Effects of the Controlled-Released Basic Fibroblast Growth Factor from Chitosan−Gelatin Microspheres on Human Fibroblasts Cultured on a Chitosan−Gelatin Scaffold

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Received October 25, 2006; Revised Manuscript Received January 28, 2007

To provide for prolonged, site-specific delivery of basic fibroblast growth factor (bFGF) to the grafted skin in a convenient manner, biodegradable chitosan−gelatin microspheres containing bFGF were fabricated and incorporated into a porous chitosan−gelatin scaffold in this study. The microspheres are an integral part of the porous three-dimensional scaffolds, and their incorporation does not significantly affect the scaffold porosity and the pore size. The release kinetics of bFGF showed a fast release (23.7%) at the initial phase in the first 2 days, and the ultimate accumulated release was approximately 71.8% by day 14, indicating an extended time course for complete release. Human fibroblasts seeded on chitosan−gelatin scaffolds with and without bFGF-loaded chitosan−gelatin microspheres (bFGF-MS) were incubated in vitro for 2 weeks and showed that, compared to chitosan−gelatin scaffolds alone, the scaffolds with bFGF-MS significantly augmented the proliferation and glycosaminoglycan (GAG) synthesis of human fibroblasts. Moreover, real-time reversed transcribed polymerase chain reaction (RT−PCR) analysis for fibroblast-related extracellular matrix (ECM) gene markers demonstrated that the transcript level of laminin was markedly upregulated by about 9-fold. These results suggest that chitosan−gelatin scaffolds with bFGF-MS possess a promising potential as a tissue engineering scaffold to improve skin regeneration efficacy and to promote vascularization.

Introduction

The full thickness skin loss is one of the oldest and still not totally resolved problems in surgical field. For patients with extensive burns, wound coverage with an autologous in vitro reconstructed skin made of both dermis and epidermis should be the best alternative to split-thickness graft. Unfortunately, various obstacles have delayed the widespread use of composite skin substitutes. Slow cell ingrowth and insufficient vascularization has been proposed as the most likely reason for their unreliable survival.[1] Therefore, many studies are turning toward the tissue engineering approach, which utilizes both engineering and life science discipline to promote skin regeneration and to sustain, recover their functions.[2−4] One crucial factor in skin tissue engineering is the construction of a scaffold. A three-dimensional scaffold provides an extracellular matrix analogue which functions as a necessary template for host infiltration and a physical support to guide the differentiation and proliferation of cells into the targeted functional tissue or organ.[5,6] An ideal scaffold used for skin tissue engineering should possess the characteristics of excellent biocompatibility, suitable microstructure, controllable biodegradability, and suitable mechanical properties.

Scaffolds can be fabricated using a variety of materials including synthetic and natural polymers. Among these materials, chitosan (poly-1, 4-D-glucosamine), a partially deacetylated derivative from chitin, is chemically similar to glycosaminoglycan (GAG) and has many desirable properties as tissue engineering scaffolds.[7,8] Gelatin is the partial derivative of collagen and has been processed into composites by blending with other materials for promoting cell adhesion, migration, differentiation, and proliferation.[9,10] With a similar chemical structure to GAGs and collagen, the polyelectrolyte complex derived from chitosan and gelatin has been developed and used as the supporting scaffolds, mimicking the natural extracellular matrix (ECM), for tissue engineering skin with promising results.[11,12] Nevertheless, many studies have indicated that a porous scaffold in itself is insufficient to induce vascularization and rapid skin regeneration at the initial stages of wound healing.[13−15] On the other hand, a combination of the use of growth factors and a porous scaffold might substantially improve skin regeneration efficacy.

It has been reported that various growth factors function in the process of wound healing. Specifically, basic fibroblast growth factor (bFGF) is a multifunctional protein that promotes angiogenesis and regulates many aspects of cellular activity, including cell proliferation, migration, and ECM metabolism, in a time- and concentration-dependent manner.[16−19] bFGF had been shown to enhance the proliferation of the periodontal ligament cells in a dose-dependent manner, and the response reached plateau at the concentration of 10 ng/mL. In addition, the proliferative response was decreased when the concentration

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10.1021/bm061025e CCC: $37.00
Published on Web 03/22/2007

Table 1. Real-Time RT−PCR Primer Sequences

genes	forward primer sequences	reverse primer sequences
collagen I	5′-CAGCCGCTTCACCTACA GC-3′	5′-TTTTGTATTCAATCACTG TCTTGCC-3′
collagen III	5′-ACACGTTTGGTTTGGAG AGTCC-3′	5′-CTGCACATCAAGGACAT CTTCAG-3′
fibronectin	5′-CAATCCAGAGGAACAA GCATGTCTC-3′	5′-GCTTTCCTATTGATCCCA AACCAAA-3′
laminin	5′-GTGCTTGGCTGGTTACT ATGGCGAC-3′	5′-CAATGTATCCAGGATCA CAAACACA-3′
GAPDH	5′-ATGGGGAAGGTGAAGG TCG-3′	5′-TAAAAGCAGCCCTGGTG ACC-3′

of bFGF was over 100 ng/mL.[20] It is known that the half-life of bFGF is very short. Moreover, bFGF added directly to the cell culture media or injected in vivo may be inhibited by binding proteins or the ECM before reaching the desired target cells.[21] The most important concern regarding the delivery of bFGF is whether or not the released protein actually retains its biological activity. Therefore, the best way to enhance the efficacy of bFGF is to achieve sustained release and to maintain suitable concentration over an extended time period.

In the present study, chitosan−gelatin microspheres loaded with bFGF were fabricated and incorporated into a porous three-dimensional chitosan−gelatin scaffold for skin tissue engineering. Human fibroblasts were cultured on the scaffolds with and without bFGF-loaded chitosan−gelatin microspheres (bFGF-MS) for 2 weeks. The objective of this study was to investigate the effect of the controlled-released bFGF from chitosan−gelatin microspheres on human fibroblasts cultured on chitosan−gelatin scaffolds. Cell morphology, cell proliferation, GAG synthesis, and gene expression were examined in vitro and were compared to those on chitosan−gelatin scaffolds alone.

Materials and Methods

Materials. Human recombinant bFGF, 1-ethyl-(3,3-dimethylaminopropyl) carbodiiminde (EDC), *N*-hydroxysuccinimide (NHS), Dulbecco's phosphate-buffered saline (DPBS), bovine serum albumin (BSA), 5,5′-tetramethylbenzidine (TMB), gelatin, and glutaraldehyde were all purchased from Sigma Chemical Co. (St. Louis, MO). Chitosan, which was produced from snow crab shell, was purchased from Qingdao Haihui Biology Engineering, China (degree of deacetylation > 85%; Mw 2×10^5). 2-Morpholinoethane sulfonic acid (MES) was purchased from Shanghai Shenggong Biology Engineering, China. Other chemicals were purchased from Shanghai Chemical Co., China.

Microsphere Preparation. Two-and-a-half milliliters of 10 wt % gelatin aqueous solution preheated at 40 °C was mixed with 2 mL of 4 wt % chitosan acetic solution under magnetic stirring at 450 rpm to prepare a homogeneous aqueous solution. While the stirring was continued, the mixed aqueous solution was added dropwise into 350 mL olive oil for 10 min at 40 °C to prepare a water-in-oil emulsion. The emulsion was cooled to 20 °C, and stirring was continued for 30 min to complete the gelation of gelatin and chitosan in the water phase. After addition of 100 mL acetone, the emulsion was further stirred at 300 rpm for 1 h. The resulting microspheres were washed five times with acetone and twice with isopropyl alcohol, were collected by centrifugation (5000 rpm, for 5 min, at 4 °C), and finally were air-dried at 4 °C to obtain non-cross-linked chitosan−gelatin microspheres. The non-cross-linked microspheres prepared were placed in a 2 wt % glutaraldehyde aqueous solution containing 0.1 wt % Tween 80, and the cross-linking was allowed to proceed at 4 °C for 12 h. The microspheres were placed in 100 mL of 10 mM glycine aqueous solution containing 0.1 wt % Tween 80 at 37 °C for 30 min to block unreacted residual glutaraldehyde. The resulting microspheres were washed twice with 0.1 wt % Tween 80-solution, were centrifuged at 4000 rpm for 5 min at 4 °C, and were freeze-dried for 3 days at room temperature.

Microsphere Loading and in Vitro bFGF Release Study. The concentration of bFGF solution was quantitatively measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Briefly, 100 μL/well of 2 μg/mL anti-bFGF as capture antibody was immobilized on a 96-well high-binding polystyrene plate (NUNC, Denmark) by incubation at room temperature overnight. The plate wells were then blocked with 200 μL/well DPBS, including 1% BSA, 5% sucrose, and 0.05% NaN_3 for 1 h. One hundred microliters per well of bFGF solutions was added to the plate, and the plate was incubated for 1 h, also at room temperature. At the same time, a dilution series of known concentrations of human bFGF were prepared on each plate as standards. One hundred microliters per well of biotin-anti-bFGF (0.5 μg/mL) as detection antibody was then added, and the plate was incubated for another hour. The plate wells were then emptied and washed three times with 200 μL/well wash buffer (DPBS with 0.05% Tween-20, pH 7.4), followed by addition of 100 μL/well of a 1:1000 dilution of streptavidin-horseradish peroxidase. After 1 h incubation at room temperature, each well was loaded with 100 μL of TMB, and a blue color gradually appeared. The plate wells were then washed again, after which the color-forming reaction was stopped by addition of 100 μL/well 1 M sulfuric acid. Finally, the plate was read at 450 nm to determine the bFGF level.

The freeze-dried chitosan−gelatin microspheres were loaded with bFGF using a method described in the literature.[22] Briefly, a solution of human recombinant bFGF in phosphate-buffered saline (PBS) was sterilized using a 0.22-μm filter (Millipore, Bedford, MA). After the bFGF concentration was determined using ELISA, the solution was diluted to 5 μg bFGF/mL with PBS. Subsequently, the aforementioned freeze-dried microspheres were rehydrated in the prepared bFGF solution at 4 °C overnight. Finally, the bFGF-loaded microspheres were washed in 5 mL PBS (twice for 20 min) to remove all unbound bFGF. The morphology of the bFGF-MS was examined under scanning electron microscopy (SEM; Hitachi S-520, Japan), and the size distribution was determined by the laser light scattering technique (AccuSizer 780 model, Particle Sizing Systems Inc., Santa Barbara, CA). To measure the loading ratio, 1 mg of bFGF-MS was dispersed in 10 mL of distilled water and then was ultracentrifuged (4 °C, 12 000 rpm) for 30 min. bFGF in the suspension was quantitatively measured using ELISA. The loading ratio of bFGF was calculated by the difference between the amount of bFGF loaded in microspheres and the weight of microspheres. To measure the release kinetics of bFGF from chitosan−gelatin microspheres, 10 mg of bFGF-MS was placed in 5 mL of PBS (pH 7.4) and was incubated in a shaking bath (135 rpm) at 37 °C for various time periods, up to 14 days. Periodically, the microsphere suspension was centrifuged and the supernatant was collected for bFGF analysis. The pellets obtained were resuspended in fresh PBS. The amount of bFGF in releasing media was determined using ELISA.

Incorporation of bFGF-MS into Chitosan−Gelatin Scaffolds. Two percent chitosan acetic solution and 1% gelatin aqueous solution were prepared. Each solution was mixed to be a weight ratio (*r*) of 3:7 (chitosan to gelatin) and was stirred with a magnetic bar at room temperature for 60 min. The mixed solutions were poured into polystyrene Petri dishes, were quickly frozen at −40 °C overnight, and were lyophilized at −20 °C for 36 h. The sponges obtained were immersed in 20 mL of 90% (v/v) ethanol containing 50 mM MES at room temperature for 0.5 h. Subsequently, the scaffolds were cross-linked by immersion in 20 mL of 90% (v/v) ethanol containing 50 mM MES, 30 mM EDC, and 8 mM NHS. After reaction at room

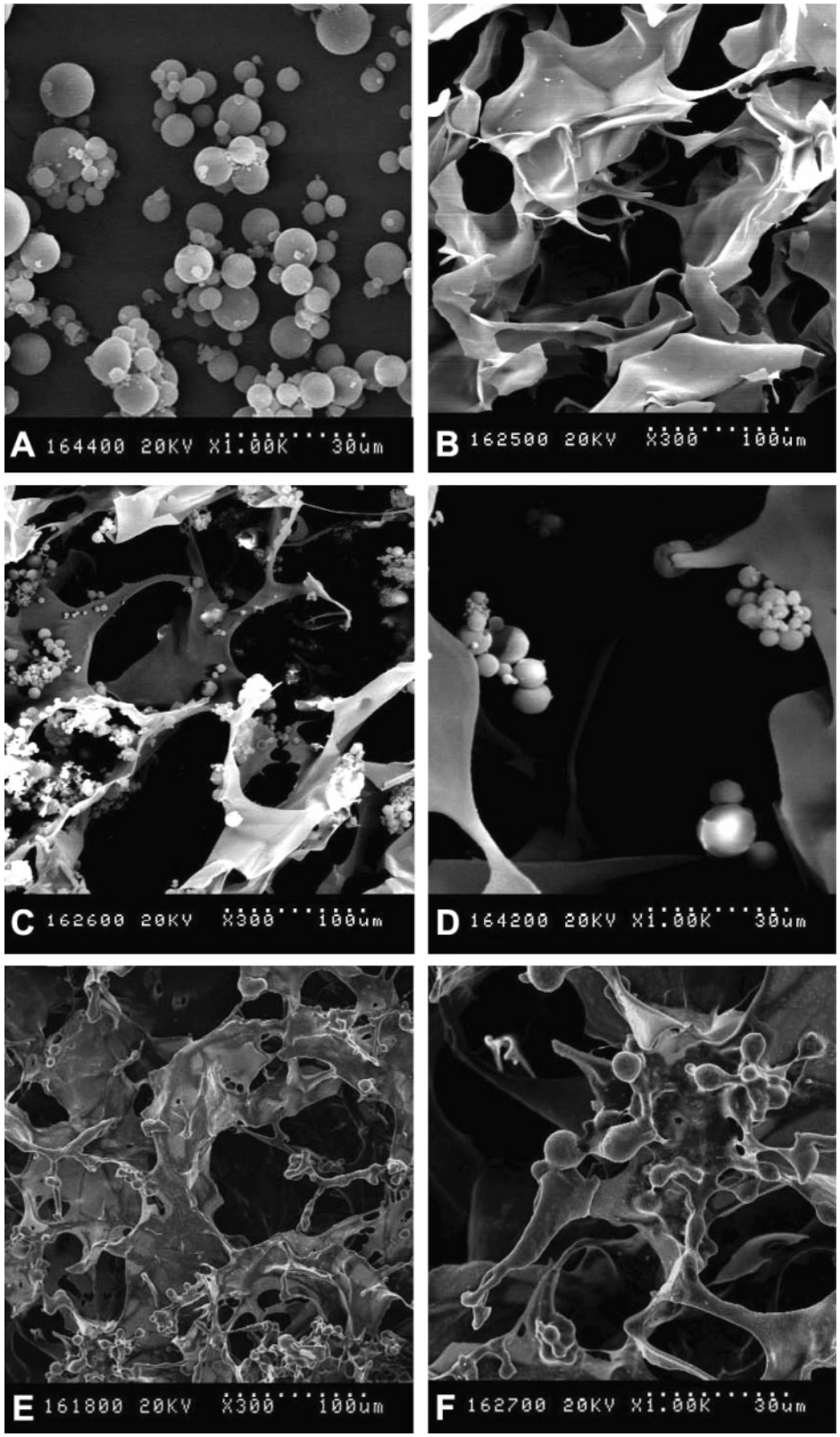

Figure 1. Morphological observations by SEM. (A) bFGF-MS (×1000), (B) chitosan−gelatin scaffold (×300), (C) bFGF-MS-incorporated chitosan−gelatin scaffold immediately after preparation (×300), and (D) its enlarged view (×1000), (E) bFGF-MS-incorporated chitosan−gelatin scaffold after incubation in a PBS solution at 37 °C for 2 weeks (×300), and (F) its enlarged view (×1000).

temperature for 2 h, the scaffolds were washed with double-distilled water until the matrix pH value returned to the physiologic range (7.0−7.4) and then were freeze-dried in the same conditions. The scaffolds were then prepared in cylinders (0.5 cm in thickness and 1 cm in diameter) for in vitro study. bFGF-MS (∼0.50 mg) dispersed in 90% ethanol (100 μL) were injected into several portions of the inner of each scaffold and were immediately lyophilized,[23,24] in which the amount of bFGF loaded into each scaffold was 10 ng. To observe the morphology of the scaffolds, the specimens were coated with gold-palladium and were examined using SEM (Hitachi S-520, Japan). To

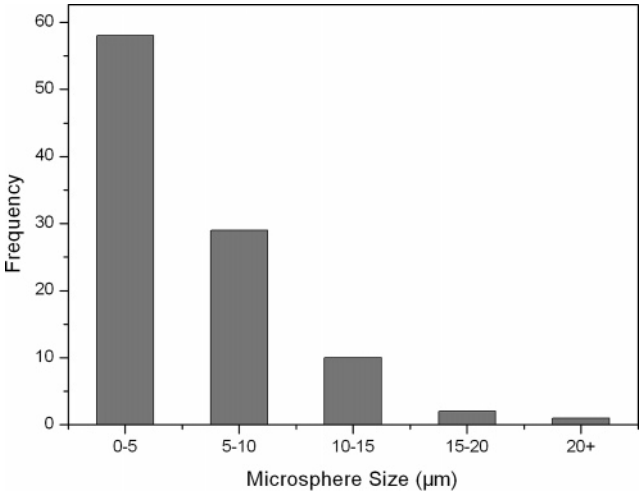

Figure 2. Size distribution of bFGF-MS.

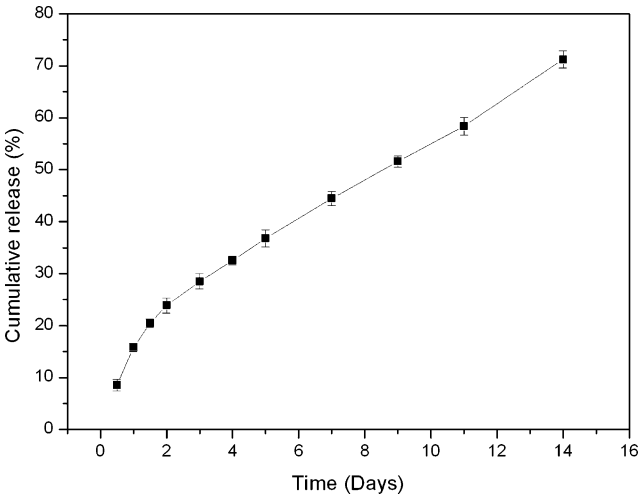

Figure 3. Cumulative bFGF release from chitosan−gelatin microspheres. Individual points represent the mean values ± standard deviation from five replicate microsphere samples.

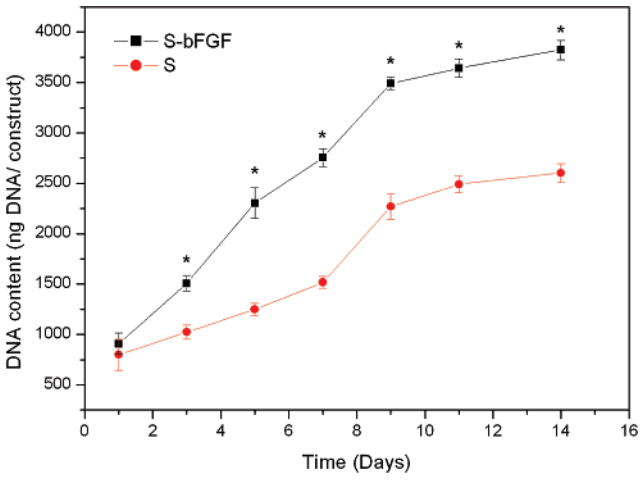

Figure 4. DNA content of human fibroblasts cultured on the scaffolds with and without bFGF-MS (S-bFGF, S). Results are expressed as ng DNA per cell−scaffold construct. Data are shown as means ± SD ($n = 5$). *$p < 0.05$ vs control without bFGF-MS at each time point.

observe the degradation of the scaffolds, the specimens were immersed in a PBS solution at 37 °C for 2 weeks and then were examined by SEM.

Cells and Cell Culture. Primary cultures were grown by the method of Rheinwald and Green.[25] Fibroblasts were isolated from human

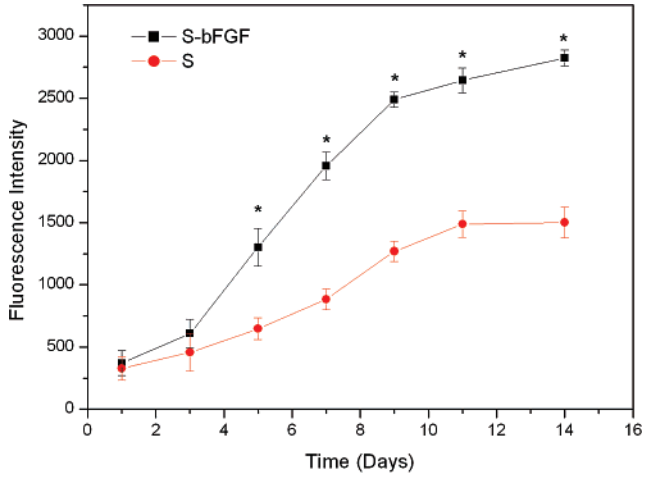

Figure 5. Cell viability of human fibroblasts cultured on the scaffolds with and without bFGF-MS (S-bFGF, S). Data are shown as means ± SD ($n = 5$). *$p < 0.05$ vs control without bFGF-MS at each time point.

foreskins by sequential trypsin and collagenase digestion. About 3×10^6 cells were cultured in 75-cm^2 tissue culture plates (Falcon) in Dulbecco's Modified Eagle Medium (DMEM; Gibco) with 10% fetal calf serum containing L-glutamine and penicillin-streptomycin. They were incubated at 37 °C in air containing 5% CO_2. The culture medium was changed every 3 days. At confluence, fibroblasts were harvested and subcultivated in the same medium.

Cell Culture on Scaffolds. Fibroblasts were seeded in the scaffolds at a cell density of 2×10^5 cells/scaffold using a syringe under mild negative pressure and then were incubated at 37 °C in 5% CO_2 for 3 h. Culture medium (1.0 mL) was then added to each well, and incubation was continued for 24 h. To remove the cells present at the bottom of the wells, the scaffolds were placed in new culture plates. The culture medium was changed two times a week. After 2 weeks, the cell−scaffold constructs were fixed with 10% formalin and were stained with hematoxylin and eosin (HE) for histological observation. To identify ECM proteins produced by fibroblasts, immunohistochemical staining was performed on the paraffin sections with a labeled streptavidin-biotin immunoenzymatic antigen detection system (DAKO LSAB2 System, Dako Co., Carpinteria, CA). The paraffin sections were digested enzymatically with pepsin (1 mg/mL in 0.01 N of HCl) for 30 min at 37°C and then were incubated with a mouse antihuman type I collagen monoclonal antibody or a mouse antihuman type III collagen monoclonal antibody (both from Chemicon, Temecula, CA) before the horseradish peroxidase (HRP) labeled secondary antibody. Sections were ounterstained with hematoxylin.

To observe the morphologies of fibroblasts cultured on the scaffolds, the cell−scaffold constructs were fixed with 2.5% glutaraldehyde in PBS at 4 °C. After thorough washing with PBS, the samples were dehydrated through a series of graded alcohols and then were critical point dried. Finally, the samples were gold-sputtered under vacuum and were observed by SEM (Hitachi S-450, Japan).

Cell Labeling and Detection. Fluorescein diacetate (FDA) molecule probe is well recognized as a viable cell marker.[26] Therefore, in this study, this technique was used to investigate the growth of human fibroblasts on the scaffolds. On the seventh day, the cell−scaffold construct was incubated with 15 μg/mL FDA solution for 15 min and then was washed with PBS. The positive cells were observed under laser confocal microscopy (Leica TCS SP2, Germany) immediately.

Cell Proliferation Assay. The proliferation of fibroblasts seeded on the scaffolds was determined by DNA assay using a fluorimetric dsDNA quantification kit (PicoGreen, Molecular Probes). Briefly, samples collected on days 1, 3, 5, 7, 9, 11, and 14 were washed twice with a sterile PBS solution and were transferred into 1.5-mL microtubes containing 1 mL of ultrapure water. Constructs were incubated for 1 h at 37 °C in a water bath and then were stored in a −80 °C freezer until

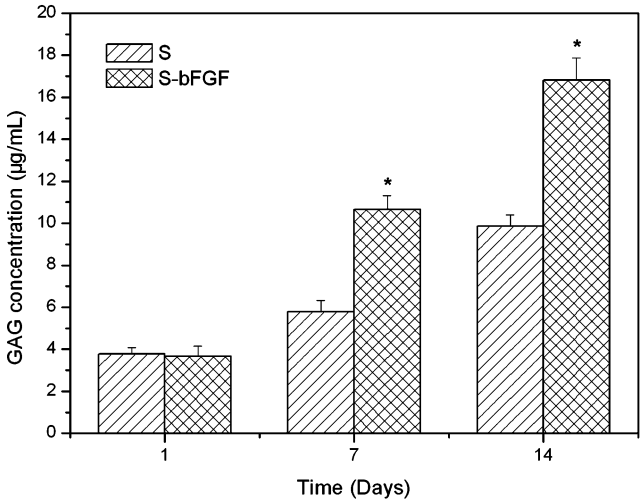

Figure 6. The total GAG synthesized by human fibroblasts cultured on the scaffolds with and without bFGF-MS (S-bFGF, S). Data are shown as means ± SD ($n = 5$). *$p < 0.05$ vs control without bFGF-MS at each time point.

testing. Prior to DNA quantification, constructs were thawed and sonicated for 15 min. Samples and standards (ranging between 0 and 2 μg/mL) were prepared per each well of an opaque 96-well plate. To each well were added 28.7 μL of sample or standard plus 71.3 μL of PicoGreen solution and 100 μL of Tris−EDTA buffer. Triplicates were made for each sample or standard. The plate was incubated for 10 min in the dark and fluorescence was measured on a microplate reader (Fluostar Optima, bMG Labtechnologies) using an excitation wavelength of 490 nm and an emission of 520 nm. A standard curve was created and sample DNA values were read off from the standard graph.

Cell Viability Assay. Cell viability was assessed by alamar blue assay following the vendor's instructions. Alamar blue is a nontoxic aqueous fluorescent dye that does not affect phenotype, viability, and proliferation of the cells.[27] Briefly, scaffolds seeded with cells were incubated in medium supplemented with 10% (v/v) alamar blue fluorescent dye (Biosource, United States) for 2 h. One hundred microliters of medium from each sample was read at 560/590 nm in a fluorescence microplate reader (Fluostar Optima, bMG Labtechnologies). Medium supplemented with 10% alamar blue dye was used as a negative control and its fluorescent intensity was subtracted from the experimental groups containing cells.

Glycosaminoglycan (GAG) Assay. After fibroblasts were cultured on the scaffolds for 1, 7, and 14 days, respectively, the total sulfated GAG contents were determined quantitatively using a 1,9-dimethylmethylene blue (DMB, Sigma, United States) method[28] with shark chondroitin sulfate C (Sigma, United States) as a standard. Briefly, the samples were digested with papain (Sigma, United States) in buffer containing 0.1 M NaH_2PO_4, 5 mM Na_2EDTA, and 5 mM cysteine·HCl at pH 6.0, which was continued for 6 h at 60 °C before adding the dye. The dye solution was prepared by dissolving 16 mg of DMB in 1 L of distilled water containing 3.04 g glycine, 2.37 g NaCl, and 95 mL 0.1 M HCl. A 25-μL aliquot of the papain digest of each sample was added to 96-well plates, and then 250 μL of DMB solution was added. The absorbance was measured at 525 nm on a microplate reader.

Real-Time RT−PCR Analysis. Human fibroblasts were cultured on the scaffolds for 1 week, were harvested by trypsinization, and were washed twice by PBS. Total RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, United States) following the supplier's instructions. Briefly, the scaffolds seeded with cells were washed with PBS, were cut into small pieces, and were disrupted and lysed with supplied buffer (Qiagen, United States). A QIAshredder spin column was used to homogenize the lysate, and ethanol was added before transfer to an RNeasy spin column. The final elute was stored at −80 °C. The RNA samples were reverse transcribed into cDNA according to the manufacturer's protocol (iScript cDNA synthesis Kit, BioRad). Real-time PCR was performed in a real-time PCR machine (STRATAGENE Mx3000P QPCR System) using the QuantiTect RT-PCR Kit (Qiagen, United States). The primer sequences of selected genes for real-time PCR were obtained from published literatures[29−31] and are summarized in Table 1. At least three replicates were performed on each sample, and each experimental gene was tested by three PCR runs. In each run of PCR, human housekeeping gene GAPDH was used as the reference transcript. All cDNA samples were analyzed for the transcript of interest and the housekeeping gene in independent reactions. Data were analyzed by MxPro QPCR Software version 3.00 supplied by the vendor (STRATAGENE). The Ct value for each sample was defined as the cycle number at which the fluorescence intensity reached a certain threshold where amplification of each target gene was within the linear region of the reaction amplification curves. Relative expression level for each gene of interest was normalized by the Ct value of human housekeeping gene GAPDH using an identical procedure ($2^{\Delta Ct}$ formula, Perkin-Elmer User Bulletin #2). Each sample was analyzed in triplicate.

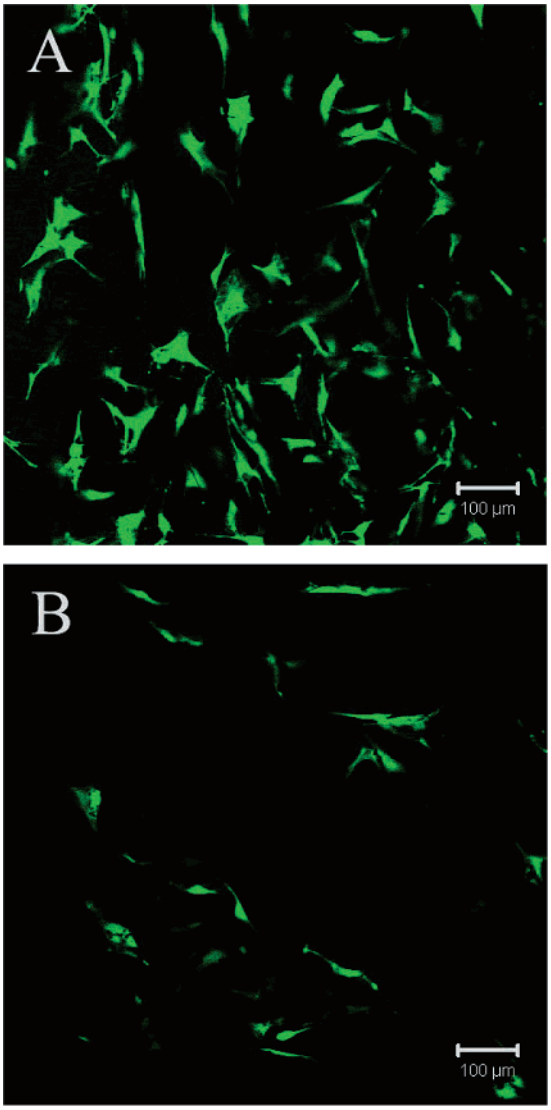

Figure 7. Laser confocal microscope photographs of human fibroblasts cultured on the scaffolds with (A) and without (B) bFGF-MS for 1 week.

Statistical Analysis. All data were expressed as means ± standard deviation (SD) for $n = 4$. Single-factor analysis of variance (ANOVA) technique was used to assess the statistical significance of results between groups. The statistical analysis was performed with the software OriginPro (version 6.1) at a confidence level of 95%. A value of $p < 0.05$ was accepted as statistically significant.

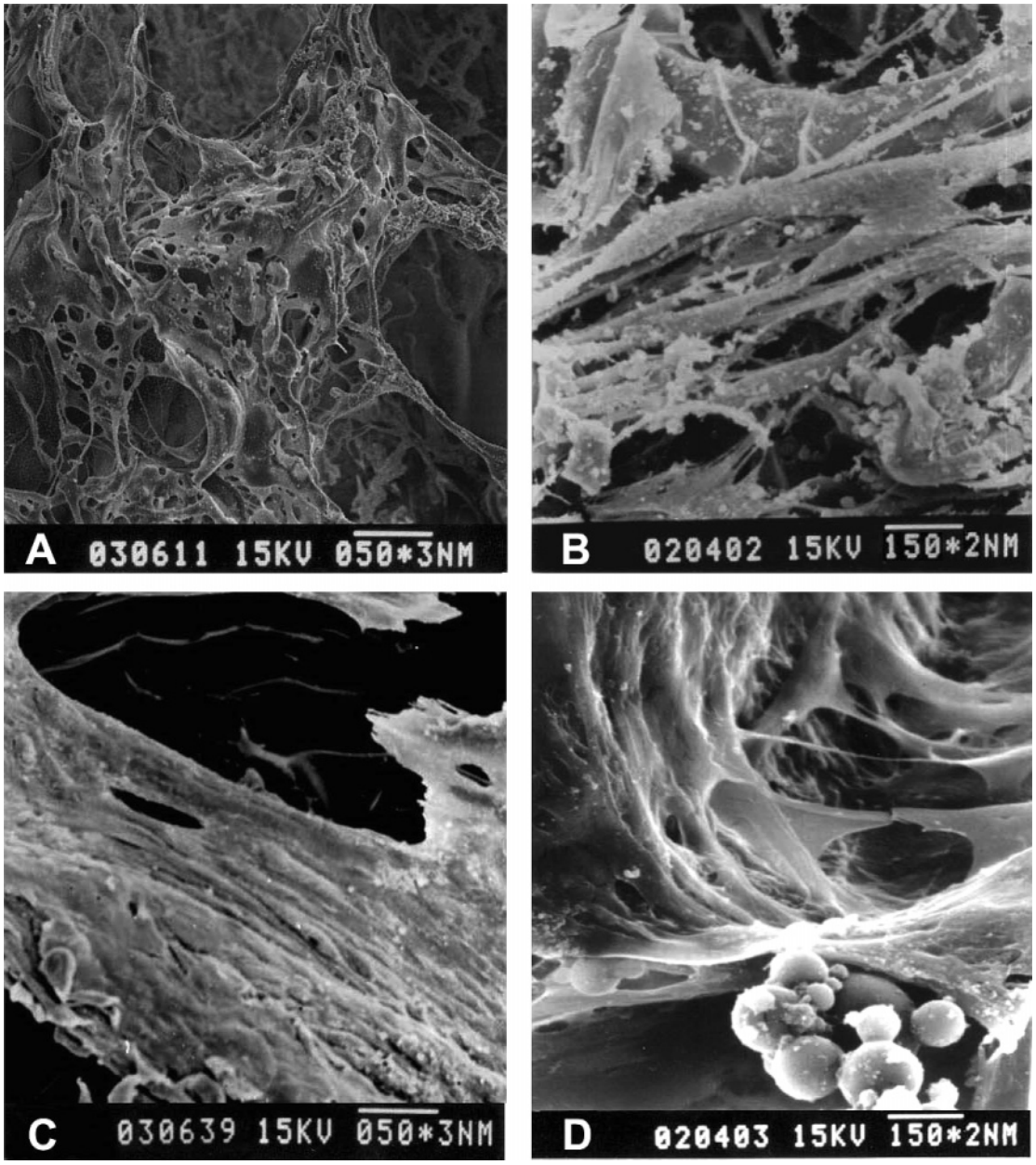

Figure 8. SEM images of human fibroblasts cultured on the scaffolds with (C and D) and without (A and B) bFGF-MS for 1 week. (A, C) Original magnification × 300; (B, D) original magnification × 1000.

Results and Discussion

Characterization of Microspheres and Scaffolds. Chitosan is a natural polysaccharide comprising copolymers of glucosamine and *N*-acetyl-glucosamine. Its cationic nature and high charge density allow chitosan to form insoluble polyelectrolyte complexes with various anionic substances in an acidic environment and to dissociate a portion of the complexed ones in a physiological solution (pH 7.4). This characteristic may be beneficial to retain or accumulate biologically active molecules for their sustained release from chitosan-based matrix. It is well-known that gelatin is the partial derivative of collagen and is composed of a unique sequence of amino acids. Structurally, gelatin molecules contain repeating sequences of glycine−X−Y triplets, where X and Y frequently are praline and hydroxyproline.[32] In addition, the isoelectric point (IEP) of acidic gelatin is 5.0. Therefore, in this study, we chose chitosan and gelatin to fabricate microspheres by a water-in-oil emulsion method and being cross-linked with glutaraldehyde. Figure 1A shows the morphology of bFGF-MS. The microspheres were spherical and had smooth surfaces without cracks or wrinkles. Particle size distributions ranged between 2.55 and 20.62 μm, and the mean diameter was 4.60 ± 2.28 μm (Figure 2). The loading ratio of bFGF-MS was 20 ng/mg. The release kinetics of bFGF from chitosan−gelatin microspheres was monitored for 14 days (Figure 3). Growth factor was released in a biphasic fashion, characterized by a fast release phase at initial 2 days followed by a slower one for the remaining days. The amount of bFGF released at the initial phase was 23.7%. After 2 days, the cumulative mass of bFGF released reached a plateau. Although the initial release burst appeared in the first 2 days, the percentage of cumulative release was approximately 71.8% by day 14, indicating an extended time course for complete release.

Transport issues such as nutrient delivery, waste removal, protein transport, gaseous exchange, general vascularization, and guided tissue regeneration are governed by the pore structure of the scaffold.[33] Pore sizes recommended for skin tissue

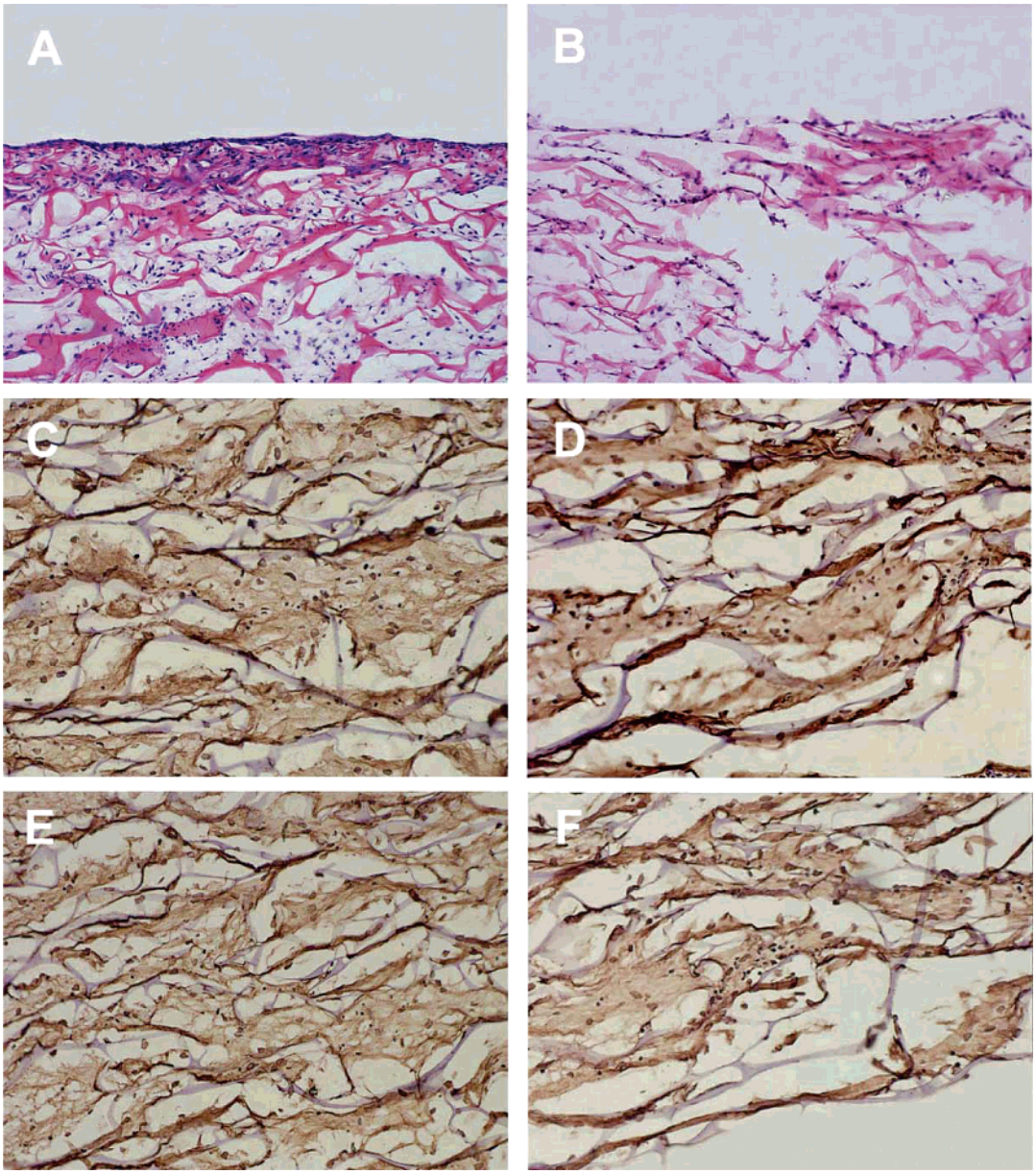

Figure 9. Histological (A, B) and immunohistochemical (C−F) observations of human fibroblasts cultured on the scaffolds with (A, C, E) and without (B, D, F) bFGF-MS for 2 weeks. Images A and B were for hematoxylin and eosin staining (×100); images C and D were for type I collagen staining (×200); images E and F were for type III collagen staining (×200).

engineering scaffold ranges are 100−200 μm with a desired porosity of 90% to provide sufficient space and surface for cell seeding into temporary scaffold prior to implantation.[34,35] In the present study, chitosan−gelatin three-dimensional scaffolds were fabricated by freeze-drying method using water as a porogen. The cross-sectional morphology of chitosan−gelatin scaffolds (Figure 1B) revealed that the scaffolds were interconnected with well-controlled pore size varying from 95 to 160 μm. As shown in Figure 1C and D, bFGF-MS were well distributed on the inner surface of chitosan−gelatin scaffolds. The microspheres were an integral part of the porous three-dimensional scaffolds, and their incorporation did not significantly affect the scaffold porosity and the pore size. After 2 weeks of incubation, the microspheres were approximately spherical, whereas they appeared highly eroded (Figure 1E and F).

Biochemical Analysis. bFGF in culture is a mitogen for several cell types such as fibroblasts, endothelial cells, and smooth muscle cells. It belongs to the heparin-binding fibroblast growth factor family and stimulates wound healing and tissue repair by promoting cell proliferation, angiogenesis, and synthesis of noncollagenous protein via heparin and heparin-like binding receptors.[36] In our study, human fibroblasts were seeded on the scaffolds with and without bFGF-MS and then their growth, proliferation, and cellular function were compared in vitro.

The DNA content of human fibroblasts seeded on the scaffolds with and without bFGF-MS increased with culture time. After the first day, cell proliferation on the scaffolds with bFGF-MS was significantly faster than that on the scaffolds alone under the same cultivation conditions ($p < 0.05$), resulting in a 1.47 times higher final DNA content over a 14-day period (Figure 4). The cell viability of human fibroblasts cultured on the scaffolds with bFGF-MS was consistently higher than that of the scaffolds alone as indicated by alamar blue assay (Figure 5). Compared to chitosan−gelatin scaffolds alone, no statistical difference in viability was detected on the scaffolds with bFGF-

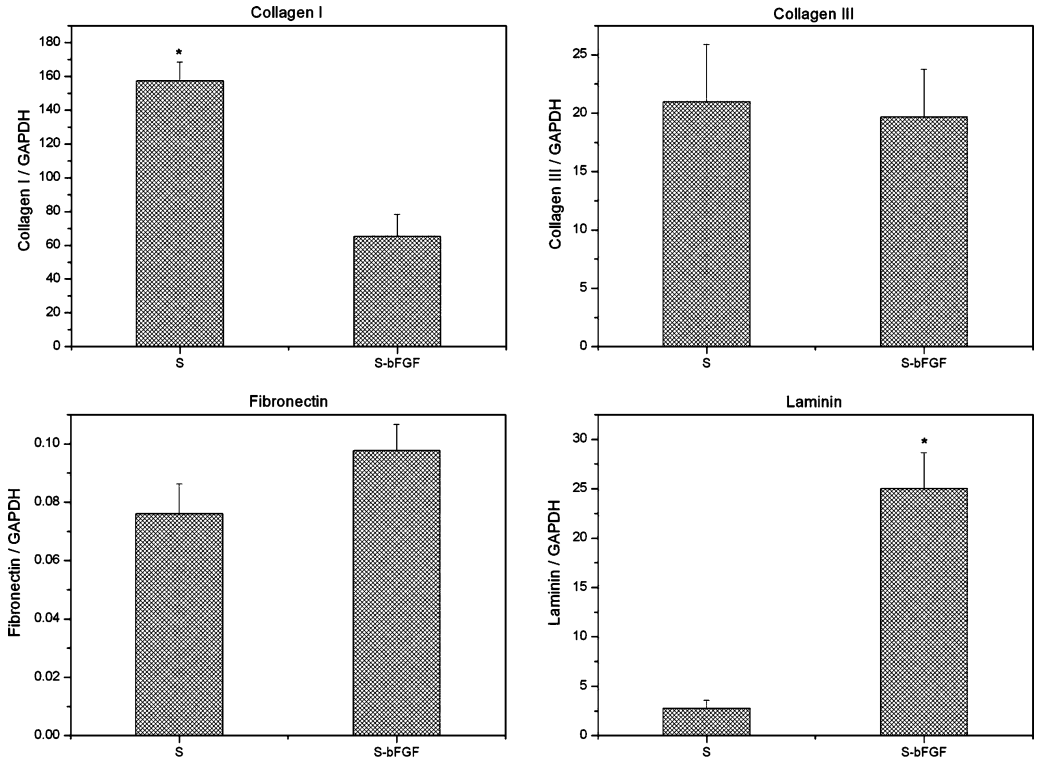

Figure 10. Expression of fibroblast-related ECM genes by human fibroblasts cultured on the scaffolds with and without bFGF-MS (S-bFGF, S) for 1 week. All results were normalized to GAPDH within the linear range of amplification (*Y* axis). Data are shown as means ± SD ($n = 3$). * Significant difference between two groups at $p < 0.05$.

MS on day 1 and 3 ($p > 0.05$), while after 5 days, the difference increased greatly ($p < 0.05$). Quantitation of the GAG synthesized by human fibroblasts cultured on different scaffolds was determined to evaluate the cellular function. As shown in Figure 6, there was also a significant increase in GAG production on day 7 and 14 on the scaffolds with bFGF-MS ($p < 0.05$). In addition, after normalization with respect to the total DNA content, the synthesized GAG revealed the same trend as that of non-normalized ones.

The biological activity of bFGF during the procedure of microsphere preparation is very important. It is possible that the biological activity of bFGF is attenuated since most proteins are known to be denatured when exposed to harsh environmental conditions such as heating or exposure to sonication and organic solvents.[37] Although the biological activity of bFGF loaded into the chitosan−gelatin microspheres was not quantitatively confirmed in this study, the results from in vitro tests demonstrated that its biological activity was preserved during microsphere preparation and still acted on the cells after 2 weeks. Compared to chitosan−gelatin scaffolds alone, the scaffolds with bFGF-MS significantly promoted the cell proliferation, viability, and production of GAG, indicating the role of bFGF released from chitosan−gelatin microspheres.

Cell Morphology on Scaffolds. The positive effect of bFGF-MS on human fibroblast proliferation was also confirmed by the laser confocal microscope, SEM, histological, and immunohistochemical images (Figures 7−9). Cell−scaffold constructs based on the scaffolds with bFGF-MS maintained their initial dimensions without significant contraction or collapse. After 1 week in culture, laser confocal microscopy after fluorescent staining with FDA showed a more profuse cell proliferation on the scaffolds with bFGF-MS (Figure 7). SEM observations indicated that human fibroblasts attached and spread well on the scaffolds with bFGF-MS. After 1 week, the cells formed large-cell aggregates and bridged across the scaffold (Figure 8C). Magnified SEM image (Figure 8D) showed that the cells adhered and grew on the inner surface of the pores in the scaffold and bFGF-MS were still embedded in the scaffold. By week 2, histological images showed that the cells occupied the space in the highly interconnective pores in the scaffolds in the presence of bFGF-MS but left large vacancies thus lowering the cell density in the absence of bFGF-MS. The high cell density in cell−scaffold constructs with bFGF-MS helped the scaffolds maintain structural integrity during histological processing, indirect evidence for improved mechanical properties in contrast to the scaffolds without bFGF-MS (Figure 9A and B). By the end of 2 weeks, the scaffolds were still structurally intact from gross observation. Immunohistochemical staining of type I and type III collagen revealed that significant new ECM production occurred in the highly interconnected porous space (Figure 9C−F). The structural stability of the scaffolds with bFGF-MS helped the cells achieve a relatively homogeneous distribution over time, which in turn resulted in a relatively homogeneous ECM accumulation. This is necessary for achieving stable mechanical properties and could eventually be beneficial for tissue integration after in-vivo implantation.

Real-Time RT−PCR Analysis. Comparisons of mRNA transcript levels of fibroblast-related ECM genes were made between human fibroblasts cultured on the scaffolds with and without bFGF-MS. mRNA transcript expressions of type I and III collagen, laminin, and fibronectin were evaluated by real-time RT−PCR after 1 week in culture (Figure 10). In comparison to the scaffolds without bFGF-MS, the transcript level of type I collagen was significantly downregulated ($p < 0.05$). In contrast, the transcript level of laminin was markedly upregulated by about 9-fold ($p < 0.05$). On the other hand, the transcript levels of type III collagen and fibronectin were almost

unaffected by the presence or absence of bFGF-MS, and no significant differences were found between the two scaffolds ($p > 0.05$).

In this study, we revealed that bFGF downregulated the gene expression of type I collagen which is one of the most common ECM proteins produced by fibroblasts. Our finding of inhibition of type I collagen gene expression is similar to those of previous studies of bFGF-treated fibroblasts, smooth muscle cells, and osteoblastic cells.[38−40] Yayon et al. suggested that cell surface GAGs such as heparin sulfate proteoglycan can bind bFGF to present the growth factor to the high-affinity FGF receptors on the cells.[41] bFGF mediates alterations of collagen metabolism in cells by stimulating the expression of collagenase, and the increased activity of the enzyme is related to an elevation in collagenase mRNA level.[38]

Angiogenesis is critical for wound healing. Newly formed blood vessels participate in provisional granulation tissue formation and provide nutrition and oxygen to growing tissues. In previous study, although capillary ingrowth is often noted in the implanted cell−scaffold constructs over time, the vascularization is either too slow or limited to provide sufficient nutrient transport for most of the transplanted cells.[42,43] In addition, skin substitution in patients showed that slow ingrowth of vascular structures into a dermal component often resulted in a second operation step in which the epidermal equivalents were transplanted to achieve wound closure.[44] Thus, stimulation of blood vessel ingrowth into the implant would ensure tissue survival and function. In the present study, the gene expression of laminin was significantly upregulated by bFGF. Laminin is one of the most important biological noncollagenous glycoproteins which participate in angiogenesis.[45] Laminin is highly expressed in blood vessels around skin wounds. It promotes dermal endothelial cell attachment, migration, and tubule formation.[46,47] Furthermore, some in-vivo studies have demonstrated that many capillaries were newly formed around the area where bFGF had been applied at the early phase of the wound-healing process.[13,48,49] The result suggests that the increased production of laminin by human fibroblasts which is a reaction for the stimulation of controlled-released bFGF from chitosan−gelatin microspheres may be helpful to angiogenesis in skin rapid regeneration and may solve the problems we faced in clinic.

Conclusion

In this study, to achieve a sustained release of bFGF, chitosan−gelatin microspheres loaded with bFGF were prepared by a water-in-oil emulsion method and were incorporated into a porous chitosan−gelatin scaffold. The scaffold offering the controlled release of bFGF was seeded with human fibroblasts and was developed as a skin tissue engineering scaffold. Compared to chitosan−gelatin scaffolds alone, the scaffolds with bFGF-MS significantly augmented the cell proliferation, GAG synthesis, and mRNA transcript level of laminin, possessing a promising potential to promote rapid skin regeneration and vascularization.

Acknowledgment. This study was supported by the National Basic Science Research and Development Grant (Grant No: G199954305).

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BM061025E