III contents showed a significant difference. In the normal rabbit skin, collagen I contents amounted to 97%, so the collagen I fibers newly secreted by the transplanted fibroblasts were not significantly changed. However, newly secreted collagen III by the transplanted fibroblasts was significantly different on the basis of the 3% collagen III that exists in normal rabbit skin. The fibroblasts apparently secrete other extracellular matrix proteins, such as elastin, thromospondin, fibronectin, and glycosaminoglycans, etc. These extracellular matrix proteins commonly arouse significant differences of the dermal depth between the experimental and the control subgroup.

In addition, lots of embryo fibroblasts were found and converged in the experimental subgroup in the unlabeled cell group, which implied that embryo fibroblasts would become mature in the future. During the procedure, more and more extracellular matrix will be secreted by the transplanted fibroblasts, which also proves the lasting effect of the treatment in clinical trials. The apparent absence of macrophages and lymphocytes surrounding the transplanted cells is suggestive of good biocompatibility of the filler.

The preliminary study shows that the implanted autologous cultured fibroblasts could survive in vivo for a long time (more than 5 months), and the implanted active fibroblasts could secrete the new collagen. This strongly supports the possibility that the autologous cultured fibroblast is an ideal filler of soft substance to treat dermal and subcutaneous defects on the face. Intensive trials are currently still in progress.

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