Microencapsulated nerve growth factor-expressing NIH3T3 cells-incorporated tissue engineering skin: a preliminary study.

Hu Z H, Chen S Z, Jin Y, Xiong Y, Wang W, Ma X J, Song M

ABSTRACT

Introduction: In order to find a suitable carrier to deliver the product of gene transfection to improve the performance of bioengineered dermis, we used microencapsulation and gene transfection technology together for the first time and found that it was feasible.

Methods: We used a recombinant nerve growth factor (pcDNA3.1+/NGF) to modify NIH3T3 cells genetically. Control of NIH3T3-NGF cells were encapsulated within microspheres composed of alginate-poly-L-lysine-alginate and cultivated in-vitro. The concentration of NGF released from the microencapsulated NIH3T3-NGF cells was confirmed using ELISA assay. We co-cultivated microencapsulated NIH3T3-NGF cells, NIH3T3 cells (control) with human keratinocytes and fibroblasts, and tested the percentage of cycle of these cells. The alkaline hydrolysis method was used to analyse the content of hydroxyproline (Hyp). Immunohistochemistry method was used to calculate the transformation efficiency from fibroblasts to myofibroblasts.

Results: The concentration of NGF released from the microencapsulated NIH3T3-NGF cells lasted about six weeks in the supernatant of bioengineered dermis in-vitro. The proliferation of keratinocytes, as well as the concentration of Hyp in supernatant of fibroblasts, were promoted about three times. Transformation efficiency from fibroblasts to myofibroblasts was increased approximately two-fold because of the bio-effects of NGF. Two kinds of microencapsulations were seeded into collagen which contained human fibroblasts to form bioengineered skin. Microencapsulated NIH3T3-NGF cells formed a thicker dermis. The concentration of Hyp in the bioengineered skin which indicated the level of collagen synthesis was increased due to existing NGF.

Conclusion: Microencapsulated NIH3T3-NGF cells can be used to enhance performance of bioengineered dermis and it also can be deduced that other cytokines can be used to treat local wound areas.

Keywords: dermis, gene transfection, microencapsulation, nerve growth factor, tissue engineering

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INTRODUCTION

Bioengineered skin substitutes are a potential treatment for the closure of skin defects, especially in chronic skin ulcers such as diabetic ulcer, venous ulcer, decubital ulcer. In order to construct bioengineered skin, extracellular matrix scaffold and seed cells are essential ingredients. Recently, many gene modified cell lines have been used as seed cells to enhance performance of tissue-engineered skin[1-6]. The wound healing process requires a sophisticated interaction between inflammatory cells, biochemical mediators including growth factors, extracellular matrix molecules, and microenvironmental cell populations[7,8]. Nerve growth factor (NGF) is produced by many types of cells, including fibroblasts[9,10], keratinocytes[11], mast cells[12], and T cells[13]. Therefore, there is a possibility that NGF produced at the wounded site may regulate the healing of the cutaneous wounds. Although NGF is a neurotrophic polypeptide mandatory for the development and function of peripheral and central neurons[14], recent findings have shown that NGF regulates immune and inflammatory responses through direct and/or indirect effects on immunocompetent cells[15] and promotes regeneration of keratinocytes at the edge of the wound and fibroblasts in the granulation tissue during a wound healing process. It is also demonstrated that topical application of NGF to cutaneous wounds accelerates the rate of wound healing in normal and diabetic mice[16].

Although gene modified cell line transplantation has been used so extensively, immunologic rejection

is one of the barriers to limit efficient use of this technology. An alternative form of gene therapy involves immunoisolation of a nonautologous cell line engineered to secrete a therapeutic product. Encapsulation of these cells in a biocompatible polymer serves to protect these allogeneic cells from host-versus-graft rejection while recombinant products and nutrients are able to pass by diffusion. As a new delivery system for gene therapy, APA microcapsules have been used extensively for different applications, particularly for the encapsulation of pancreatic islet cells and insulin delivery[17]. This method has also been used for the encapsulation of cells that release growth hormone, b-endorphin, endostatin and other agents for gene therapy[18-21]. The APA membranes allow the free exchange of nutrients and oxygen between the implanted cells, and could prevent the escape and elimination of encapsulated cells. More importantly, this approach provides a prolonged sustained delivery of recombinant protein produced by the cells, thus maintaining high levels of the agent[22]. In the present study, NIH3T3 cells engineered to continuously secrete high levels of NGF were encapsulated with APA. The ability of this system to secrete biologically-active NGF capable of promoting the regeneration of human keratinocytes and secretion of collagen of fibroblasts, was investigated. Above all, the performance of bioengineered dermis incorporated with microencapsulations which secrete NGF was enhanced.

METHODS

Mouse fibroblasts (NIH3T3) were donated by the Institute of Molecular Genetics, Fourth Military Medical University (FMMU), Xi'an, China, and cultivated in Dulbecco's Modified Eagle's medium (DMEM)(Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal calf serum (Gibco-BRL, Gaithersburg, MD, USA), 2 mM glutamine, penicillin 100 U/ml, and streptomycin 100 μg/ml. Normal human keratinocytes and fibroblasts derived from neonatal foreskins were isolated and cultivated following the method described by Rheinwald and Green[23]. Keratinocyte culture medium (KCM) was a 3:1 mixture of DMEM and Ham's F12 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% foetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA), adenine 1.8×10^{-4} M (Sigma Chemical Co, St Louis, MO, USA), cholera toxin 10^{-10} M (Vibrio cholerae, Type Inaba 569 B; Calbiochem, La Jolla, CA, USA), hydrocortisone 0.4 μg/ml (Calbiochem), insulin 5 μg/ml (Novo Nordisk, Princeton, NJ, USA), transferrin 5 μg/ml (Boehringer Mannheim Co.), triiodo-L-thyronine 2×10^{-9} M (Sigma), and penicillin-streptomycin 100 IU/ ml - 100 μg/ml and was changed every three to four days.

Beginning with the first medium change, EGF (Collaborative Biomedical Products, Bedford, MA, USA) was added at 10 ng/ml. Fibroblasts were cultivated in DMEM supplemented with 10% heat-inactivated foetal calf serum, 2 mM glutamine, penicillin 100 U/ml, and streptomycin 100 μg/ml and all of above cells were incubated in a humidified 10% CO_2 atmosphere at 37ºC. 2.5S NGF isolated from murine submaxillary glands was purchased from AM Stanisz and J Bienenstock (McMaster University, Hamilton, Ontario, Canada)[26]. Rabbit anti-mouse 2.5S NGF polyclonal Ab and goat anti-rabbit IgG (H+L) polyclonal Ab conjugated with peroxidase were respectively obtained from Sigma Chemical (St Louis, MO, USA) and BioMakor (Kirat Weizmann, Rehovot, China). Mouse anti-2.5S NGF mAb was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Rabbit anti-mouse α-SMA polyclonal Ab was purchased from Biotech (Wuhan, HB, China). All chemicals used were purchased from Sigma Chemical, unless otherwise indicated.

Murine 2.5S NGF cDNA were subcloned into pcDNA3.1 plasmids (kindly provided by Prof. Han Hua, Institute of Molecular Genetics, FMMU, China) containing human IL-2 signal peptide sequence code immediate-early enhancer promoter and a G418 selected gene. NIH3T3 cells were stably transfected with these expression plasmids (pcDNA3.1+/ NGF) using FuGENE™6 Transfection Reagent Kit (Boehringer, Mannheim, USA). To obtain stably transfected clones (NIH3T3-NGF), transfected cells were grown in G418 containing medium (600 mg/L, Sigma, separated by Huamei Inc, China) for 14 days, and resistant clones were propagated separately. Subsequent secretion of NGF was determined by ELISA kit (LifeKey Inc, USA). Immunostaining was used to confirm the expression of NGF (unmodified NIH3T3 cells acted as negative control). Two kinds of microencapsulations were co-cultivated with keratinocytes and fibroblasts respectively and used a modified MTT assay to detect the difference of cell proliferation. Basic Hydrolisation Assay Kit (Jiancheng Co, Nanjing, JS, China) was used to determine the concentration of hydroxyproline (Hyp) in the culture medium followed the method of Lange and Malyusz[24]. Immunohistochemistry method was used to determine the difference of positive rates of α-SMA in fibroblasts.

NIH3T3-NGF cells were encapsulated within microspheres composed of APA. Briefly, cells were

resuspended in sodium alginate-saline (1.5% wt/vol, purified by Syringe Driven Filter Unit) (Sigma, St Louis, MO, USA) to a final ratio of 0.5×10^9 cells/L of alginate. The suspension was sprayed through an high-power-voltage pulse droplet-forming apparatus (Dalian Physical and Chemical Research Institute of Academia Sinica, LN, China), into a solution of 4.9% barium chloride (pH 7.4, Sigma), where they were allowed to gel for ten minutes, washed three times with PBS, resuspended into a solution of poly-L-lysine (Sigma) and alginate-saline to form alginate-poly-L-alginate sandwich structure membrane, then cultivated in the conditioned medium (described above) and with neomycin (G418, 300 mg/L, Sigma). The number of cells encapsulated and the viability of the cells in the microcapsules were evaluated weekly using a modified MTT assay. Microencapsulated NIH3T3-NGF cells were suspended in the conditioned medium described above at a density of 1×10^5 cells/well. The medium was collected every 24 hours and assayed for NGF using ELISA Kit (LifeKey Inc, USA). Medium from NIH3T3-NGF monolayer cells and normal NIH3T3 cells were used as a positive control and negative control.

Collagen came from neonatal calf skin and was extracted according to the method of Mariappan Nithya et al[25]. Prepared collagen was treated with trypsin in the ratio of 100:1. The helical collagen molecules after precipitation methods were redissolved in 0.5 M acetic acid and dialysed exhaustively against 0.05 M of the same acid and then lyophilised. 8 mcg of calf collagen was dissolved in 1 ml of 0.05 M acetic acid and mixed with collagen I (8 mg/ml , Sigma) together in a ratio of 4:1. The pH value of this admixture was adjusted at 7.4, and then subjected to gamma irradiation to suppress antigenicity and impart sterility. The sterile gel was supplemented with 10% FCS and 10× DMEM (Gibco-BRL, Gaithersburg, MD, USA) and stored at 0°C to prevent coagulation.

Bioengineered skin was prepared by using a modification of the technique described by Liu et al[26]. After the collagen gel was prepared, fibroblasts (1.5×10^6 cells/ml) and microencapsulations of NIH3T3-NGF or unmodified NIH3T3 control cells, respectively, were added in a ratio of 100:1 and mixed together. Then, 3 ml of complex gel were added into 35 mm tissue culture dish and incubated in a humidified 10% CO_2 atmosphere at 37°C for two hours until the gel coagulated. The culture medium (mentioned above) was added into the dish without G418. After being cultivated for four days, keratinocytes were seeded onto each piece of dermis (5×10^7 cells/ml) in a seeding medium composed of DMEM/F12 (3:1), FBS 1%, cholera toxin 10^{-10} M, hydrocortisone 200 ng/ml, insulin 5 μg/ml, ascorbic acid 50 μg/ml (Sigma), and penicillin – streptomycin 100 IU/ml – 100 μg/ml. The next day, the culture medium was changed to priming medium, which was the same as seeding medium but supplemented with bovine serum albumin (BSA) 24 μM (Sigma), fatty acid cocktail (oleic acid 25 μM, linoleic acid 15 μM, arachidonic acid 7 μM, palmitic acid 25 μM) (Sigma), L-carnitine 10 μM (Sigma), and L-serine 1 mM (Sigma). Bioengineered skin was submerged in this medium for an additional two days. On the seventh day, the skins were placed on a stainless steel mesh and raised to the air-liquid interface for seven days. The air-liquid interface medium was composed of serum-free priming medium supplemented with 1 ng/ml EGF. The medium was changed every two days.

After seven days of cultivation, about 0.3 g tissue was cut from the tissue engineered skin flap and the content of Hyp was also determined by alkaline hydrolysis assay kit described earlier. Small pieces were cut from bioengineered skin tissues gently smoothed and flattened onto a piece of thick filter, and were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 12 hours at 4°C. Skin tissues were embedded in paraffin wax (m.p. 56°C) and cut at 5μm; the sections were placed on silane-coated glass slides, and stained with haematoxylin and eosin. The result was analysed by HPIAS-1000 high-resolution colour pathological diagram-writing analysing system (Wuhan Champion Image Technology Corporation Limited, HB, China) under ×100 magnification to measure the thickness of the dermis and epidermis.

Data were expressed as mean ± standard deviation (SD), and analysed using Statistical Package for Social Sciences (SPSS) version 11.0 (Chicago, IL, USA). Analysis of variance (ANOVA) followed by the S-N-K multiple comparison test was used to determine the significant differences among the groups. P-values of less than 0.05 were considered significant.

RESULTS

NIH3T3 cells were transfected with pcDNA3.1+/NGF (Fig. 1) and clonal populations of stably transfected NIH3T3 cells were obtained (NIH3T3-NGF). The microcapsules have an average diameter of 0.25 mm to 0.35 mm (Fig. 2). Both encapsulated and non-encapsulated NIH3T3-NGF cells were cultivated in-vitro, and the conditioned medium was collected every week for six weeks. The encapsulated cells were viable in culture as determined by

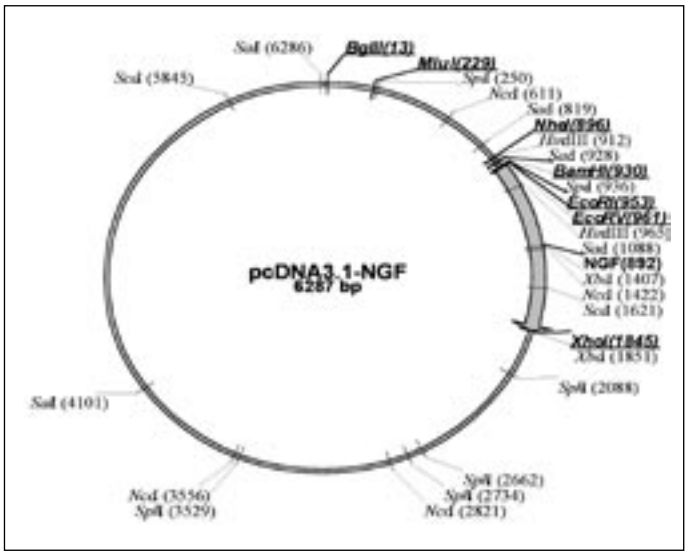


Fig. 1 Diagram shows the structure of pcDNA3.1+/NGF.

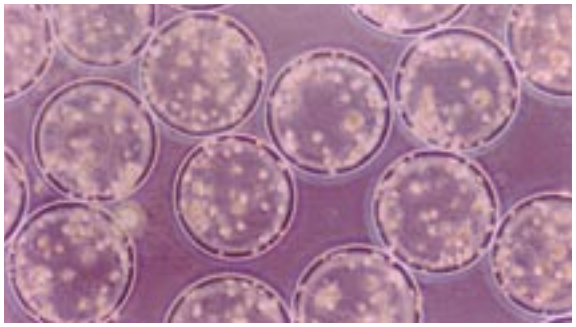


Fig. 2 Inverted phase contrast microscopic image shows NIH3T3-NGF cells-loaded microcapsules (average capsule diameter 0.3 mm) (×40).

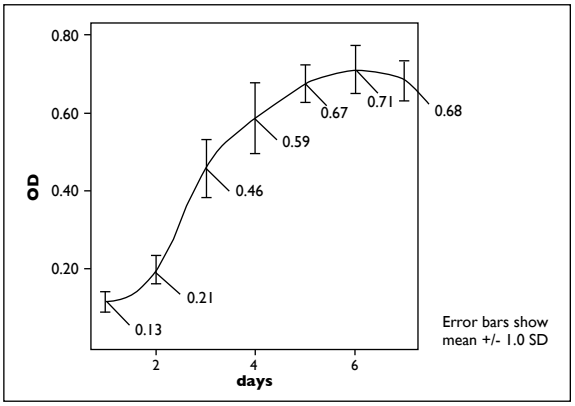


Fig. 3 Graph shows the growth curve of NIH3T3 cells after encapsulation in APA membranes determined by MTT test. The cells proliferation was observed in the microencapsulation from the second day until the sixth day.

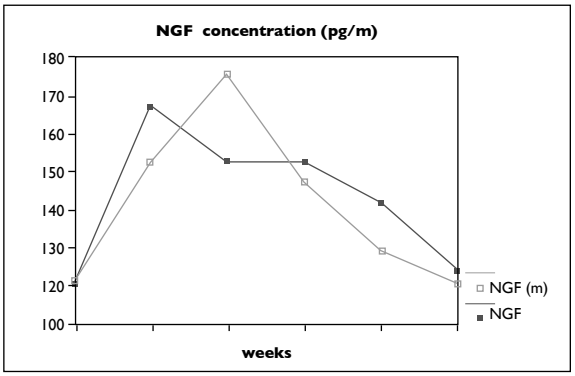
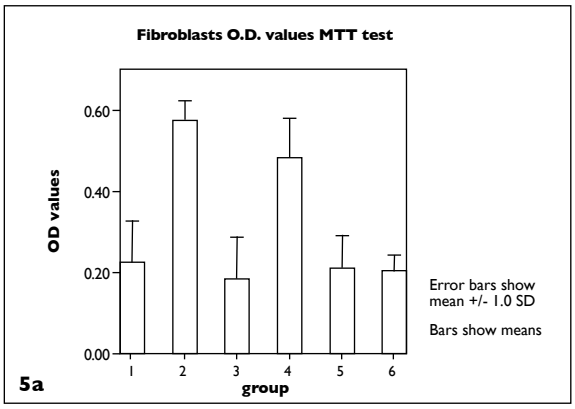


Fig. 4 Graph shows the average concentration of NGF secreted by NIH3T3-NGF cells (NGF) and microencapsulated NIH3T3-NGF cells (NGF (m)) cultivated in six weeks. NGF levels in culture medium were measured by a sandwich ELISA. Each point represents the mean of concentration of NGF (pg/ml).

MTT assay (Fig. 3). ELISA method was used to determine NGF in the medium collected at all time points. The average concentration of NGF secreted by 1×10^5 cultivated encapsulated NIH3T3-NGF cells or nonencapsulated NIH3T3-NGF cells were 143.31 ± 17.6 and 141.26 ± 20.6 pg per one week per 105 cells, respectively (Fig. 4). From Fig. 4, we found that the concentration of NGF was increased in one to two weeks after enclosed in APA microencapsulation, and then, maintained a stable level about 140 pg/ml. These results indicate that NGF protein could release freely from the microencapsulated NIH3T3-NGF cells.

Keratinocytes and fibroblasts were derived successfully from neonatal foreskins and cultivated in-vitro. According to the differences of culture condition, we set up six groups: V12 group one (normal culture medium), group two (culture medium with NGF (5 ng/ml)), group three (culture medium with NGF (5 ng/ml)+NGF Ab (50 ng/ml)), group four (co-cultivated with microencapsulations of NIH3T3-NGF), group five (co-cultivated with microencapsulations of NIH3T3), and group six (co-cultivated with empty microencapsulations). In these groups, groups one, three, five and six acted as negative controls, and group two as the positive control. Keratinocytes and fibroblasts were seeded in 96 wells plate, and 10^3/well. After co-cultivated for 24 hours with microencapsulated NIH3T3-NGF cells, the proliferation of keratinocytes and fibroblasts was tested by MTT, and promoted about three times and two times ($p<0.01$) compared with normal culture medium. The value of optical density (OD) was significantly ($p<0.01$) higher than that of negative control groups and was similar to the positive control group ($p>0.05$) throughout the experimental period (Figs. 5a-b). The transformation efficiency from fibroblasts to myofibroblasts was tested by immunostaining of α-SMA (Fig.6), and the positive rate of α-SMA increased about two-fold ($p<0.01$) in the second and fourth groups (Fig. 7). The concentration of Hyp in supernatant of fibroblasts was also promoted about three-fold compared with negative control groups ($p<0.01$) (Fig. 8).



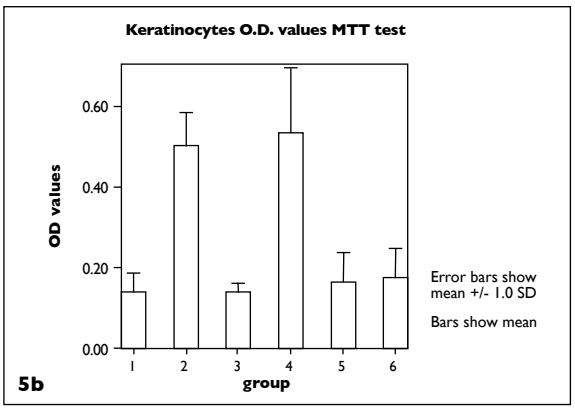


Fig. 5 Histograms show the optical density (OD) values of (a) fibroblasts and (b) keratinocytes in different groups after co-cultivation over 24 hours in different conditions. Each histogram represents mean ± SD for six groups in each individual experiment, the experiment group and positive control group were significantly different from the control group at $p<0.01$ as determined by ANOVA.

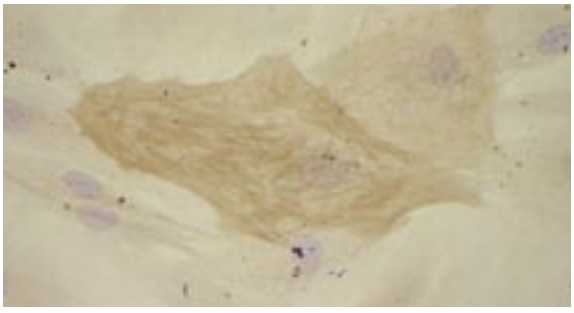


Fig. 6 Positive cell immunostaining of α-SMA in fibroblasts (×100).

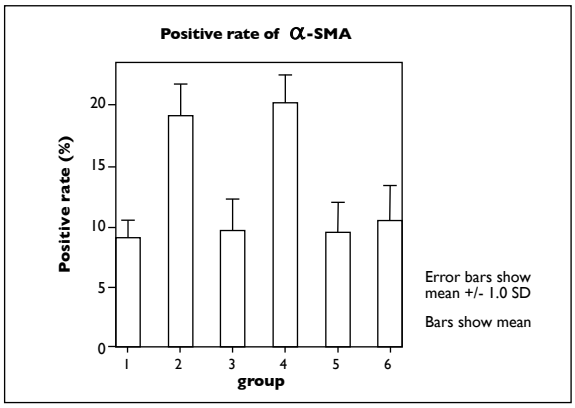


Fig. 7 Histograms show the positive cells of α-SMA in fibroblasts were calculated by HPIAS-1000 under ×100 magnification. According to HPIAS-1000 and statistical analysis, the number of positive rate in experiment group and positive control group was significantly higher than in negative control groups ($p<0.01$).

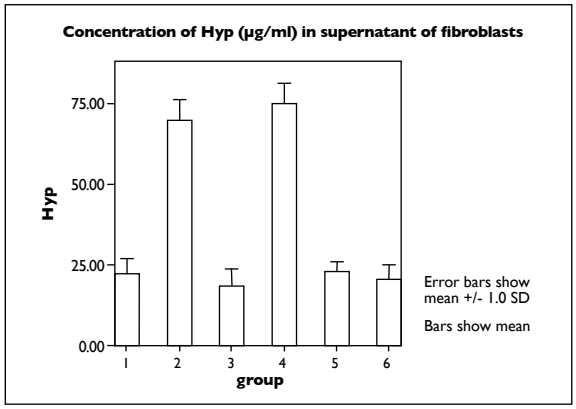


Fig. 8 Histograms show the concentration of Hyp in supernatant of fibroblasts, each point represents the mean ± SD (μg/ml) of three separate experiments using duplicate samples.

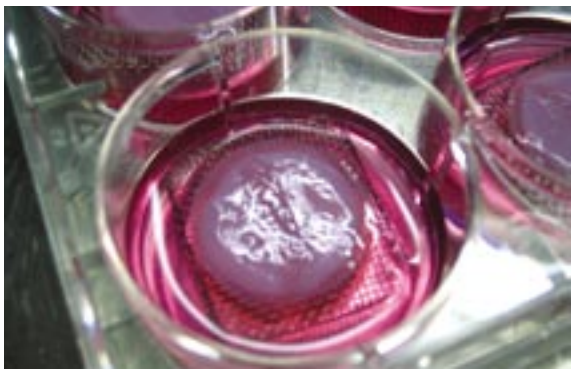


Fig. 9 Photograph shows the tissue engineered skin placed on a stainless steel mesh and raised to the air-liquid interface.

We used self-made collagen gel as extracellular matrix and scaffold, and the microencapsulations, fibroblasts and keratinocytes as seed cells of tissue engineered skin to construct gene modified tissue engineered skin, and cultivated in-vitro (Figs. 9, 10). According to the differences of ingredient and culture conditions of bioengineered skin, we set six groups which were group one (normal condition and without microencapsulations), group two (normal condition with empty microencapsulations), group three (normal condition with microencapsulations of NIH3T3), group four (normal condition with microencapsulations of NIH3T3-NGF), group five (culture medium with NGF (5 ng/ml)), and group six (culture medium with NGF (5 ng/ml)+NGF Ab (50 ng/ml)). We also measured the levels of secreted NGF in supernatant of tissue engineered skin by ELISA every week for six weeks in-vitro, and we found that the concentration of NGF was increased in one to two weeks after being seeded in the tissue engineered skin, and then maintained a stable level about

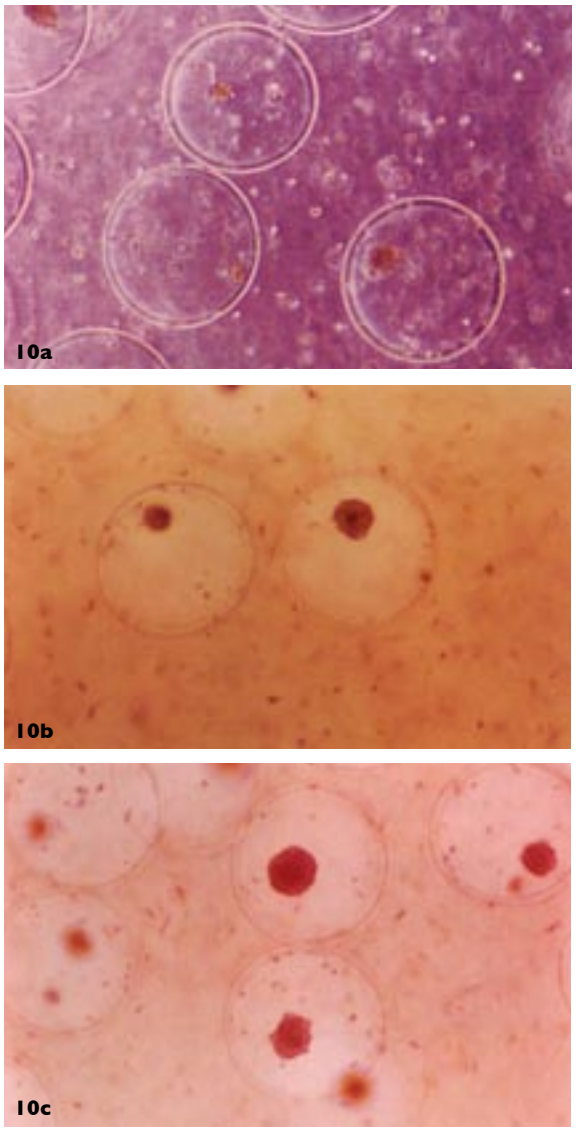


Fig. 10 Inverted phase contrast microscope images of microencapsulations seeded into the gel at (a) four hours, and cultivated after (b) three days and (c) seven days (×40). Inside the microencapsulation, cells proliferated and became cell clumping.

153.27 ± 17.61pg/ml (Fig. 11). In control unmodified groups, NGF levels were undetectable (detection limit <15 pg/ml). The concentration of Hyp in tissue engineered skin with microencapsulated NIH3T3-NGF was promoted significantly compared with negative control groups ($p<0.01$) (Fig. 12). The average thickness of epidermis and dermis was promoted significantly in microencapsulated NIH3T3-NGF cells - incorporated tissue engineered skin compared with control groups (see above) ($p<0.01$) (Figs. 13a-b). After they were incorporated with collagen gel, microencapsulated NIH3T3-NGF cells proliferated and became cells clumping. We also found that the gel can infiltrate into the APA microencapsulation sandwich membranes (Fig. 14).

DISCUSSION

Recent studies in the process of wound healing reveal that secretion of biologically-active substances like growth factors, integrins and fibronectin are inevitable for the initiation of healing process, which involves epithelialisation, contraction and connective tissue deposition[27,28]. In this process, growth factors play a very important role to promote re-epithelialisation, formation of granulation, induction of inflammation, tissue remodelling and wound contraction. It is confirmed that exogenous growth factors such as epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and NGF can accelerate the process [16,29].

Tissue engineered skin substitute can provide the indispensable ingredient for wound healing, such as fibroblasts, keratinocytes, collagen, and scaffold of cell immigration. Moreover, because of the importance of growth factors in the process of wound healing, tissue engineered skin substitute is also used as a growth factor delivery system. With the development of gene transfection techniques, genetic modification to program the cells to produce higher levels of exogenous growth factors has been associated with tissue engineering skin substitute. Many kinds of factors such as insulin-like growth factor-1 (ILGF-1), platelet-derived growth factor (PDGF), VEGF, hepatocyte growth factor (HGF), EGF, and fibroblast growth factor-7 (FGF-7) have been made[1-6,29]. The use of NGF, with all its achievements of re-epithelialisation, skin regeneration, collagen metabolism, fibroblast proliferation, activation and vascularisation hastens the process of wound healing [30,31]. Therefore, it is suggested that the use of NGF-delivery system as a wound healing material would be a promising one.

Encapsulation of living cells in a protective, biocompatible, and molecular weight cut-off polymeric membrane has been proven to be an effective method for immuno-isolation of desired cells, regardless of the type of recipient involved (allograft, xenograft)[32]. APA microcapsules have been applied for various purposes, and the molecular cut-off of APA microcapsule membrane is 75 kDa[33], so the 2.5S NGF protein (a molecular weight of 26 kDa) can pass through the membrane. In the present study, we observed that NGF protein could release freely from the microencapsulated NIH3T3-NGF cells. After the microencapsulated NIH3T3-NGF cells were incorporated into the tissue engineered skin, the cells can still secrete NGF stably and the proliferation of fibroblasts and keratinocytes were significantly enhanced (Figs. 5a-b). The increased content of Hyp (Figs. 8 & 12) indicates that NGF,

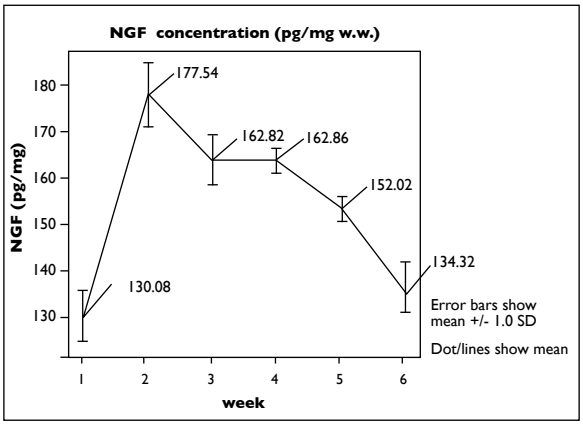


Fig. 11 Graph shows the average concentration of NGF secreted by microencapsulated NIH3T3-NGF cells incorporated tissue engineering skin cultivated in six weeks. NGF levels in culture medium were measured by a sandwich ELISA. Each point represents the mean ± SD of three separate experiments using duplicate samples.

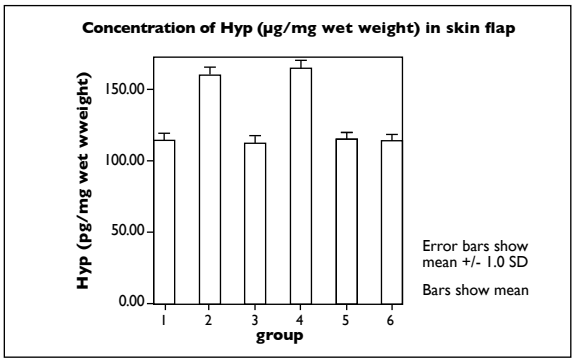
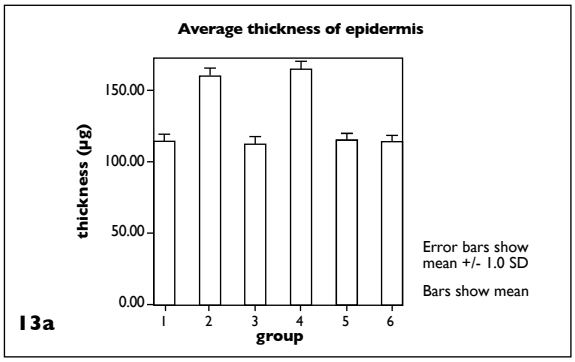


Fig. 12 Histogram shows the content of Hyp in six groups described above, each point represents the mean ± SD (μg/g wet weight) of three separate experiments using duplicate samples.



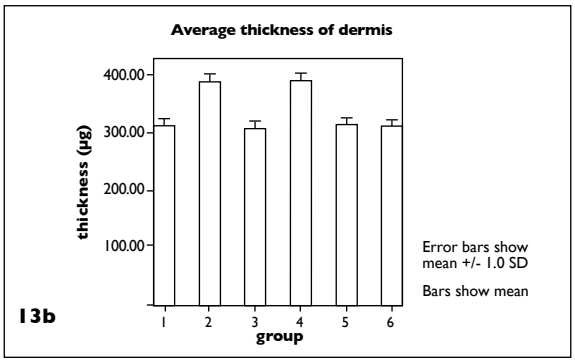


Fig. 13 Histograms show the average thickness of (a) epidermis and (b) dermis determined by HPIAS-1000, Each histogram represents mean ± SD for six groups in each individual experiment, the experiment group and positive control group were significantly different from the control group at $p<0.01$ as determined by ANOVA.

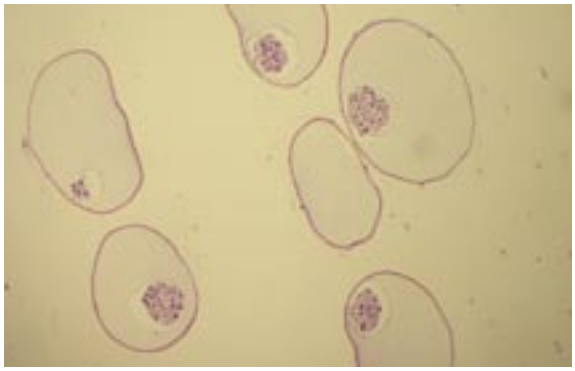


Fig. 14 Photomicrograph shows the histological features of microencapsulated NIH3T3-NGF cells incorporated tissue engineered dermis cultivated seven days in-vitro (Haematoxylin and eosin, ×40).

probably in combination with collagen, indirectly enhances the biosynthesis of collagen by directly stimulating the proliferation of the fibroblasts[34].

It is evident from histological studies that the NGF plays a vital role in regulating responses to tissue engineered skin performances. A prior study demonstrated that exogenous application of NGF (1 ng/cm^2) accelerates all the phases of wound healing, which includes re-epithelialisation, matrix formation and remodelling[25]. As evident from the present study, the thickness of epidermis and dermis in the NGF-existed groups are relatively thicker when compared to that of negative control groups (Figs. 13a-b). Similar results are obtained and thus provide the histological evidence from the present study that strongly demonstrates the bio-effects of NGF in fibroblasts and keratinocytes[35]. These studies have demonstrated the feasibility of this approach and have shown that growth factor production can influence the performance of bioengineered skin. In this study, we developed an alternative approach for local long-term delivery of NGF by a single administration of APA microcapsules containing cells secreting NGF. Using this system, the microencapsulated engineered cells could supply the appropriate doses of effective NGF protein in a paracrine fashion to induce potent wound healing and promote the performance of tissue engineered skin substitute. This system differs from other gene delivery systems which utilise engineered autologous somatic cells[29], local injection or induction of growth factor.

Considering the difficulties of prolonged culture and transduction of human autologous somatic cells for each patient, and in contrast, the ready availability of microencapsulated cells, the use of

microencapsulated engineered cells for prolonged growth factor administration is an attractive alternate method for clinical application of growth factors. With respect to the finding that local secretion of NGF at the wound site might induce the inflammation without severe immunological rejection that often limited the availability of gene modified cells, this system has potential advantages for initiating studies of the prolonged delivery effect of NGF with microencapsulated engineered cells on wound healing.

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