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Induction of Mineralized Nodule Formation in Rat Bone Marrow Stromal Cell Cultures by Silk Fibroin

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ABSTRACT

Background: Silk fibroin is a suitable protein for osteogenesis by inducing markers of bone formation in the cultures of osteoblasts, so we examined the ability of this protein to induce mineralized nodules in the rat bone marrow stromal cell cultures. **Methods:** Bone marrow stromal cells obtained from 4 to 6 weeks old Spruge-Dawely male rats were grown in primary culture for seven days and then subcultured for 21 days. The secondary cultures were done on either silk fibroin-coated polystyrene plates or free-silk fibroin ones. After 21 days of grow up, the cultures were examined for nodule formation by scanning electron microscopy, for mineralization by alizarin red S staining and for expression of gene markers of osteoblast maturation by reverse transcription PCR (RT-PCR). **Results:** The stromal cells were observed to form three-dimensional nodules when cultured on the silk fibroin and compared to the stromal cells cultured on the free-silk fibroin polystyrene plates, where no nodules were observed in the time-frame studied. These nodules were also found to be mineralized and expressed the gene markers of osteoblast. **Conclusion:** Silk fibroin could serve as suitable inducing factor by stimulating stromal cell differentiation to form mineralized nodules. *Iran. Biomed. J. 10 (3): 133-138, 2006*

Keywords: Bone marrow stromal cells, Mineralization, Fibroin, Culture

INTRODUCTION

Silkworm silk consists of two protein components: fibroin which is the structural protein and sericins which are the water-soluble glue-like proteins that bind the fibroin fibers together [1]. Silk fibroin consists of heavy and light chain polypeptides, connected by a disulfide link [2, 3] and it is a protein dominated in composition by the amino acids glycine, alanine and serine which form antiparallel β-sheets in the spun fibers [4, 5]. After removal of the sericin protein, purified silk fibroin as a natural polymer exhibits many of the prerequisites required of a scaffolding material for tissue engineering [6-9]. However, few studies have been carried out to examine the growth and differentiation of bone marrow stromal cells on this material. Silk scaffolds are suitable for osteogenesis of bone marrow stromal cells [10, 11]. Also, silk films have potential to induce markers of bone formation in the cultures of osteoblasts [12]. On the basis of these findings, silk fibroin would be expected to conduct osteoblast differentiation of rat bone marrow stromal cells. To address this issue, the ability of silk fibroin to form mineralized nodules *in vitro* was examined by light and scanning electron microscopy and by reverse transcription PCR (RT-PCR) for expression of gene markers of osteoblast differentiation (Bone morphogenetic protein-2 [BMP-2], Type 1 collagen, and osteocalcin).

MATERIALS AND METHODS

Primary cultures. Bone marrow cells were obtained from the femur of 4-6 weeks old male Spruge-Dawely rats. Animals were killed by overdose chloroform. Both femora were removed and the soft tissues were detached aseptically, metaphyses from both ends were resected and bone

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marrow cells were collected by flushing the diaphisis with a culture medium. A suspension of bone marrow cells was obtained by repeated aspiration of the cell preparation through a 20-gauge needle, cells obtained from each femur, plated into 60 mm tissue culture dishes (Falcon, USA) , and incubated at 37ºC for 1 h to promote adherence to the substrate. Growth medium (2 ml), [alpha minimal essential medium (alpha-MEM, GIBCO-BRL, USA), pH 7.2 (containing 2.2 g/l sodium bicarbonate +15% FCS (Atlanta Biologicals, USA) +100 units/ml of penicillin/100 µg/ml of streptomycin (GIBCO-BRL, USA) was added to each dish and then an additional 2 ml was added after 24 h and cultured for seven days as primary cultures.

Preparation of silk fibroin. Raw silkworm silk was degummed twice with 0.4 g/l $NaHCO_3$ solutions at 100°C for 1 h that removed the sericin and then washed with distilled water. Degummed silk was dissolved in 9.5 mol/l LiBr (Sigma, USA) solution at a ratio of 3 g/l. After dialysis against distilled water for three days, the solution was filtered, and the silk fibroin solution was obtained [13]. A 5-ml volume of the silk fibroin solution (5 mg/ml) was added to each well of a six-well polystyrene plate (Falcon 1018, USA) and incubated at 25°C for 5 min, followed by decantation of the solution. After keeping the plate at 30°C for 2 h, 5 ml of the fibroin solution was added again to the well, and the plate was incubated at 25°C for 5 min. The plate was dried at 50°C for 1 h. A 5-ml volume of 50% methanol was added to the well and incubated at 25°C for 10 min. After drying the plate again at 50°C for 1 h, the silk fibroin-coated plate was prepared.

Secondary cultures. After seven days in primary cultures, the confluent monolayer was released with 0.25% trypsin-EDTA (Sigma, USA), and then 4 ml of medium (alpha-MEM) containing 1-3 × 10^6 cells which supplemented with 10 mM sodium beta-glycerophosphate (GIBCO-BRL, USA) and 50 µg/ml ascorbic acid (Sigma, USA) was placed onto silk fibroin-coated plates. The plates with cells were incubated in 5% CO_2, at 37°C for 21 days. Growth medium was changed every second day. For control groups, 4 ml of the above mentioned medium containing 1-3 × 10^6 cells was directly placed onto a six-well polystyrene plate (Falcon 1018, USA) and incubated at 37°C, 5% CO_2 for 21 days. Each experiment was repeated six times in each group.

Culture examination. Cultures were examined daily using inverted microscope (Olympus, Japan). Some cultures were fixed in 10% neutral buffered formalin and stained with either H and E or toluidine blue (Sigma, USA).

Scanning electron microscopy. After 21 days, cells in secondary cultures were fixed in 1.5% glutaraldehyde at 4°C for 30 min and then, post-fixed in 1% osmium tetroxide at 4°C for 1 h. Then were dehydrated through a series of increasing concentrations of ethanol and dried using hexamethyldisilazane. Samples were sputter coated with gold and viewed using a scanning electron microscope (S-4500, Hitachi, Japan).

Mineralization assay. Mineralization of nodules was determined using alizarin red S staining which is a common histochemical technique used to detect calcium deposits in mineralized tissues and cult [14, 15]. Cells in secondary cultures after 21 days were fixed in 70% ethanol and stained with 1% alizarin red S (Sigma, USA), pH 6.4, for 5 min. Cells were then washed with distilled water and viewed under the inverted microscope.

RT-PCR. Total RNA was isolated from each silk fibroin-coated plate and after 21 days, controls subcultured using Trizol reagent (GIBCO-BRL, USA). The RNA pellet is washed with 70% ethanol. RNA Concentration was determined at 260 nm and was analyzed for markers of osteoblast maturation: BMP-2, Type 1 collagen, and osteocalcin. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as a housekeeping gene. Equivalent amount of RNA samples (0.5 mg) was initially reverse transcribed for first strand cDNA synthesis (GIBCO-BRL, USA). Synthesis of cDNA entailed using oligo (dT) as a RT primer, which binds to the poly A tail of the mRNA. After the RT reaction, remaining RNA was removed by RNase treatment. Template DNA was then used in gene specific PCR for GAPDH, BMP-2, Type I collagen, and osteocalcin [16]. cDNA was amplified by PCR with oligonucleotide primers (Table1). All RT-PCR products were visualized on 1.5% agarose gel with 0.5 mg/ml ethidium bromide. Photographs were taken under ultraviolet illumination (Bio-Rad, USA) and qualitative assessments were made of relative gene expression.

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Table 1. Primers sequences and cycle conditions used for the RT-PCR.

Gene	Sequence	Cycling conditions	Amplicon
Type I collagen	F: 5-CCTGGTAAAGATGGTGCC-3 R: 5-CACCAGG TTCAC CTTTCGCACC-3	25 cycles; 94°C 30 s; 58°C 30 s; 72°C 1 min	222 bp
BMP-2	F: 5-AGTTCTGTCCCCAGTGACGAGTTT-3 R: 5-GTACAACATGGAGATTGCGCTGAG-3	36 cycles; 95°C one min; 63°C 30 s; 72°C 1 min	708bp
Osteocalcin	F: 5-CCTCAGTCCCCAGCCCAGATCC-3 R: 5-CAGGGCAGAGAGAGAGGACAGG-3	25 cycles; 94°C 30 s; 58°C 30 s; 72°C 1 min	219 bp

BMP-2, Bone morphogenetic protein-2.

RESULTS AND DISCUSSION

Subcultures. In the fibroin-coated cultures, nearly all of the stromal cells attached to silk fibroin up to 6-8 h of culture and formed cell colonies representing early nodule formation at day 14 (Fig. 1) while in the control groups, the stromal cells attached to floor of culture dish up to 24 h and they did not form any colonies up to 21 days but they formed a monolayer of cells (Fig. 2).There were not any meaningful differences between cultures in each group.

It has shown that three-dimensional silk fibroin nets support the attachment, spread and growth of a variety of human cell types of diverse tissue origins. These includes epithelial, fibroblast, glial, keratinocyte and osteoblast cells [17, 18]. Cell adhesion is important because it directly influences cell growth and differentiation. In the present study, the silk fibroin improved cell adhesion when compared to the control cultures. This improvement can be attributed to the changes in surface texture.

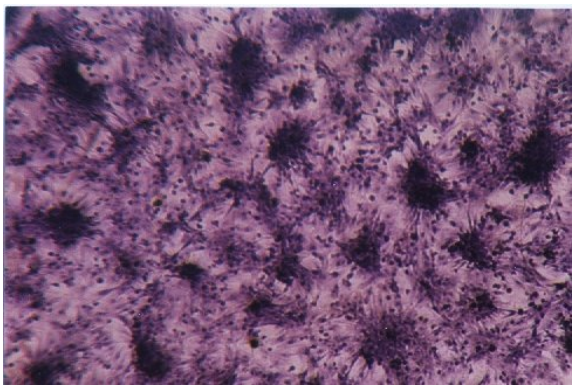


Fig. 1. Cell colonies in secondary cultures on silk fibroin-coated plates at day 14 (Tolidine blue staining, × 100).

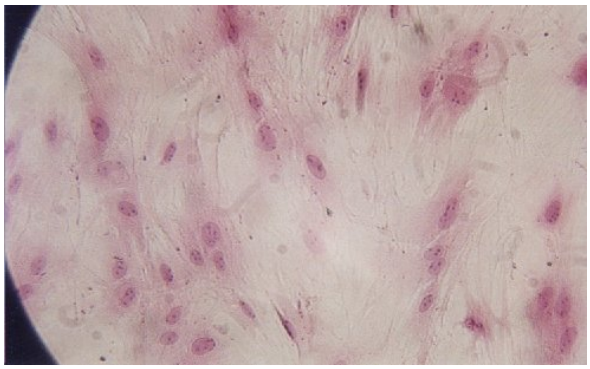


Fig. 2. Stromal cells in secondary cultures of control groups at day 14. They are forming a monolayer of cells and no cell colonies were seen (H and E staining, × 400).

Also fibroin is considered to be hydrophilic protein [18], and this property might be favorable for the interaction with the negatively-charged surface of animal cells. Since the outermost surface of biomaterials directly interfaces with the cells, the biocompatibility of silk fibroin would be different from that of the polystyrene plate as the control. Therefore, the behavior of the stromal cells cultured on the free-silk fibroin plates and the silk fibroin-coated plates would be different.

Nodule formation and mineralization. At day 21, three-dimensional nodule formation was observed in the fibroin-coated cultures as shown in Figure 3 by scanning electron microscopy. No nodule formation was observed in control groups at day 21. As shown in Figure 4 the nodules in the fibroin-coated cultures were stained heavily with alizarin red S. It was found that control cultures showed no alizarin red S staining.

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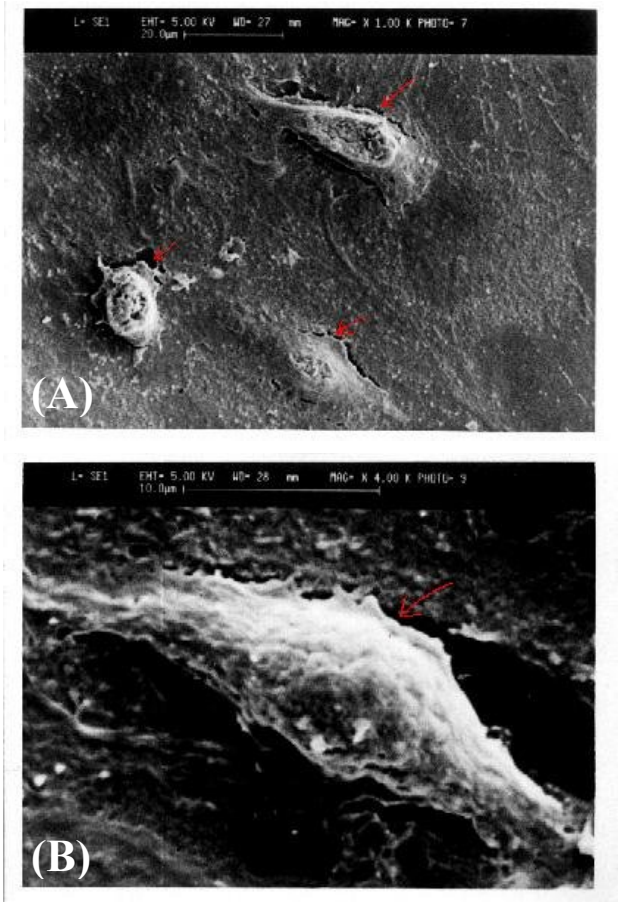


Fig. 3. Scanning electron micrographs of three-dimensional calcified nodules from stromal cells cultured for 21 days on silk fibroin-coated plates: **(A)**, Low power of three nodules (× 200); **(B)**, Higher power of one nodule (× 1200).

RT-PCR. RT-PCR analysis was performed to assess progression of BMSC towards the osteoblast lineage at the mRNA gene expression level. Expression of genes coding for BMP-2, Type I collagen, and osteocalcin in BMSC after 21 days of culture was assessed. Remarkably, the osteoinductive effects of silk fibroin-coated plates were obvious. In the control cultures, there was no expression or weak expression of these genes (Fig. 5). Previous studies have shown that stromal cell cultures only form mineralized nodules in the presence of additional factors like dexamethasone [20-23]. In the present study, mineralized nodules were observed in the absence of dexamethasone demonstrating the remarkable ability of the silk fibroin to cause mineralized nodule formation and indicate that this protein serve as suitable mineralized nodule-inducing factor. This is important with regard to developing materials for bone repair or bone tissue engineering/regeneration because many materials are biocompatible with regard to certain cell types but often require addition of growth factors to improve the cell responses [24].

An interesting finding is the significant increase in TGFß1; one of the primary cytokines associated with the growth and regulation of repair of bone [25]; observed in the silk fibroin cultures compared to the synthetic polymeric material controls [26].

Increased differentiation of osteoblast precursors by inducing mesenchymal cells to differentiate into osteoblasts [26] and proliferation of osteoblasts [28], and increased collagen synthesis [29] has been shown in the presence of TGFß1 in culture. Thus, the effect of silk fibroin on osteoblast differentiation and formation of mineralized nodule in part may be due to stimulation of stromal cells to express TGFß1 and it should be studied in the future. Weak expression of the genes in the control groups indicate that the stromal cells have potential to differentiate to osteoblasts without any inducer supplements but it is not enough to produce mineralized nodules.

Collectively, these results indicate that silk fibroin could serve as suitable inducing factor by stimulating stromal cell differentiation to form mineralized nodules.

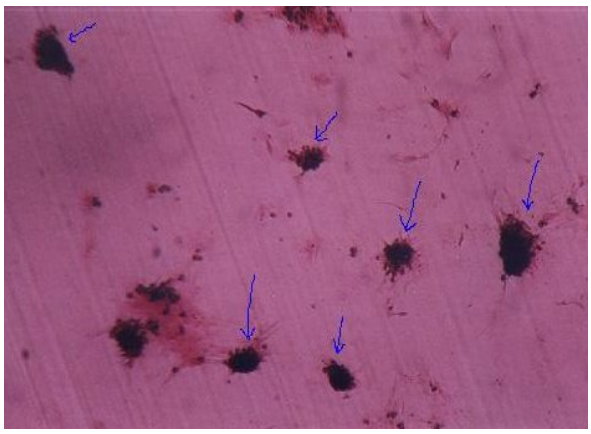
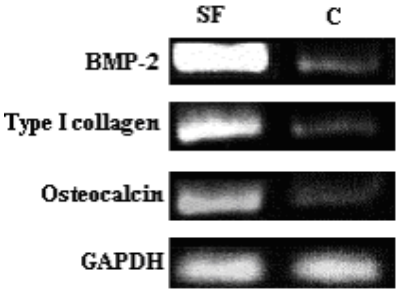


Fig. 4. Light micrograph of bone nodules from stromal cells cultured for 21 days on silk fibroin-coated plates. Positive dark red staining shows presence of calcium deposits i.e. mineralization (alizarin red S staining, × 100).

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5. Gene expression of BMP-2, Type I collagen, and osteocalcin using RT-PCR. GAPDH was used as a housekeeping gene. Note to strong expression in the silk fibroin-coated plates and weak expression in the control plates. SF, silk fibroin-coated plates; C, Control plates.

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