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Original article

Expression of TGF-β3 in Isolated Fibroblasts from Foreskin

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Abstract

Background: The multifunctional transforming growth factor beta (TGF-β) is a glycoprotein that exists in three isoforms. TGF-β3 expression increases in fetal wound healing and reduces fibronectin and collagen I and III deposition, and also improves the architecture of the neodermis which is a combination of blood vessels and connective tissue during wound healing. Fibroblasts are key cells in the wound healing process. TGF-β3 plays a critical role in scar-free wound healing and fibroblast actions in the wound healing process. The aim of this study was to express the TGF-β3 gene (tgf-b3) in human foreskin fibroblasts (HFF's).

Methods: We obtained HFF's from a newborn and a primary fibroblast culture was prepared. The cells were transfected with TGF-β3-pCMV6-XL5 plasmid DNA by both lipofection and electroporation. Expression of TGF-β3 was measured by enzyme-linked immunosorbent assay (ELISA).

Results: The highest TGF-β3 expression (8.3-fold greater than control) was obtained by lipofection after 72 hours using 3 µl of transfection reagent. Expression was 1.4-fold greater than control by electroporation.

Conclusions: In this study, we successfully increased TGF-β3 expression in primary fibroblast cells. In the future, grafting these transfected fibroblasts onto wounds can help the healing process without scarring.

Keywords: Fibroblasts, Gene expression, TGF-β3

Introduction

Transforming growth factor-β (TGF-β) is a glycoprotein that acts as a cytokine. This multifunctional cytokine has a pivotal role in the regulation of many cellular activities, such as proliferation, differentiation, and other functions, and also plays a role in immunity, cancer, heart disease, diabetes, Marfan syndrome, Loeys–Dietz syndrome, Parkinson's disease, and AIDS (1).

Three TGF-β isoforms have been found in mammals: TGF-β1, 2, and 3, which are structurally and functionally similar (2). TGF-β is a chemo-absorbent for macrophages and can mediate collagen synthesis (3, 4). It can also increase granulation tissue formation (5). TGF-β3 has a role in embryogenesis and cell differentiation (6). Wound healing can be accompanied by keloid and hypertrophic scar formation.

The healing process is the same and predictable for cuts, burns, and infarcted tissue (7).

Healing occurs in four phases: inflammatory, proliferative, granulation tissue formation, and remodelling. Multiple cell types cooperate in these phases (8). Inordinate collagen type III synthesis in the proliferative phase increases scar formation. Keratinocytes and fibroblasts migrate during wound healing. Keratinocytes migrate to the wound edge within hours, but fibroblast migration is undetectable until several days later.

TGF-β3 plays an important role in this process. TGF-β3 halts dermal migration and consequently causes wound re-epithelialization prior to wound remodeling (9). In fetal tissue, wound healing phases are less clear; however, the inflammatory phase increases and wounds heal without scarring. TGF-β3

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expression increases during fetal wound healing. TGF-β3 reduces fibronectin, collagens type I and III deposition (10), and also improves architecture of the neodermis during wound healing. TGF-β3 also reduces scarring, in contrast to TGF-β1 and 2, which promote scar formation (6).

Fibroblasts are the key cells in the wound-healing process. Fibroblasts have an essential role in angiogenesis during wound healing via extracellular matrix (ECM) protein production. They produce collagen, therefore they form collagen scaffolds and also function as energy reservoirs in wound healing. Recently, these cells (11), and TGF-β3, due to its role in scar-free wound healing, have been extensively used for wound treatment. Growth factor production is essential during wound healing; however, the lifetime of growth factors in the wound environment is short (12). The aim of our study was to increase TGF-beta 3 expression in allogenic fibroblasts, which can be used to promote scar-free wound healing. Our long-term goal is to treat wounds with these fibroblasts without leaving scars.

Materials and Methods

Skin preparation

With parental consent, we obtained a foreskin sample from a newborn under sterile conditions. The sample was placed in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% L-glutamine (Sigma, USA), and 10% penicillin/streptomycin (Sigma , USA), and dermis separation was completed under a laminar hood.

Fibroblasts cultivation

The sample was washed with phosphate buffered saline (PBS) (Sigma, USA). Dermis was separated from epidermis in 0.25% Trypsin-EDTA (1X) (Gibco, USA). The dermis was cut into smaller than 1 mm pieces with sterile scissors and explanted into a 100-mm cell culture dish (Corning, USA) containing complete medium (DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin and incubated at 37 ˚C with 5% CO_2. Each three days, the medium was replaced with the fresh complete medium. Confluent cells were harvested from the tissue culture dish using short-term trypsinization. Harvested cells were collected and pipetted into centrifuge tube. After centrifuge at 350×g for 5 minutes, supernatant was removed and collected fibroblasts were subcultured in 25 cm^2 cell culture flasks (CELLSTAR, USA). Cells from the fifth subculture were stored at -80˚C.

Lipofection

The HFF's were transfected with Fibroblast Transfection Reagent (Altogen Biosystems, USA) by both reverse and direct transfection. In reverse transfection, the cells were cultivated in dishes for 30 minutes, and then the transfection mixture was added. In the direct transfection method, the transfection mixture was added to the plates first, and then the cells were added.

To prepare the transfection mixtures, 40 µl of DMEM without serum and 750 ng TGF-β3-pCMV6-XL5 plasmid DNA (Origene Technologies, MD, USA) were combined and then 1.5, 2, 2.5, or 3 µl of transfection reagent were added to each sample. Mixtures were incubated at room temperature for 15-30 minutes. For reverse transfection, 2×10^4 - 3×10^4 fibroblasts were seeded in 24-well plates (Corning, USA) with 0.5 ml complete medium, and after 30 minutes the transfection mixtures were added. The cells were incubated at 37 ˚C and 5% CO_2 and checked hourly. If cell viability was affected, the medium was changed. If the cell viability was unaffected after 16-24 h, the transfection enhancer reagent was added. Supernatants were assayed for TGF-β3 after 24, 48, 72, and 96 hours by ELISA (R&D Systems).

Electroporation

Confluent fibroblasts were dissociated from the wells using 0.25% trypsin (Invitrogen) and suspended in complete medium. The cell suspension was pelleted by centrifugation at 350×g for 5 minutes. OptiMEM (Invitrogen) was added to the cell pellet and the suspension was again centrifuged at 350×g for 5 minutes. Twenty µg of TGF-β3-pCMV6-XL5 plasmid DNA and OptiMEM were added to the cell pellet. The cell suspension was incubated for 10 minutes at room temperature and then added into an electroporation cuvette (Gene Pulser, 0.2 cm electrode, Bio-Rad). Electroporation was performed in a BioRad Gene Pulser™ (Bio-Rad Laboratories, CA, USA) with 350 V and 500 µF for 12.7-15.1 mS.

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Assessing TGF-B3 production

Seventy-two hours post-transfection TGF-β3 production in transfected and control cells was assayed using a human TGF-beta 3 DuoSet ELISA Development kit (R&D Systems). Optical densities were read by a microplate reader (TECAN, SunRise) at 450 nm. Diagrams were drawn and concentrations were calculated using Magellan software (Tecan).

Statistics

Data are presented as means ± standard deviations (SD). Statistical significance for comparisons was determined by the Student's two-tailed *t-test. P* value equal to or less than 0.05 was considered statistically significant.

Results

In this research fibroblasts were transfected with TGF-β3-pCMV6-XL5 plasmid DNA via lipofection and electroporation.

To determine the optimum the lipofection method, we tested different amounts of fibroblast transfection reagent by both reverse and direct methods.

The results showed that TGF-β3 expression was greater with reverse transfection than with direct transfection.

We found that TGF-β3 expression was greatest, at 8.3-fold over controls, in the reverse transfection method using 3 µl of transfection reagent after 72 hours of incubation (Fig. 1).

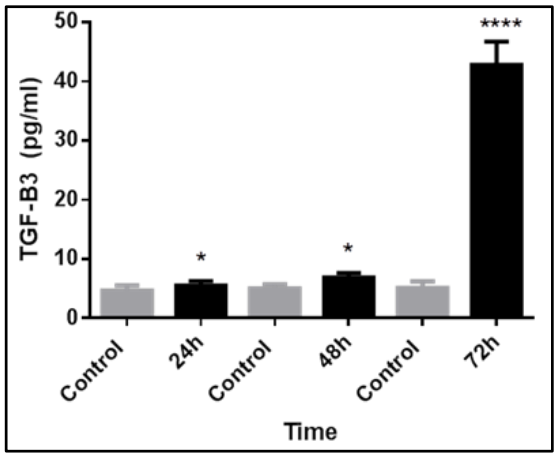

Fig. 1. Time-dependent expression of TGF-β3 in fibroblasts after transfection with 3µL of transfection reagent via reverse transfection. TGF-β3 expression was significantly greater in transfected fibroblasts than control cells. The data represent the means ± SEMs of five independent experiments. (*p<0.05 and ****p<0.001).

TGF-β3 expression increased with time and was greatest at 72 hours, but decreased after that. TGF-β3 expression was 1.5-fold greater than controls after 72 hours in the direct transfection method using 3 µl of transfection reagent (Fig. 2). Using 2.5 µl of transfection reagent in direct transfection method, after 72 hours, TGF-β3 expression in transfected cells was significantly greater, at 1.3-fold, than control cells (Fig. 3). Using 2.5 µl of transfection reagent in the reverse transfection method, after 72 hours TGF-β3 expression in transfected cells was significantly greater by more than 1.5-fold than control cells (Fig. 4). In the electroporation method, cell mortality was high and TGF-β3 expression in transfected cells after 72 hours was 1.4-fold greater than in control cells (Fig. 5).

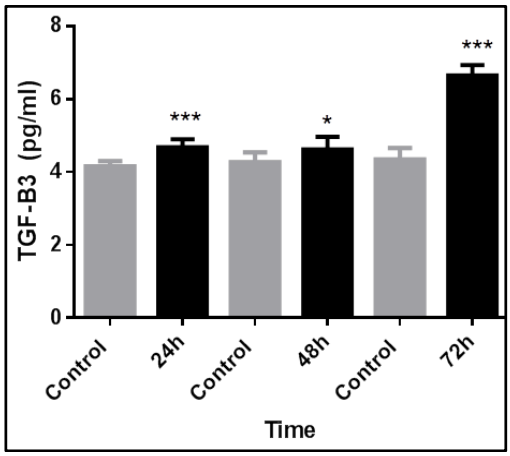

Fig. 2. Time-dependent expression of TGF-β3 in fibroblasts after transfection with 3µL of transfection reagent via direct transfection. TGF-β3 expression was significantly greater in transfected fibroblasts than control cells. The data represent the means ± SEMs of five independent experiments. (*p<0.05 and ***p<0.001).

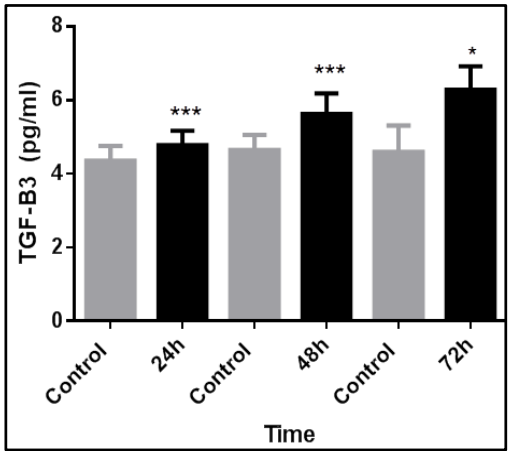

Fig. 3. Time-dependent expression of TGF-β3 in fibroblasts after transfection with 2.5µL of transfection reagent via direct transfection. TGF-β3 expression was significantly greater in transfected

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fibroblasts than control cells. The data represent the means ± SEMs of five independent experiments. (*p<0.05 and ***p<0.001).

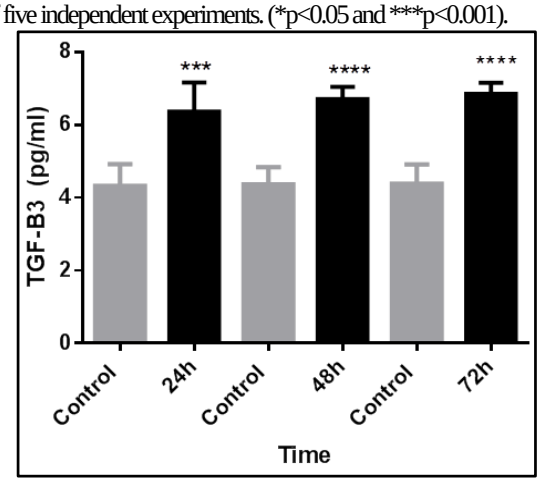

Fig. 4. Time-dependent expression of TGF-β3 in fibroblasts after transfection with 2.5µL of transfection reagent via reverse transfection. TGF-β3 expression was significantly greater in transfected fibroblasts than control cells. The data represent the means ± SEMs of five independent experiments. (***p<0.001 and ****p<0.0001).

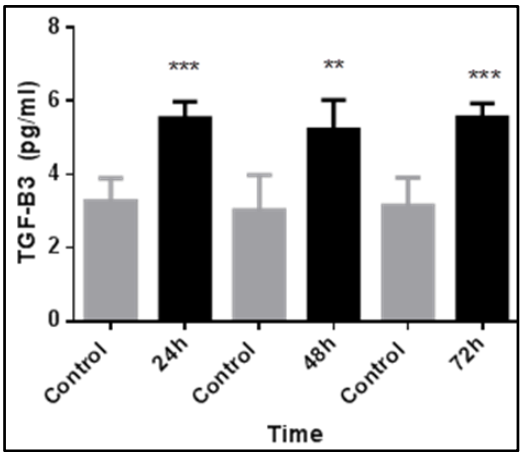

Fig. 5. Time-dependent expression of TGF-β3 in fibroblasts after transfection with electroporation method. TGF-β3 expression was significantly greater in transfected fibroblasts than control cells. The data represent the means ± SEMs of five independent experiments. (**p<0.01 and ***p<0.001).

Discussion

Wound healing is a complex process in which different skin cell types, extracellular matrix, and soluble growth factors are all involved (8). Other methods, such as using recombinant growth factors and skin substitute products have been successful, but are not cost-effective (13). Scar-free wound healing is one of the most important goals of researchers and clinicians; however, inflammation inhibits fast wound healing and contributes to scar formation. Fetal wound healing is scar-free due to the lack of inflammation (6). Immediately after scar formation, remaining skin cells encounter serum, and when wounds heal, cells encounter plasma again. Human serum, unlike plasma, induces migration of keratinocytes via TGF-β3 (14). Replacement of plasma with serum regulates cell migration. TGF-β3 in serum regulates this migration and the TGF-β receptor II (TbRII) level on the cell surface works as a sensor for recognition of skin cell migration (15).

Recent research has focused on genetic and cell-based technologies to treat wounds. Tang et al. (16) transfected stable NIH3T3 fibroblasts using Lipofectamine 2000 to deliver pcDNA3.1(-)/TGF beta3 gene into the cells. Their results showed an increase of TGF-β3 production in 7 out of 11 transfected cell lines; however, MTT assays showed proliferation of the transfected cells was inefficiently slow. Stable transfection of TGF-β3 in fibroblasts by this method was costly, had undesirable side effects on the transfected cells, and the cells could not be used clinically.

Ferguson et al. reported that Avotermin (recombinant TGF-β3) can accelerate wound and scar healing (17). Although gene transfection in skin cells seems relatively difficult compared to other methods of tissue injury treatment, it is essential for good wound healing (18). In this research we attempted to optimize TGF-β3 gene transfection in HFF's by transient transfection. Transient transfection is superior to recombinant protein production or stable transfection because the process is carried out in a single step, is easily scalable, achieves high expression quickly, and is safe and cost-effective (19). Many previous studies used viral vectors (20), such as adenovirus (21-22) or retrovirus (23) for gene transfection in skin cells, but viral vectors have some undesirable effects, such as such as harmful immune responses, toxicity, or tumor induction (24-27).

We used two safe methods: lipofection and electroporation. In the electroporation method, because of cell membrane sensitivity, cell mortality was high and TGF-β3 expression in the transfected cells was only 1.4 fold greater than in the control cells. However, by the lipofection method, 3 µL of the transfection reagent after 72 hours showed an 8.3-fold increase in TGF-β3 production in transfected cells compared to controls. As the lifetime of growth

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factors in the wound environment is short (8), we transfected allogenic fibroblasts with the TGF-β3 gene to use them in wound healing in future studies. In this way, we can heal wounds without scarring. Roland (28) reported differences between in vivo and in vitro gene expression. Therefore, grafting of these transfected allogenic fibroblasts on wounds can result in greater TGF-β3 concentrations than in vitro methods. Cell therapy is an effective method for wound healing.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

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