Biodegradable honeycomb collagen scaffold for dermal tissue engineering

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Abstract: Tissue engineering requires a mechanically stable, biocompatible, and biodegradable scaffold that permits cell adherence and proliferation, allows preservation of cell-specific properties, and suitable for surgical implantations. In this study, honeycomb collagen sheet was used for three-dimensional (3D) cultures of human skin fibroblasts and characterized as an effective and suitable scaffold for dermal tissue engineering. About 1-mm-thick honeycomb collagen sheets, prepared from bovine dermal atelocollagen, cross-linked by UV-irradiation, and sterilized by heat, were placed on the proliferating fibroblasts on day 3 of the culture. The cells attached quickly to the collagen scaffold, proliferated inside the honeycomb pores, and formed a structure similar to dermis within 60 days. On day 60, total cellular DNA content of the 3D cultures was 12-fold higher when compared with the 2D control cultures without the scaffold. Measurement of procollagen type I in the media demonstrated a 20-fold increase. Scanning electron microscopy of the 3D cultures showed a well-formed structure similar to dermis and biodegradation of the honeycomb collagen scaffold. Our study proved that honeycomb collagen sheet is a mechanically stable, biocompatible and biodegradable scaffold for dermal tissue engineering, and also potentially useful for other cell-based therapies and tissue engineering applications.
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Key words: tissue engineering; honeycomb collagen; biodegradable scaffold; dermal substitute; cell-based therapy

INTRODUCTION

Tissue engineering is an emerging field with the prospect to provide functional replacement of impaired tissues or organs to patients. It is an interdisciplinary field that brings together the principles of the life sciences and medicine with those of engineering. This technology involves the implantation of an engineered biological substitute, which is either functional at the time of implantation or has the potential to integrate and form the expected functional tissue at a later stage. There has been substantial progress recently in the development of increasingly complex tissue engineered structures.[1–3] Continued progress in tissue engineering will depend on the further development and integration of several classes of enabling tools that will allow not only precise and reproducible fabrication of scaffolds, but also quantitative characterization of the biological integration of the tissue engineered constructs.[4] Furthermore, advances in *in vivo* imaging, such as positron emission tomography, make it possible to provide a noninvasive monitoring of the development and incorporation of the engineered tissues.

Cells are the key unit for tissue regeneration and repair, because they possess extensive proliferation and multiplication capabilities, cell-to-cell signaling, biomolecule production, and formation of extracellular matrix (ECM).[5] The cells cultured on a three-dimensional (3D) matrix behave almost the same as they behave *in vivo*. 3D cell cultures on a biodegradable cell scaffold are the basis of tissue engineering where the specific cells can grow and multiply into a structure similar to the tissue or organs in the living body.[6,7] Various 3D matrices are currently employed for the proliferation and multiplication of various progenitor cells, including adult mesenchymal stem cells. However, most of these matrices do not provide the inherent and unique biological environment where cells can proliferate and multiply in the same fashion as in *in-vivo* systems.

Development of a mechanically stable, biocompatible, and biodegradable scaffold for 3D cell culture poses a significant challenge in dermal tissue engineering. Current scaffolds for skin tissue engineering

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are not ideal because they do not provide the optimal environment for cell adherence, proliferation, and multiplication. The proliferation and multiplication of the transplanted cells is greatly influenced by the composition, architecture, and 3D environment of the scaffold, and biocompatibility of the biomaterial. The ECM is a vital component of cellular microenvironments, providing cells and tissues the appropriate 3D architecture for normal growth and development. Various studies demonstrated that the ECM promotes key cell signaling pathways, influences and enables cell proliferation, differentiation, and proper cell-to-cell and cell–matrix interactions.[8,9] Reproduction of these conditions and structures *in vitro* would yield an opportunity for rapid and accurate studies of molecular and biological events happening *in vivo*. Moreover, ECM and a tuned 3D microenvironment are critical for any successful clinically relevant studies of tissue engineering, cell-based therapy, or stem cell biology.

It has been reported that honeycomb collagen sponge prepared from bovine dermal atelocollagen is a suitable scaffold for 3D cell cultures, which has enormous potential in the field of various tissue engineering applications.[10–12] The biodegradable honeycomb collagen sheet can be cut into suitable thickness and various sizes depending on the application. The aim of our investigation was to employ the honeycomb collagen sheet for proliferation and multiplication of human skin fibroblasts into a dermal-like structure and to characterize the scaffold for dermal tissue engineering. Our study was also aimed to evaluate the use of honeycomb collagen scaffold for biomedical engineering and cell-based therapy.

MATERIALS AND METHODS

Preparation of honeycomb collagen scaffolds

The honeycomb collagen scaffolds used for the studies of skin tissue engineering were prepared from highly purified bovine dermal atelocollagen solution as described earlier.[10] In brief, 1% atelocollagen solution in 1 m*M* HCl (pH 3.0) was transferred to a clean polystyrene shallow tray to a thickness of about 12 mm. The collagen solution was neutralized using ammonia gas evolved from 5% ammonia solution in a closed chamber for 20 h. During this process, the collagen solution turned into a white gel, and the honeycomb structure was generated. The pore size of the honeycomb collagen scaffold was adjusted to 200–400 μm, by altering the concentration of collagen solution and ammonia gas. The collagen gel was then placed in running tap water for 72 h in order to remove the excess ammonia and salt produced during neutralization. It was rinsed twice in distilled water and lyophilized in a slow process. The lyophilized honeycomb collagen sponge was sliced on a slicing machine (Omas, Italy) as 1-mm-thick sheets. About 25, 50, and 100-mm^2 sheets were cut from the 1-mm-thick honeycomb collagen sheet. The collagen sheets were cross-linked under UV irradiation at a dose of 550 $\mu w/cm^2$ for 40 min on each side in a UV chamber. The collagen sheets were then dried at 110°C for 30 min and sterilized at 121°C for 6 h in an oven. The heat dried and sterilized honeycomb collagen sheets were tested for cytotoxicity with human skin fibroblasts and used for dermal tissue engineering studies.

Phase contrast and scanning electron microscopy of honeycomb collagen scaffold

Phase contrast and scanning electron microscopic studies were carried out to determine the structural features of the 1-mm-thick honeycomb collagen sheet prepared from bovine atelocollagen solution. An inverted microscope (model IX71; Olympus, Tokyo, Japan) attached with an Olympus DP71 digital camera was used to examine the honeycomb collagen scaffold and obtain phase contrast pictures. Scanning electron microscopy was carried out using a Jeol (model JSM-5310LV) microscope. A sample of about 3-mm^2 honeycomb collagen sheet was used to obtain scanning electron microscopic pictures after gold coating through sputtering.

Cell culture conditions

Normal human skin fibroblast cells were procured from American Type Culture Collection (ATCC, Manassas, VA) and cultured on 60-mm Falcon polystyrene cell culture dishes (BD Biosciences, NJ). The cells were propagated in Eagle's minimum essential medium (Invitrogen, Tokyo) supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (100 μg/mL streptomycin and 100 units/mL penicillin) in a humidified incubator containing 5% CO_2 on air at 37°C. About 25-mm^2 and 1-mm-thick sterile honeycomb collagen sheets were placed on the proliferating human skin fibroblasts on the third day of the culture. Only one 25-mm collagen sheet was placed on each culture dish. A set of two-dimensional control cultures without honeycomb collagen sheets was also prepared and studied simultaneously. Both control and experimental culture dishes were about 50% confluent when the honeycomb collagen sheet was placed. After placement of the honeycomb collagen scaffold, the amount of culture media was reduced to 50% of normal volume in order to avoid floating the collagen sheet, and also to accelerate the attachment of proliferating fibroblasts to the honeycomb collagen sheet. The cells attached quickly to the collagen scaffold and proliferated through the honeycomb walls. The media was changed carefully every 48 h without disturbing the scaffold. The proliferating cells anchored the honeycomb collagen sheet to the culture dish within 5 days of placement. The ECM synthesized by the proliferating fibroblasts reenforced the attachment of the collagen scaffold to the culture dish. When the collagen sheets were firmly attached to the culture dish, the amount of culture media was increased to the normal level. Afterwards, the media was changed every other day. The proliferating fibroblasts along with the honeycomb collagen sheets were examined twice a week using an Olympus phase contrast microscope attached with a digital camera and photographed. A few dishes of control cultures and cultures with honeycomb collagen sheets were

terminated on days 15, 30, and 45 for biochemical analyses. All cultures were terminated on day 60.

Morphological analyses

To demonstrate the prominent growth of fibroblasts on the honeycomb collagen scaffold and surrounding areas, the cultures were photographed using a phase contrast microscope (Olympus, Tokyo, Japan) at the edges of the scaffold on days 30 and 45 after the placement of the honeycomb collagen sheet. On day 45, a portion of the culture surrounding the scaffold was cut and removed using a sterile scalpel to depict the thickness of the fibroblast growth around the scaffold. The cultures were incubated for 2 more days and photographed. Cultures were again photographed around day 50 and on day 60 in order to demonstrate the marked growth of fibroblasts, to depict the distortion and biodegradation of the honeycomb collagen scaffold, and to show the formation of a dermal-like structure.

Quantification of cell growth

Assay of total cellular DNA

The total cellular DNA contents of both control and honeycomb collagen sheet cultures were measured on days 15, 30, 45, and 60 after placing the collagen scaffold. The media was removed, and cells were washed twice with phosphate-buffered saline (PBS). The cells along with the honeycomb collagen scaffold were scraped and collected in 2 mL of 0.05*M* phosphate buffer (pH 7.4) containing 2.0*M* NaCl. About 1 mL of this cell suspension was sonicated using an ultrasonic disruptor (UD 201; Tomy, Tokyo, Japan). It was centrifuged at 3000 rpm for 5 min at 4°C, and the supernatant was collected. The DNA content in the supernatant was determined using Hoechst 33258 reagent (Polysciences, Warrington, PA), which follows the fluorometric method of Labarca and Paigen.[13] In brief, 1 μg of Hoechst reagent in 0.05*M* phosphate buffer containing 2.0*M* NaCl was mixed with 100 μL of suitably diluted cell preparation. The resultant fluorescence was measured using a Hitachi F-2000 fluorescence spectrophotometer with excitation at 356 nm and emission at 458 nm. Denatured DNA from Salmon testes (Wako, Osaka, Japan) was used to prepare the standard curve. The results are represented as mg DNA/culture dish.

Assay of procollagen type I C-peptide

The procollagen type I C-peptide released into the culture media was determined as a measure of collagen synthesis and deposition by the fibroblasts on honeycomb collagen scaffold. On days 14, 29, 44, and 59, the regular media was removed from both control and honeycomb collagen cultures. The cells were then washed twice with serum-free media and replaced with serum-free media containing 50 μg/mL ascorbic acid. Ascorbic acid is an essential component for collagen biosynthesis and stimulates procollagen production. The media was collected after 24 h and stored at −80°C until assayed. Type I procollagen C-peptide released into the serum-free media of both control and honeycomb collagen cultures were measured using a precoated procollagen Type I C-peptide EIA kit (Takara Bio, Shiga, Japan) as per manufacturer's instructions. Because of very high concentrations of procollagen C-peptide, the media from honeycomb collagen cultures were diluted up to 100-fold using serum-free media. The results are represented as μg/mL media.

Scanning electron microscopy

To demonstrate the formation of dermal-like structures on cultures with the honeycomb collagen scaffold, scanning electron microscopy was performed after termination of the cultures on day 60. In brief, the cells with the honeycomb collagen scaffold were carefully removed with forceps, washed in PBS at 37°C and fixed in 2% electron microscopic grade glutaraldehyde (pH 7.5) at 4°C for 2 h. The honeycomb collagen scaffold with the cells was then washed three times, 10 min each in 0.1*M* sucrose prepared in 0.1*M* phosphate buffer, pH 7.4. It was dehydrated in 30, 40, 50, 60, 70, 80, and 90% ethanol for 30 min each at room temperature and then transferred to 100% ethanol. The 100% ethanol was changed three times at 15 min interval. Finally, the scaffold was transferred to 1,1,1,3,3,3-Hexamethyldisilazane (Aldrich, Milwaukee, WI) for 30 min at room temperature. The scaffold with the cells was cut into a size of about 3 mm^2 and fixed to the electron microscopic copper grid using double sided carbon tape facing the cell surface to the top. It was coated with gold using a sputtering machine (JFC-1200; Jeol, Tokyo, Japan) and examined on a scanning electron microscope (JSM-5310LV; Jeol) and photographed.

Statistical analysis

Arithmetic mean and standard deviation were calculated for all quantitative data. The control culture data were compared with the honeycomb collagen culture data on respective days using Student's t test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Phase contrast and scanning electron microscopy of honeycomb collagen scaffold

Phase contrast and scanning electron microscopic pictures of 1-mm-thick honeycomb collagen sheet prepared from highly pure bovine atelocollagen solution are presented in Figure 1(A,B), respectively. Figure 1(A) reveals the 3D phase contrast image of the honeycomb collagen sheet including the smooth wall structure and pore size. The picture also depicts the honeycomb framework and collagen fibers. Figure 1(B) demonstrates the scanning electron microscopic picture of the honeycomb collagen sheet and also the honeycomb pores. As evident from the

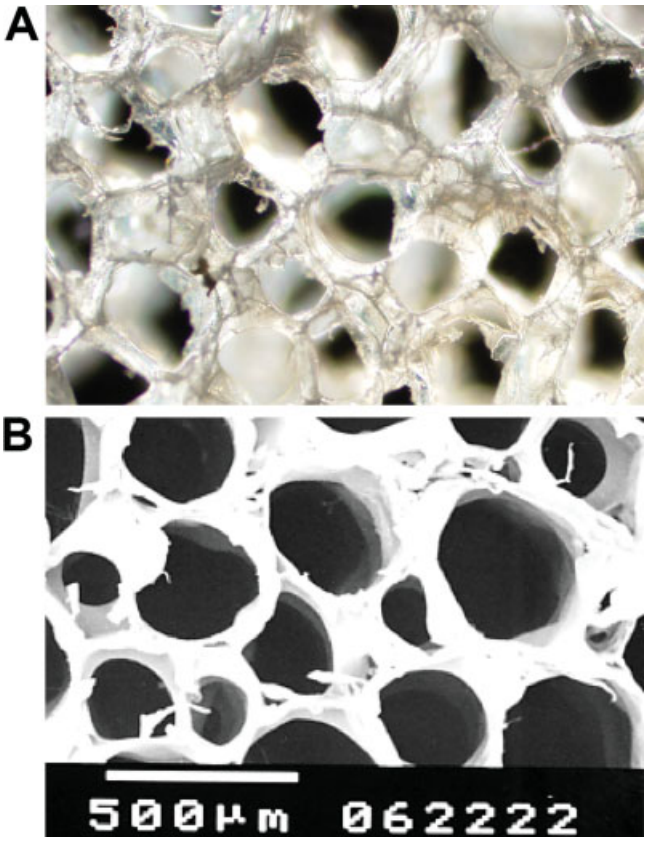

Figure 1. A: Phase contrast micrograph of 1-mm-thick honeycomb collagen sheet prepared from bovine atelocollagen solution (×40). B: Scanning electron micrograph of 1-mm-thick honeycomb collagen sheet (×50). The average pore size of the honeycomb collagen scaffold was around 300 μm.

micrograph, the average pore size of the honeycomb collagen scaffold was about 300 μm.

Cell attachment and cell growth on honeycomb collagen scaffold

Figure 2 demonstrates the proliferation and multiplication of human skin fibroblasts on the honeycomb collagen sheet. The honeycomb collagen scaffold has appropriate pore size for the proliferation of fibroblasts through the interior walls of the honeycomb. The fibroblasts proliferated inside the honeycomb pore in a circular manner and finally filled the pore and the entire honeycomb scaffold. The arrow in Figure 2 indicates the proliferating fibroblasts inside the honeycomb pore. The fibroblasts maintained their characteristic spindle-like shape inside the honeycomb pore during proliferation. The entire honeycomb collagen scaffold was covered with the fibroblasts by day 21 of culture. As evident from Figure 2, fibroblasts grow rapidly on honeycomb collagen scaffold. The structure of the scaffold was found to be suitable for adherence, proliferation, and multiplication of human skin fibroblasts.

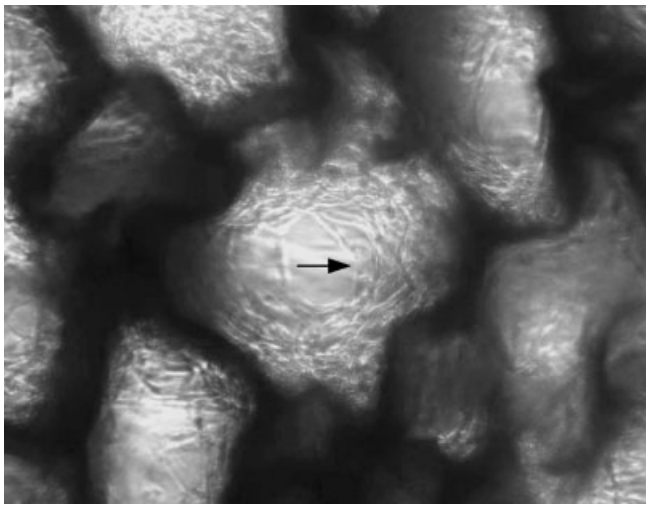

Figure 2. Phase contrast micrograph of 14-day-old culture of human skin fibroblasts on honeycomb collagen sheet (×100). All honeycomb pores are partially or fully filled with the proliferating fibroblasts. The fibroblasts proliferated inside the honeycomb well in a circular manner from the wall toward the center of the well. The arrow indicates the proliferating fibroblasts filling the honeycomb pore in a circular manner. The black area represents the walls of honeycomb scaffold.

Morphological features of fibroblasts on honeycomb collagen scaffold

Figure 3 demonstrates the prominent growth of human skin fibroblasts on honeycomb collagen scaf-

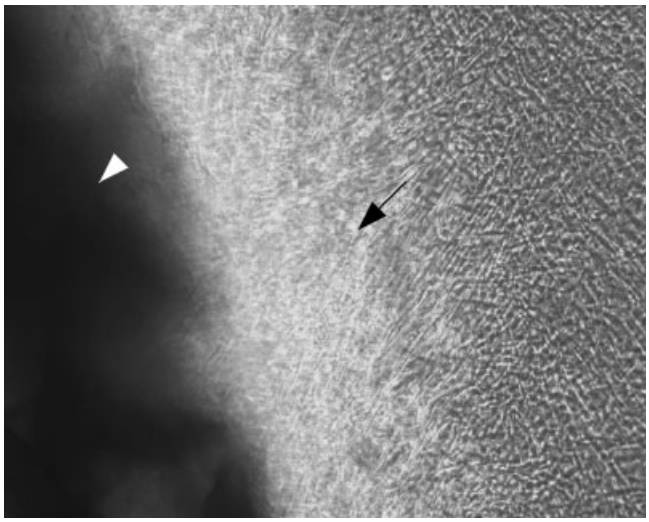

Figure 3. Phase contrast micrograph of 30-day-old culture of human skin fibroblasts on honeycomb collagen sheet (×100). The fibroblasts completely filled the whole scaffold and proliferated out from interior and top of the scaffold. The arrow indicates the prominent three-dimensional growth of fibroblasts surrounding the scaffold. The black area indicates edge of the honeycomb scaffold (white arrowhead).

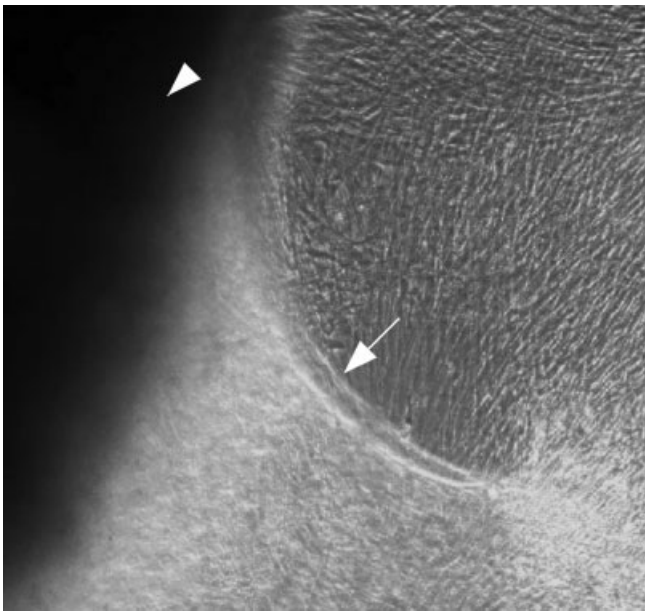

Figure 4. Phase contrast micrograph of about 45-day-old culture of human skin fibroblasts on honeycomb collagen sheet (×100). A portion of the culture surrounding the scaffold was cut and removed to show the thickness (1 mm) of the culture and also to demonstrate the marked three-dimensional growth of fibroblasts around the honeycomb collagen scaffold (arrow). The black area indicates the honeycomb scaffold (arrowhead).

fold. It also shows the 3D effect of the scaffold. After filling the entire scaffold, the fibroblasts proliferated out to surrounding areas (arrow). The proliferation of fibroblasts exterior to the scaffold may be suitable for anchoring the engineered dermal tissue with healthy skin in patients. Figure 4 further demonstrates the 3D effect of the honeycomb collagen scaffold for the multiplication and proliferation of human skin fibroblasts to form a structure similar to dermis. The cut and removed area of the culture clearly demonstrates the thickness of fibroblast growth in the plane culture dish surrounding the scaffold (arrow). This picture further indicates the suitability of honeycomb collagen scaffold for dermal tissue engineering.

Biodegradation of honeycomb collagen scaffold

The ideal scaffold for any tissue engineering application should be not only mechanically stable to hold the cells, but also biocompatible and biodegradable. Figure 5(A) demonstrates the honeycomb collagen scaffold fully covered and overgrown with human skin fibroblasts on day 50 of the culture. As evident from the picture, the honeycomb scaffold still retains its original structure along with the scaffold walls. This proves the mechanical stability of the honeycomb collagen scaffold to hold the cells in fully loaded condition. Figure 5(B) clearly demonstrates the biodegradability of the honeycomb collagen scaffold. As evident in the figure, the honeycomb walls are fragmented and fully or partially degraded. The continuation of the fibroblast cultures on honeycomb collagen scaffolds leads to the biodegradation of the scaffold by the proliferating fibroblasts. This occurs at a point where there is no more space in the scaffold for further growth and multiplication of the cells. Also, at this juncture the fibroblasts form a network and initiate the formation of a structure similar to dermis (Fig. 7).

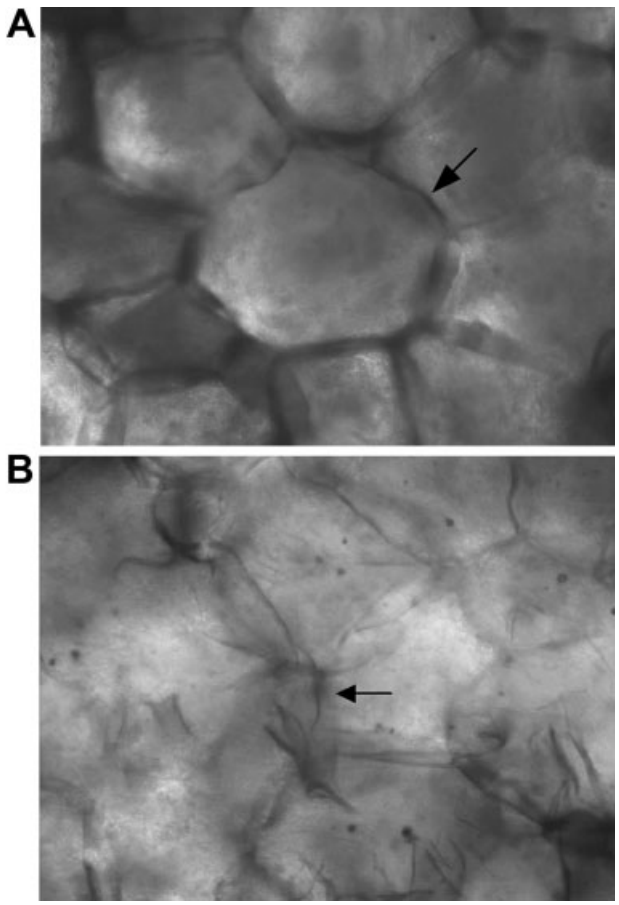

Figure 5. A: Phase contrast micrograph of around 50-day-old culture of human skin fibroblasts on honeycomb collagen sheet (×100). The honeycomb collagen scaffold pores are totally filled with the fibroblasts. In addition, the fibroblasts formed layers covering the entire scaffold including the honeycomb walls (arrow). The honeycomb structure was still intact. B: Phase contrast micrograph of about 60-day-old culture of human skin fibroblasts on honeycomb collagen sheet (×100). The honeycomb scaffold is completely filled and covered with fibroblasts. The entire structure is almost distorted and has started degrading. The arrow indicates biodegradation of the scaffold walls. The cultures were processed for scanning electron microscopy at this stage.

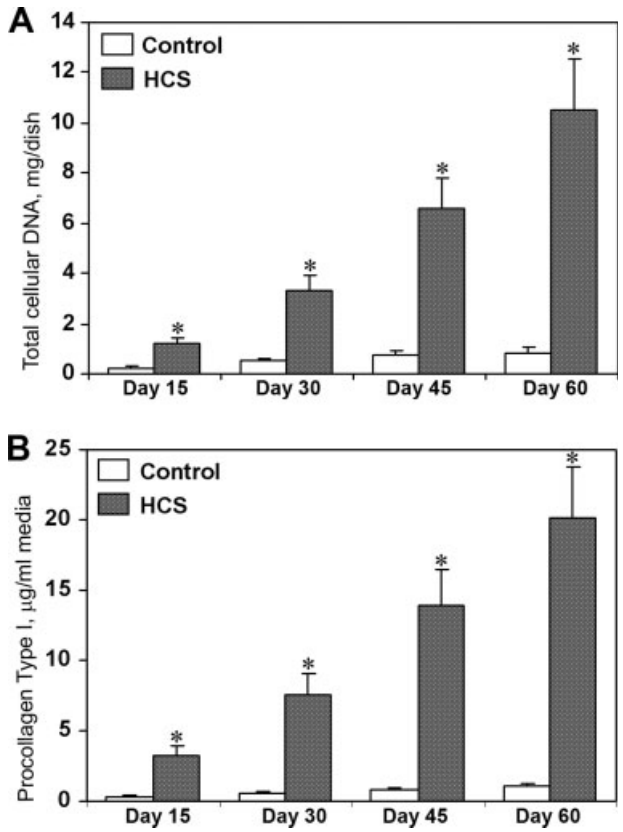

Figure 6. A: Total cellular DNA content of control cultures and cultures with honeycomb collagen scaffold on days 15, 30, 45, and 60 after placing the honeycomb sheet. The DNA content was more than 12-fold higher when compared with the corresponding control cultures on day 60 ($*p < 0.001$, $n = 5$). B: Procollagen Type I C-peptide present in the media of control cultures and cultures with honeycomb collagen scaffold on days 15, 30, 45, and 60 after placing the honeycomb collagen sheet. On day 60, the procollagen type I content in the serum-free media was 20-fold higher compared with the corresponding control cultures without the scaffold ($*p < 0.001$, $n = 5$). HCS, honeycomb collagen scaffold.

DNA measurements

The total cellular DNA content of control cultures and 3D cultures on various time periods are presented in Figure 6(A). The total DNA was determined as a measure of the quantification of cell proliferation and multiplication. The DNA content of the cultures with honeycomb collagen scaffold was significantly higher ($p < 0.001$) when compared with the respective controls without the scaffold on all days studied. On day 60, more than a 12-fold increase was observed in the DNA content of the cultures with the honeycomb collagen scaffold. In the 3D environment on the honeycomb collagen scaffold, the fibroblasts proliferated and multiplied in a high-density manner in contrast to the conventional flat bed culture on dishes.

Collagen biosynthesis by fibroblasts on honeycomb collagen scaffold

The procollagen type I C-peptide measured in the serum-free media of both control and honeycomb collagen scaffold cultures using a one-step sandwich enzyme immunoassay is presented in Figure 6(B). Synthesis of collagen is a characteristic feature of human skin fibroblasts. The amount of the free procollagen released into the media during collagen biosynthesis is a measure of the rate of collagen biosynthesis by the fibroblasts. Because the honeycomb collagen scaffold is made up of 100% pure atelocollagen, the degraded collagen molecules from the honeycomb scaffold do not cross react in the procollagen assay. A dramatic increase ($p < 0.001$) was observed in the amount of procollagen type I C-peptide in the serum-free media of cultures with the honeycomb collagen scaffold when compared with the respective controls without it. At termination, a 20-fold increase was observed in the procollagen type I content of the cultures with the honeycomb collagen scaffold.

Tissue-engineered dermal reconstruction on honeycomb collagen scaffold

The scanning electron microscopic picture of a 2-month-old culture of human skin fibroblasts on the honeycomb collagen scaffold is presented in Figure 7. As evident from the picture, the fibroblasts formed a structure similar to dermis in more than 50% of the area. Spindle-shaped united fibroblasts are visible in other areas. A portion of the biodegrading honeycomb collagen scaffold is also visible (arrow). This

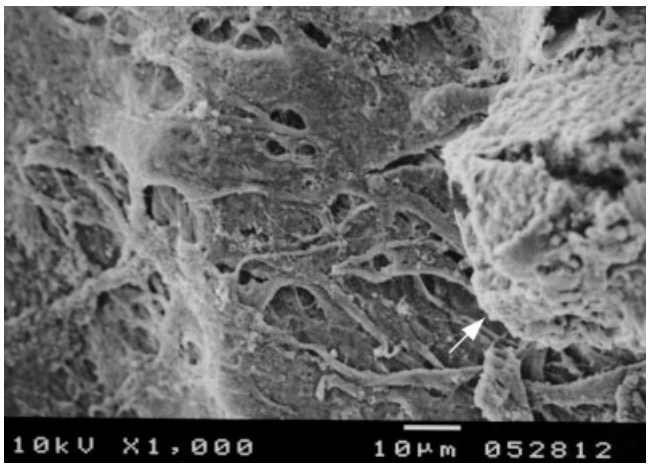

Figure 7. Scanning electron micrograph of about 60-day-old culture of human skin fibroblasts on honeycomb collagen sheet (×1000). The fibroblasts formed a network similar to dermis on honeycomb collagen scaffold. A part of the degrading scaffold is also visible (arrow).

picture demonstrates that fibroblasts grown on a honeycomb collagen scaffold can form a structure similar to dermis within 2 months of the culture.

DISCUSSION

Biomaterials used for tissue engineering as cell carriers must have the unique properties of mechanical stability, biodegradability, and biocompatibility. They should also be free from cytotoxins and promote cell adherence, cell proliferation, and multiplication. In this investigation, we have proved that honeycomb collagen scaffold prepared from bovine dermal atelocollagen has all the above unique properties and characteristics for dermal tissue engineering. The integrins, cell surface collagen receptors, may play a significant role in the quick attachment and proliferation of fibroblasts on the collagen scaffold.[14] This is the first study to demonstrate that honeycomb collagen scaffold is a useful and suitable material for dermal tissue engineering. Even though several materials have been proposed previously for dermal tissue engineering, they lack the unique characteristics and biocompatibility of a honeycomb collagen scaffold for dermal tissue engineering and related applications.

Collagen is an excellent and appropriate material for various biomaterial and biomedical applications.[15–17] In biological systems, collagen promotes cell attachment, proliferation, differentiation, organogenesis, tissue regeneration, and wound healing. Collagen is mechanically stable with high tensile strength and can be altered through various physical and chemical modifications.[18,19] Atelocollagen prepared from highly pure bovine dermal collagen has been used for several clinical and biomedical applications including lacrimal duct occlusion,[20] gene transfer,[21] and siRNA delivery.[22] We have previously demonstrated that bone marrow mesenchymal stem cells can be differentiated into osteoblasts on honeycomb collagen scaffolds.[10] The honeycomb collagen scaffold prepared from atelocollagen has several special characteristics such as mechanical stability under various physical and chemical conditions, capability to exchange nutrients and waste products through the honeycomb membranes, and ability to retain its honeycomb structure throughout the study without deformity or collapse until it is biodegraded. The pore size and thickness of the honeycomb collagen scaffold can be controlled by altering the concentrations of collagen solution and ammonia gas. This has the great advantage of creating different types of honeycomb collagen scaffolds suitable for various tissue engineering applications. In this study, the pore size of the honeycomb collagen scaffold used was about 300 μm, which is most suitable for the attachment, proliferation, and multiplication of fibroblasts inside the honeycomb pore. Furthermore, because of its unique 3D environment and unlike other dermal tissue engineering scaffolds, the honeycomb collagen sheet guarantees adequate perfusion of nutrients and oxygen that facilitates the development of microvascular network. These advantages make the honeycomb collagen sheet a suitable scaffold for dermal tissue engineering and other cell-based therapies.

Tissue-engineered dermal reconstructions on a honeycomb collagen scaffold have several advantages such as low antigenicity, biodegradability, biocompatibility, mechanical stability, and long-term preservation of live cells. Because the honeycomb collagen scaffold is prepared from atelocollagen molecules, which do not contain the antigenic telopeptides, the antigenicity of atelocollagen is extremely low. Atelocollagen is extensively used in medical, cosmetic, and tissue repair applications, with very little or no hypersensitivity reactions.[23] The antigenicity from human dermal fibroblasts is negligible because dermal fibroblasts are not potent antigen presenting cells.[24] The antigenicity due to the heterologous sources can be further reduced by employing dermal fibroblasts prepared from the umbilical cord in which the antigenicity is relatively low when compared with the adult human skin fibroblasts. As demonstrated in this study, honeycomb collagen scaffold is fully biodegradable by the proteases, especially the matrix metalloproteinases synthesized and released by the dermal fibroblasts on the scaffold. The honeycomb collagen scaffold is biocompatible without any cytotoxic or injurious effects *in vivo*. The cross-linking of collagen molecules by UV-irradiation during the preparation of the honeycomb collagen sheet provides the mechanical stability to hold the cells and also to maintain the structural integrity of the scaffold until it is biodegraded by proteases. The honeycomb collagen scaffold can also be prepared using purified collagen from other mammals to avoid potential concerns over bovine-derived products, a concern that stems from mad cow disease or bovine spongiform encephalopathy.

Biosynthesis of collagens and other ECM proteins are the primary functions of dermal fibroblasts. Type I and type III collagens are the major proteins in the dermal tissue, and their unique triple helical structure provides mechanical stability and elasticity to the dermis. About 90% of total dermal protein consists of collagen, accounting for about 75% of the skin's total dry weight.[25] Collagens are synthesized as precursor molecules called procollagens, which contain additional peptide sequences, the propeptides at both the amino- and carboxy-terminal ends.[26] The propeptides are cleaved off from the collagen triple helix molecule during its secretion. A 20-fold increase in the rate of collagen biosynthesis observed

in this study indicates the enhanced ability of fibroblasts to synthesize collagen on the honeycomb collagen scaffold. Ascorbic acid stimulates collagen biosynthesis and metabolism.[27,28] We have treated our cultures with ascorbic acid in serum-free media. This could explain the 20-fold increase of the procollagen type I C-peptide compared with the 12-fold increase of total cellular DNA on day 60. It was also reported that ascorbic acid accelerated deposition of several basement membrane proteins such as type IV and VII collagens, nidogen, laminin, procollagens I and III, tenascin C, and fibrillin-1 in an *in vitro* human reconstructed skin model leading to a structure closer to that of normal human skin.[29] Ascorbic acid treatment led to a better organization of basal keratinocytes, an increase in fibroblast number and faster formation of the dermal-epidermal junction.[29]

There are few reports about reconstruction of dermal-like structures on collagen matrices or biopolymer scaffolds. Bell et al.[30] constructed a living-skin equivalent for skin replacement, which consists of two components—a dermal equivalent made up of fibroblasts in a collagen matrix and an epidermis that develops from keratinocytes seeded on the dermal equivalent. However, this construct lacked a 3D environment for optimum cell proliferation and multiplication as in living dermal tissue, and the dermal grafts to rat-skin failed to produce hair and sebaceous glands even after 1 year. Tonello et al.[31] developed an *in vitro* coculture system using three human cell types—keratinocytes, fibroblasts, and endothelial cells in a 3D biomaterial scaffold produced from sodium hyaluronate and benzyl alcohol. They successfully developed a dermal-like structure with a microvascular network. However, the scaffold employed lacks the unique honeycomb environment, which would be more ideal for the reconstruction of a similar skin equivalent with coculture of three human cell types. Besides, the 3D scaffold used by Tonello et al.[31] releases benzyl alcohol which is toxic both *in vitro*[32] and *in vivo*,[33] and the product would not be suitable for clinical applications.

Honeycomb collagen scaffold is a unique product for various applications in the field of biomedical and tissue engineering. It is possible to make diverse scaffolds of various sizes and shapes according to different organs or tissues such as skin, liver, kidney, or cartilage. The formation of an implanted tissue or organ is greatly influenced by composition, architecture, the 3D environment of the scaffold, and its biocompatibility. It is important that the scaffold support the formation of bioengineered tissue that mimics the mechanical properties of the tissue or organ that is being repaired or replaced. Several *in vitro* studies demonstrated specific interaction between cells and the ECM mediated by collagen molecules.[34,35] The specific honeycomb structure and the porosity of the honeycomb walls allow transportation of nutrients to the cells and also for the removal of waste products as in a dynamic culture environment. Overall, the 3D environment of the honeycomb collagen scaffold provides the natural ECM environment with the necessary mechanical support and biochemical interplay as in living systems for the formation of a dermal substitute.

The results of this investigation demonstrated that honeycomb collagen scaffold is an efficient and suitable material for cell adherence, proliferation, and multiplication of human skin fibroblasts to form a dermal substitute. Our study further proved that honeycomb collagen sheet is a mechanically stable, biocompatible, and biodegradable scaffold for dermal tissue engineering, and also potentially useful for other cell-based therapies and tissue engineering applications. Our study also provided valuable information about the use of honeycomb collagen scaffolds for long-term cell culture without deformity or collapse until it is biodegraded.

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