Differentiation of Mesenchymal Stem Cells Into Osteoblasts on Honeycomb Collagen Scaffolds

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Abstract: Tissue engineering using living cells is emerging as an alternative to tissue or organ transplantation. The adult mesenchymal stem cells can be differentiated into multilineage cells, such as adipocytes, chondrocytes, or osteoblasts when cultured with specific growth factors. In the present investigation, we have studied the effect of honeycomb collagen scaffolds for the adhesion, differentiation and proliferation of bone marrow-derived mesenchymal stem cells into osteoblasts. Mesenchymal stem cells were isolated from 6-week old albino rat femur bone marrow, and cultured in α-MEM medium without β-glycerophosphate and dexamethasone. Honeycomb collagen discs were prepared from bovine dermal atelocollagen, cross-linked by UV-irradiation and sterilized by heat. The honeycomb discs were placed on the culture dishes before seeding the stem cells. The cells attached quickly to the honeycomb collagen scaffold, differentiated and proliferated into osteoblasts. The differentiated osteoblasts were characterized by morphological examination and alkaline phosphatase activity. The osteoblasts also synthesized calcium-deficient hydroxyapatite (pseudo-hydroxyapatite) crystals in the culture. The mineralization was confirmed by Von Kossa staining and the crystals were analyzed by X-ray diffraction. Light microscopy and DNA measurements showed that the differentiated osteoblasts multiplied into several layers on the honeycomb collagen scaffold. The results demonstrated that the honeycomb collagen sponge is an excellent scaffold for the differentiation and proliferation of mesenchymal stem cells into osteoblasts. The data further proved that honeycomb collagen is an effective substrate for tissue engineering applications, and is very useful in the advancing field of stem cell technology and cell-based therapy.

Keywords: tissue engineering; mesenchymal stem cells; osteoblasts; honeycomb collagen scaffold; cell based therapy

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INTRODUCTION

Tissue engineering is a developing branch of science that merges the fields of cell biology, molecular biology, bioengineering, material science, and surgery to provide new functional tissue using living cells, biomatrices and signaling molecules. Using this technology, tissue loss or organ failure can be treated by implantation of an engineered biological substitute, that is either functional at the time of implantation or has the potential to integrate and form the expected functional tissue at a later stage. The advantage of tissue engineering is that small biopsy specimens from relatively uninvolved sites can be obtained from the patient and cells can be isolated, cultured, and expanded into large numbers (Bruder and Fox, 1999; Levenberg and Langer, 2004; Mooney and Mikos, 1999; Service, 2005). Three dimensional (3-D) cell cultures on a bio-degradable cell scaffold is the basis of tissue engineering, where the specific cells can grow and multiply into a structure similar to tissue or organs in the living body (Holmes, 2002; Liu Tsang and Bhatia, 2004; Sutherland et al., 2005).

Maniatopoulos et al. (1988) first reported that bone marrow stromal cells obtained from young adult rats can differentiate into osteoblasts and express bone like structure when cultured with α-glycerophosphate and dexamethasone. Later it was proved that bone marrow derived non-hematopoietic mesenchymal stem cells (MSCs) are pluripotent and have the ability to differentiate into multilineage cells, to form a variety of mesenchymal tissues, including bone, cartilage, tendon, ligament, muscle, and adipose tissue which serve as a potential tool for tissue engineering (Ballas et al., 2002; Gregory et al., 2005; Kassem, 2004; Mauney et al., 2005; Pittenger et al., 1999). The MSCs can be easily isolated, purified, and expanded through 3-D cell culture systems from animals and humans. Because of the ease of their isolation and their extensive differentiation potential, MSCs are among the first stem cell types to be introduced into the clinic. The differentiated MSCs such as osteoblasts or chondrocytes can be grown on a suitable 3-D cell scaffold to

form the shape of a particular organ, which would permit replacement or regeneration of a defective bone or cartilage.

It was reported that honeycomb collagen scaffold prepared from bovine dermal atelocollagen is a suitable carrier for various 3-D cell cultures and has immense potential in the field of tissue engineering (Itoh et al., 2001; Masuoka et al., 2005; Sato et al., 2003). The geometry of honeycomb collagen scaffolds provide a unique structure and environment for cell attachment and differentiation. The aim of the present investigation was to study the effect of the honeycomb collagen scaffold for the adhesion, proliferation, and differentiation of bone marrow derived mesenchymal stem cells into osteoblasts, and also to evaluate the use of honeycomb collagen scaffold for stem cell technology and tissue engineering applications.

MATERIALS AND METHODS

Preparation of Honeycomb Collagen Scaffolds

The honeycomb collagen scaffolds (discs) were prepared from highly purified bovine dermal atelocollagen. In brief, 1% atelocollagen solution in 1 mM HCl (pH 3.0) was poured into a clean polystyrene shallow tray up to a thickness of 12 mm. It 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 diameter of the honeycomb collagen sponge was adjusted to 200–400 μm, which is suitable for the optimum adhesion and proliferation of mesenchymal stem cells. The pore size was controlled by changing the concentration of collagen solution and ammonia gas. The collagen gel was placed in running tap water for 72 h in order to remove the excess ammonia and salt produced during neutralization. It was rinsed in distilled water and lyophilized in a special slow process. The lyophilized honeycomb collagen was sliced as 1 mm thick sheets using a slicing machine (Omas, Italy). About 15 mm diameter discs were cut using a mechanical punch from the 1 mm sheet. It was cross-linked under UV irradiation at a dose of 550 $\mu w/cm^2$ for 40 min on each side. Then the honeycomb collagen discs were dried at 110°C for 30 min and sterilized at 121°C for 6 h. The heat dried and sterilized honeycomb collagen discs were tested for cytotoxicity and directly used for the mesenchymal stem cell differentiation studies.

Isolation and Culture of Mesenchymal Stem Cells on Honeycomb Collagen Scaffold

About 6-week-old male Wistar rats of the albino strain were used for the isolation of mesenchymal stem cells. National guidelines for the care and use of laboratory animals were observed. The animals were sacrificed by cervical dislocation, abdominal area was shaved including legs and cleaned with 70% ethanol. The muscles present in the femoral area were removed, and both femoral bones were collected under sterile conditions and placed in a beaker containing alpha modification of minimum essential medium (α-MEM) with a 10-fold higher concentration of antibiotics (Penicillin and Kanamycin) than that generally used in cell culture media. The femoral bones were cut at both ends using sterile scissors while being held with a forceps on a sterile surface. Using a 5 mL syringe fitted with 18 G needle, 5 mL of the α-MEM containing 10-fold concentration of antibiotics was passed through the bone, and the bone marrow stromal cells were collected in a 50 mL sterile conical flask. The femoral bone was washed four times with 5 mL of α-MEM in order to collect the maximum number of stromal cells. Bone marrow stromal cells contain pluripotent stem cells along with large amounts of hematopoietic cells. The cells were mixed gently and filtered through a 52 μm membrane filter to remove the bone chips present in the preparation. The filtrate was centrifuged at 3,000 rpm for 5 min. The cells were washed again with α-MEM with 10-fold concentration of antibiotics. The purified cells were finally dispersed in α-MEM with 15% fetal bovine serum containing 100 U/mL penicillin and 60 μg/mL kanamycin sulfate, but without β-glycerophosphate and dexamethasone. The density of the cells in the preparation was adjusted appropriately using a phase contrast microscope and the cells were plated in 6-well corning polystyrene cell culture dishes. A 15 mm diameter and 1 mm thick sterile honeycomb collagen disc was placed on each well prior to seeding the cells. A set of control cultures without honeycomb collagen discs were also prepared simultaneously. The cells along with the honeycomb collagen scaffold was incubated at 37°C over 5% CO_2 in a humidified atmosphere. The cells attached quickly attached to the cell culture dish as well as to the honeycomb collagen scaffold. The media was changed after 6 h in order to remove the non-viable cells.

The amount of culture media was reduced to 50% of normal volume in order to avoid floating the honeycomb collagen disc, and also to accelerate the proliferation of mesenchymal stem cells on the scaffold. The media was changed carefully every 48 h without disturbing the honeycomb collagen scaffold. The proliferating cells anchored the honeycomb collagen discs to the culture dishes within 7 days of placement. The extracellular matrix synthesized by the proliferating cells re-enforced the attachment of the collagen scaffold to the culture dish. When the collagen discs were firmly attached to the culture dish, the amount of culture media was increased to the normal level. The proliferated mesenchymal stem cells differentiated into osteoblasts on the honeycomb collagen scaffold. The differentiated osteoblasts were examined every 48 h using an Olympus phase contrast microscope attached with Olympus digital camera and photographed. A few dishes of both the control and honeycomb collagen disc cultures were harvested on days 14 and 21. All cultures were terminated on day 28.

Morphological Analyses

Von Kossa Staining

Von Kossa staining (Bonewald et al., 2003) was carried out to characterize the biological mineralization of differentiated

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osteoblasts. The scaffold was removed and the cells and mineral deposit below the scaffold were washed twice with phosphate buffered saline (PBS). The culture dish with the mineral deposit was then fixed with 10% phosphate-buffered formalin for 10 min, washed once with distilled water and serially rehydrated from 100% to 95% to 80% ethyl alcohol to distilled water. The water was removed and 2% silver nitrate solution was added. Then the dish was exposed to direct sunlight for 20 min, after which the plate was rinsed with water. Sodium thiosulfate (5%) was added for 3 min, the plates were then rinsed in water and counter stained with acid fuchsin for 5 min. The plates were washed with deionized water, then twice with 95% ethyl alcohol and 100% ethyl alcohol and finally dried in air.

X-Ray Diffraction Analysis

The microcrystals synthesized by the differentiated osteoblasts on honeycomb collagen scaffold were characterized for the biological mineralization through X-ray diffraction analysis. The culture media was pipetted out and the microcrystals formed below the scaffold were air dried and powdered by grinding. The powder was analyzed using an X-ray diffractometer (Rint 5000 of Rigaku Co. Ltd., Tokyo, Japan) in order to determine the crystallinity of the hydroxyapatite-like powder and for the presence of other crystal phases. A few standard powder X-ray diffraction spectrums of the synthetic hydroxyapatite and biological minerals, such as bone and tooth, were used to characterize the minerals formed by the differentiated osteoblasts.

Biochemical Analyses

Determination of Hydroxyproline and Total Collagen Content of the Scaffold

In order to study the synthesis of total collagen by the differentiated osteoblasts on the honeycomb collagen scaffold, the hydroxyproline content in the scaffold was measured on days 0,14, 21, and 28 during the course of the investigation. The honeycomb collagen scaffold along with the osteoblasts and cell matrix was removed from the culture dish and gently washed in PBS. It was hydrolyzed in 6 N HCl for 20 h at 110°C in screw capped corning glass tubes. A set of fresh honeycomb collagen scaffolds were used as test controls. After hydrolysis, the acid was evaporated to dryness using a vacuum evaporator along with a hot water bath. The procedure was repeated twice after rinsing with distilled water to ensure the complete removal of the acid from the preparation. Finally it was dissolved in 2 mL of distilled water and used for the determination of hydroxyproline according to the method of Woessner (1961). In brief, 0.1 mL of the preparation was made up to 1 mL with distilled water and mixed with 1 mL of freshly prepared chloramine-T solution and allowed to stand for 20 min. It was further mixed with 1 mL of 3.15 M perchloric acid and left for 5 min. Finally, 1 mL of freshly prepared *p*-dimethylaminobenzaldehyde was added and mixed well; and the mixture was incubated in a water bath at 60°C for 20 min. The absorbance of the solution was measured in a spectrophotometer (Hitachi U-2000) at 560 nm. The total collagen content was calculated by multiplying the hydroxyproline content by the factor 7.46 as postulated by Neuman and Logan (Neuman and Logan, 1950). The test control collagen content was deducted from the total collagen content of the experimental sample.

Alkaline Phosphatase Assay

The alkaline phosphatase (ALP) activity of the differentiated osteoblasts on the honeycomb collagen scaffold was measured in control and test cultures. A set of both control and test cultures were harvested on days 14, 21, and 28 and washed twice with PBS. The cell matrix along with the honeycomb collagen scaffold was collected in 2 mL of PBS with the help of a cell scraper. About 1 mL of the cell suspension containing 0.2% IGEPAL CA-630 (Sigma-Aldrich Co.), 10 mM Tris-HCl and 1 mM $MgCl_2$ (final concentrations), pH 7.4 was sonicated gently (Ultrasonic Disruptor, UD-201, Tomy Co., Ltd., Tokyo, Japan). It was centrifuged at 3,000 rpm for 5 min at 4°C and the supernatant was collected. The alkaline phosphatase activity in the supernatant was determined using an ALP assay kit (Wako, Japan) with *p*-nitrophenyl phosphate as substrate, following the method of Garen and Levinthal (1960). A standard curve was prepared using *p*-nitrophenol. The alkaline phosphatase activity is expressed as milli units (mU)/μg DNA. One unit of alkaline phosphatase is the activity of enzyme, which hydrolyzes 1 μmol of *p*-nitrophenyl phosphate in 1 min at 37°C under the conditions of the assay.

DNA Measurements

The DNA content in the above cell preparation was determined using Hoechst 33258 reagent (Polysciences, Inc., USA), which follows the fluorometric method of Labarca and Paigen (1980). In brief, 1 μg of Hoechst reagent in 0.05 M phosphate buffer containing 2.0 M NaCl was mixed with 100 μL of diluted sample. 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, Japan) was used as the standard.

Statistical Analysis

Arithmetic mean and standard deviation were calculated for the data. The control culture data were compared with the test culture data on different days using Student's *t*-test. The value of $P < 0.05$ was considered as statistically significant.

RESULTS

The results of the present investigation are demonstrated in Figures 1–6. The structure of the honeycomb collagen

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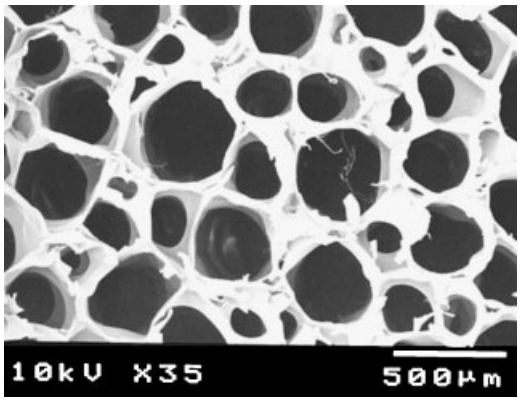

Figure 1. Scanning electron micrograph of a honeycomb collagen scaffold (×50).

scaffold prepared from the atelocollagen solution was studied by a scanning electron microscope (model JSM-5310LV, JEOL, Tokyo). Figure 1 demonstrates the scanning electron microscopic picture of the honeycomb collagen scaffold, which was cross-linked by UV-irradiation and stabilized by dry heat. As evident from the picture, the average pore size of the honeycomb collagen sponge was about 300 µm. Even though the honeycomb structure is biodegradable, it has the mechanical stability to hold the cells and did not fragment in the media during the culture period. The cross linking of collagen molecules by UV-irradiation during the preparation of the honeycomb structure helped to maintain the structural integrity of the scaffold. The structure of the scaffold was found to be very suitable for the proliferation, multiplication, and differentiation of mesenchymal stem cells into osteoblasts. The heterodimeric transmembrane glycoproteins such as integrins present on the cell surface carry negative charges, and the honeycomb collagen scaffold is positively charged. This opposite polarity would probably accelerate the quick attachment of the mesenchymal stem cells onto the collagen scaffold, which is a native extracellular matrix (ECM) protein.

The proliferation and differentiation of mesenchymal stem cells on honeycomb collagen scaffold is depicted on Figure 2.

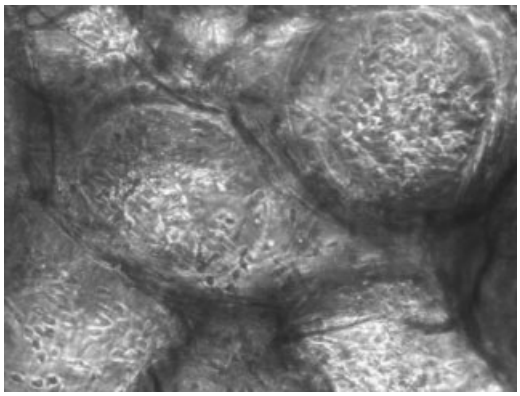

Figure 2. Proliferation of differentiated mesenchymal stem cells (osteoblasts) on a honeycomb collagen scaffold (×150). Day 14 after seeding the mesenchymal stem cells.

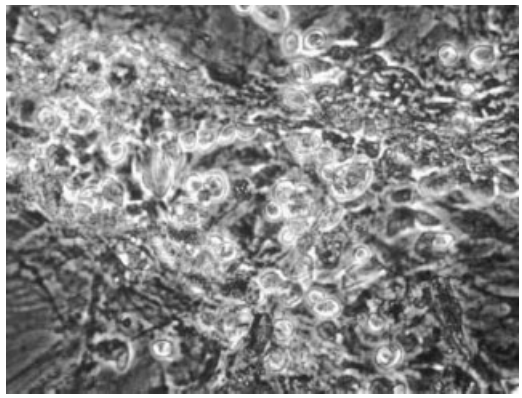

Figure 3. Low crystalline hydroxyapatite-like crystals (calcium-deficient hydroxyapatite) synthesized by the differentiated osteoblasts on a honeycomb collagen scaffold. Day 28 (×200). The image was taken using a phase contrast microscope after removing the honeycomb collagen disc from the culture for clearer visibility of the crystals.

The stem cells became spindle shaped within 24 h of plating. The spindle-shaped cells proliferated within the honeycomb structure, and slowly filled the honeycomb well. The entire honeycomb collagen scaffold was covered with differentiated osteoblasts by day 21 of culture. The synthesis of well-formed hydroxyapatite-like crystals by differentiated osteoblasts is demonstrated in Figure 3. Pure white crystals could be observed on day 28 inside the honeycomb collagen scaffold, as well as the surrounding area of the disc. Crystal formation was not observed in control cultures of mesenchymal stem cells without the honeycomb collagen scaffold. Von Kossa staining demonstrated dark brown colored nodular staining confirming the formation of minerals in the osteoblast cultures with honeycomb collagen scaffold. Von Kossa staining was negative in control cultures without the honeycomb collagen scaffold.

The X-ray diffraction spectrum of the microcrystals synthesized by the osteoblasts when cultured with honeycomb collagen scaffold is depicted in Figure 4. The powder X-ray diffraction pattern indicates a typical low crystalline hydroxyapatite similar to bone or dentine hydroxyapatite as seen in the Figure 4A with broad peaks. The chemical formula is represented as Ca_{10}-$xH_2x(PO_4)_6(OH)_2(x = 0–1)$, which is called a calcium deficient hydroxyapatite. On the other hand, the lower X-ray pattern with sharp peaks (B) indicates a typical stoichiometric hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ synthesized by a wet method using a calcium and phosphate ion solution reaction. The arrows in both figures indicate the characteristic peaks identified as hydroxyapatite.

The total collagen content synthesized by the differentiated osteoblasts during the culture on honeycomb collagen scaffold is presented in Figure 5. Synthesis of collagen is a characteristic feature of differentiated osteoblasts. Since honeycomb collagen scaffold is made up of 100% pure collagen, the total collagen synthesized by the differentiated osteoblasts is calculated by deducting the total collagen content of a fresh honeycomb collagen scaffold. A significant difference ($P < 0.001$) was observed in the total collagen

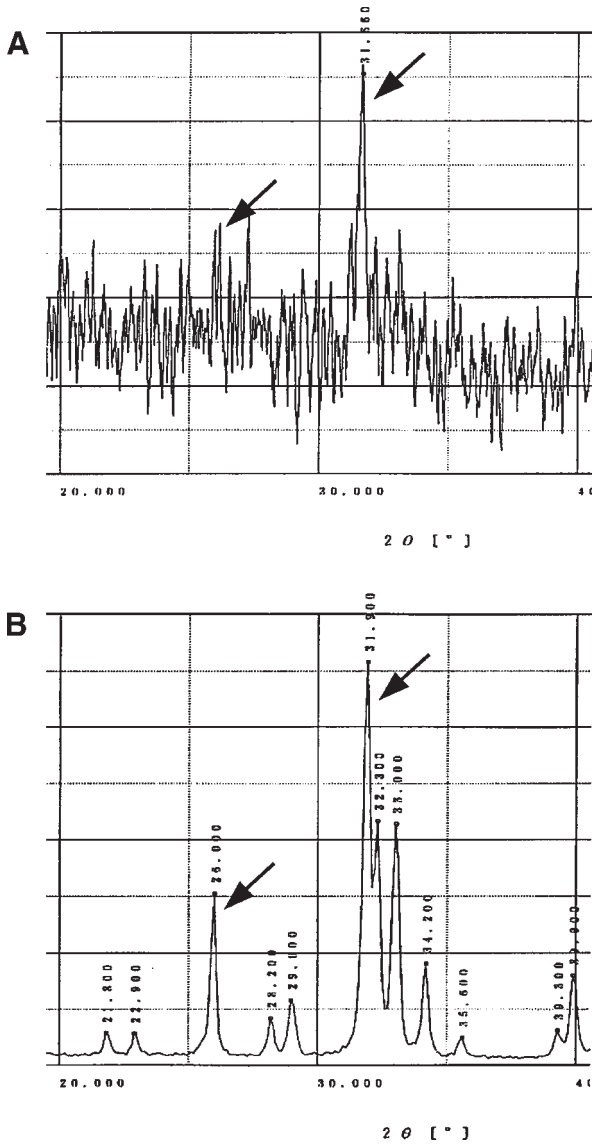

Figure 4. The powder X-ray diffraction pattern of the microcrystals synthesized by the differentiated osteoblasts on the honeycomb collagen scaffold. **A**: The X-ray diffraction spectrum demonstrated that the crystals synthesized by the osteoblasts are calcium-deficient hydroxyapatite. The lower X-ray pattern with sharp peaks (**B**) indicates a typical stoichiometric hydroxyapatite from mammalian bone. The arrows in both spectra indicate the characteristic peaks indicative of hydroxyapatite.

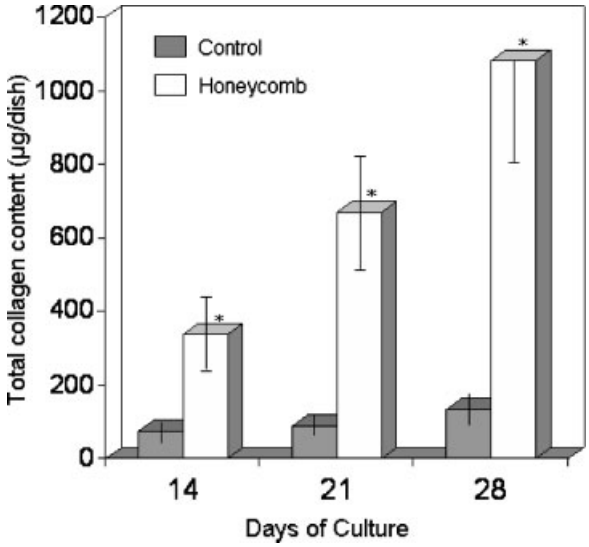

Figure 5. Total collagen content synthesized by the differentiated osteoblasts on the honeycomb collagen scaffold (*$P < 0.001$ when compared with respective controls, $n = 6$ at each time point for both control and experimental samples).

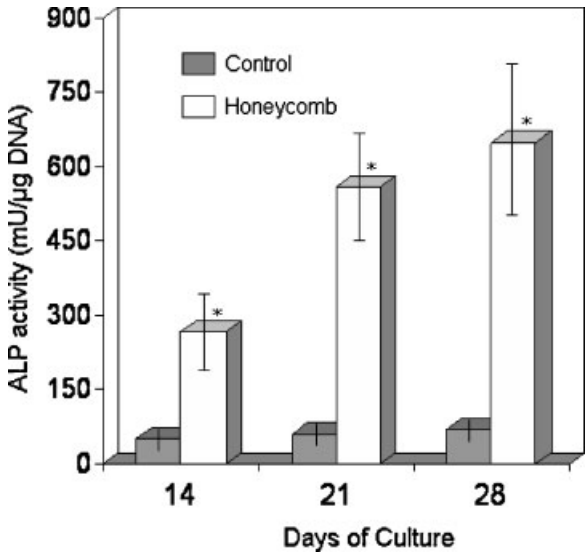

Figure 6. Alkaline phosphatase activity presented as a function of DNA during the culture of differentiated osteoblasts on a honeycomb collagen scaffold (*$P < 0.001$ when compared with respective controls, $n = 6$ at each time point for both control and experimental samples).

content on all days when compared with the respective control values. There was no significant difference in the total collagen content present in the cell harvest of control cultures without honeycomb collagen scaffold on days 21 and 28 when compared with day 14 values.

The alkaline phosphatase (ALP) activity of differentiated osteoblasts, normalized to DNA content, is illustrated in Figure 6. The alkaline phosphatase activity steadily increased in the cultures with honeycomb collagen scaffold on days 14–28. The difference was highly significant ($P < 0.001$) on all days when compared with the control cultures. About three-fold increase in the activity of ALP was observed on day 28 when compared with day 14 value. This indicated increased rates of differentiation of mesenchymal stem cells into osteoblasts in the latter periods of culture. Furthermore, the increased activity of ALP proved that the honeycomb collagen scaffolds provide an optimum environment for the normal function of osteoblasts.

DISCUSSION

Tissue engineering is emerging as a significant potential alternative to tissue or organ transplantation whereby implanted natural, synthetic, or semisynthetic tissues and

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organs are used that are fully functional from the start, or that can grow into the required functionality. Collagen, the most abundant protein in the animal body is an excellent and potential biomaterial scaffold for various tissue engineering applications. In biological systems, it provides room for cell attachment, differentiation, organogenesis, tissue regeneration, and repair. Collagen is mechanically stable with high tensile strength and can be altered into different sizes and shapes with various physical and chemical modifications. The excellent tissue compatibility, decreased antigenicity and biodegradability makes collagen a major resource in biomedical, biomaterial, and tissue engineering applications.

It has been demonstrated that the unique honeycomb collagen scaffold prepared from bovine dermal atelocollagen is a suitable carrier for various 3-D cell cultures and a compatible biodegradable material in the field of tissue engineering (Masuoka et al., 2005; Sato et al., 2003). The honeycomb collagen sponge has several distinctive characteristics such as mechanical stability under various physical and chemical conditions, for the exchange of nutrients and waste products between the honeycomb membranes, and for its ability to retain its unique structure throughout the study without deformity or collapse. 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 types of cells according to the required cell morphology and behavior. It was also reported that incorporation of a low concentration of hyaluronan in a 3-D collagen scaffold enhances matrix accumulation and cartilage specific gene expression (Allemann et al., 2001). In the present study, we demonstrated that honeycomb collagen scaffold is a suitable and biodegradable substratum for the proliferation and differentiation of rat bone marrow derived mesenchymal stem cells into osteoblasts without the presence of β-glycerophosphate and dexamethasone. The differentiated osteoblasts are capable of synthesizing the characteristic collagen and hydroxyapatite like microcrystals in culture.

Collagen is the most suitable and appropriate material for several biomaterial and biomedical applications (Lee et al., 2001; Miyata et al., 1992; Nimni, 1988; Patino et al., 2002). Various types of cells can attach, differentiate and proliferate to form a specific tissue or organ on a collagen scaffold. Different type-specific collagens may also play a role in cell attachment, differentiation, and proliferation for a particular type of cell, depending on organ or tissue of origin. Different mechanisms are involved in the attachment of cells to collagens (Rubin et al., 1981; Ruggiero et al., 1994; Schor and Court, 1979; Tandon et al., 1989). The basement membrane sulfated glycoprotein, entactin, has shown to promote cell attachment and chemotaxis (Chakravarti et al., 1990). The well-characterized arginine-glycine-aspartic (RGD) sequence is one of the major cell attachment sites in entactin and this sequence is recognized by the αvβ3 integrin receptor (Dong et al., 1995). Integrins play a major role in cell attachment and also determine how the cells interpret biochemical signals from their surrounding environment. The α1β1 and α2β1 integrins are the major collagen binding integrins, with α2β1 having a higher affinity for the fibrillar type I collagen, the major protein constituent of bone. The α2β1 integrin interaction with type I collagen is a crucial signal for the induction of osteoblastic differentiation and matrix mineralization (Mizuno and Kuboki, 2001; Mizuno et al., 2000). Furthermore, it was observed that α2β1 integrin specific collagen-mimetic surfaces supports osteoblastic differentiation (Reyes and Garcia, 2004).

The unique 3-D effects of the honeycomb scaffold may also be responsible for the attachment of mesenchymal stem cells and differentiation into osteoblasts. It was reported that the geometry of the cell scaffold is crucially important for vasculature induction and bone formation (Kuboki et al., 2001). It was also observed that honeycomb shaped hydroxyapatite tunnels, with a pore size of 300–400 μm, directly induces bone formation (Kuboki et al., 2001). In the present study, the pore size of the honeycomb collagen scaffold was within 200–400 μm, which could probably play a crucial role in the differentiation of osteoblasts. Furthermore, the wall of the honeycomb collagen scaffold may promote the attachment and deposition of autocrine cytokines and create a different environment from those of two-dimensional plastic dishes or collagen gels. Overall the 3-D cultures on a collagen scaffold provide the natural ECM environment with complex mechanical and biochemical interplay as with in living systems, which plays a vital role in the osteoblastic differentiation of mesenchymal stem cells.

Von Kossa staining and X-ray diffraction are two important tools used to examine mineralization in vitro. In the present study, staining demonstrated the formation of minerals in the osteoblast cultures with honeycomb collagen scaffold. The X-ray diffraction studies demonstrated that the microcrystals synthesized by the osteoblasts were calcium-deficient hydroxyapatite (pseudo hydroxyapatite) crystals (Fig. 5A). The X-ray patterns and the characteristic peaks correspond to those recorded for pure synthetic hydroxyapatite or mammalian bone apatite (Aoki, 1994). It is reported that bone marrow stromal cells cultured on type I collagen gel can synthesize calcified nodules in culture and can be demonstrated by Von Kossa staining and Energy-dispersive X-ray microanalysis (Hasegawa et al., 1994). Maniatopoulos et al. (1988) reported synthesis of calcium nodules in culture by rat bone marrow derived stromal cells while cultured with β-glycerophosphate and dexamethasone. The present study documented that the mesenchymal stem cells derived osteoblasts could synthesize bone-like hydroxyapatite in the presence of collagen scaffold with a unique honeycomb microenvironment.

Collagen synthesis is the primary function of differentiated osteoblasts (Koshihara and Honda, 1994). Collagen is the major constituent of bone and its unique triple helical structure provides mechanical stability for bone. In the present investigation, a steady state increase in the amount of total collagen indicated the capability of collagen synthesis

by the differentiated osteoblasts in culture. The 3-D structure and the natural ECM environment of the honeycomb collagen scaffold facilitated collagen synthesis by the differentiated osteoblasts.

Expression of alkaline phosphatase (ALP) activity is a characteristic feature of osteoblasts (Hillsley and Frangos, 1997; Rosa et al., 2003). In the present study, differentiated osteoblasts on the honeycomb collagen scaffold expressed ALP activity, which were increased to about three-fold on day 28 when compared with day 14. The increased expression of ALP activity indicated enhanced differentiation and proliferation of osteoblasts on the honeycomb collagen scaffold. In the present investigation, the cellular DNA content was increased in a steady state manner throughout the course of the study. Increase of DNA content in cell cultures was a measure of cell proliferation. In the 3-D environment on the honeycomb collagen scaffold, the cells proliferated and multiplied to a high-density manner within a short period of time, in contrast to conventional flat bed culture on dishes. This advantage of honeycomb collagen scaffold for 3-D cell cultures makes it uniquely suitable for tissue engineering applications.

Since honeycomb collagen scaffold is prepared from atelocollagen molecules, which do not contain the antigenic telopeptides, the antigenicity of atelocollagen is extremely low. Besides atelocollagen is extensively used in medical, cosmetic and tissue repair applications, with very little or no hypersensitivity reactions (DeLustro et al., 1986). It is also important that the tissue engineering scaffolds used for 3-D cell cultures should have biocompatibility and be biodegradable with little or no antigenicity (Baier Leach et al., 2003; Hutmacher, 2000). The development of an implanted tissue or organ is greatly influenced by composition, architecture and three-dimensional environment of the scaffold and its biocompatibility. The present study demonstrated that honeycomb collagen sponge is an excellent scaffold for the differentiation of mesenchymal stem cells. Using different concentrations of atelocollagen solution, it is possible to make diverse scaffolds of various sizes and shapes according to different organs or tissues such as ear, skin, liver, kidney, or cartilage. Such scaffolds can be used for 3-D cultures for specific cells either from autologous or heterologous sources. The different scaffolds prepared from atelocollagen are capable of maintaining the morphology and structural integrity, even after long-term 3-D cultures of various cells. 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. Cells are the key unit for tissue regeneration and repair, due to their differentiation, extensive proliferation and multiplication capabilities. High-density 3-D cell cultures have enormous potential in the field of tissue engineering. 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. These unique advantages make honeycomb collagen scaffolds an excellent material for high-density cell cultures and their applications to cell based therapies and tissue engineering.

In conclusion, the results of the present investigation demonstrated that the honeycomb collagen sponge is an excellent scaffold for the differentiation and proliferation of mesenchymal stem cells into osteoblasts. It also proved that honeycomb collagen is an effective substrate for tissue engineering applications and may become very useful in the rapidly advancing field of stem cell technology and cell based therapy.

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