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Low molecular weight silk fibroin increases alkaline phosphatase and type I collagen expression in MG63 cells

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Silk fibroin, produced by the silkworm *Bombyx mori*, has been widely studied as a scaffold in tissue engineering. Although it has been shown to be slowly biodegradable, cellular responses to degraded silk fibroin fragments are largely unknown. In this study, silk fibroin was added to MG-63 cell cultures, and changes in gene expression in the MG-63 cells were screened by DNA microarray analysis. Genes showing a significant (2-fold) change were selected and their expression changes confirmed by quantitative RT-PCR and western blotting. DNA microarray results showed that *alkaline phosphatase (ALP), collagen type-I alpha-1, fibronectin,* and *transforming growth factor-β1* expressions significantly increased. The effect of degraded silk fibroin on osteoblastogenic gene expression was confirmed by observing up-regulation of ALP activity in MG-63 cells. The finding that small fragments of silk fibroin are able to increase the expression of osteoblastogenic genes suggests that controlled degradation of silk fibroin might accelerate new bone formation. [BMB reports 2010; 43(1): 52-56]

INTRODUCTION

Silk protein spun by the silkworm *Bombyx mori* mainly consists of fibroin and sericin (1). Due to the presence of sericin, silk may cause immunological and allergic reactions (2). However, silk fibroin is reported to induce minimal inflammatory reactions (3). Silk fibroin is a biomaterial that has been used in surgical sutures for several thousand years due to its good mechanical and biological properties, which, in addition to its low potential for inflammatory reaction, include high strength and biocompatibility (3). In addition, silk fibroin has other advantageous characteristics, including good water vapor and oxygen permeability (4, 5) and blood compatibility (6). Recently, silk fibroin has been studied for use as a wound dressing and as a scaffold for bone and cartilage regeneration (7-10).

Silk is a high molecular weight (300 kDa) natural protein polymer that has been approved as a biomaterial by the U.S. Food and Drug Administration (FDA), which has classified it as a nonabsorbable material according to US Pharmacopeia (1). However, silk proteins produced by insects have been shown to be enzymatically degradable and absorbed *in vivo* (1). It has been recently reported that degradation of silk is dependent on the morphology of the silk material as well as treatment methods (11). For example, some silk materials have been degraded by more than 70% of their original weight within 15 days at 37°C using protease (12). If silk fibroin decomposes *in vivo*, the small molecular weight protein decomposition products may affect the cellular activity. The impacts of such degraded products on osteoblasts have not been previously reported.

The objective of this study was to screen for changes in gene expression when a small fragment of silk fibroin was applied to human MG-63 osteoblast-like cells. DNA microarray analysis was used to search for affected genes. Among the MG-63 genes, osteoblastogenic gene expressions increased following silk fibroin introduction, and their expressions were subsequently confirmed using a quantitative reverse transcription polymerase chain reaction (RT-PCR) or by western blot analysis.

RESULTS AND DISCUSSION

The DNA microarray results are shown in Table 1. The expressions of alkaline phosphatase (*ALP*), collagen type *I-alpha1 (Col1A1)*, and *transforming growth factor-β1 (TGF-β1)* were significantly increased (fold ratio > 2.0, Table 1). In addition, there was a significant increase in the expressions of *fibroblast growth factor receptor-1 (FGFR1), fibronectin, and interleukin 16* (fold ratio > 2.0, Table 1). In the subsequent real-time RT-PCR and ALP assays, the small fragments of silk fibroin increased both *ALP* expression (Fig. 1A) and enzyme activity (Fig. 1B). Compared with the untreated control cells, MG-63 cells

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Table 1. The results of cDNA microarray

Title	GenBank	Chromosome	Fold-ratio
Alkaline phosphatase	NM_001632	2q37.1	2.055
Collagen type I, alpha 1	NM_000088	17q21.33	2.221
Collagen type V, alpha 3	NM_015719	19p13.2	2.054
Fibroblast growth factor 7	NM_002009	15q15-q21.1	3.945
Fibroblast growth factor receptor 1	NM_023110	8p11.2-p11.1	2.027
Fibronectin 1	NM_054034	2q34	5.735
Insulin-like growth factor 2	NM_001007139	11p15.5	6.719
Interleukin 16	BC040272	15q26.3	3.348
TIMP metallopeptidasse inhibitor 3	NM_000362	22q12.3	3.967
Transforming growth factor, beta 1	NM_000660	19q13.2; 19q13.1	3.072
Bone morphogenetic protein 8b	NM_001720	1p35-p32	−2.300
Interleukin 7	NM_000880	8q12-q13	−2.053
Matrix metallopeptidase 3	NM_002422	11q22.3	−9.009

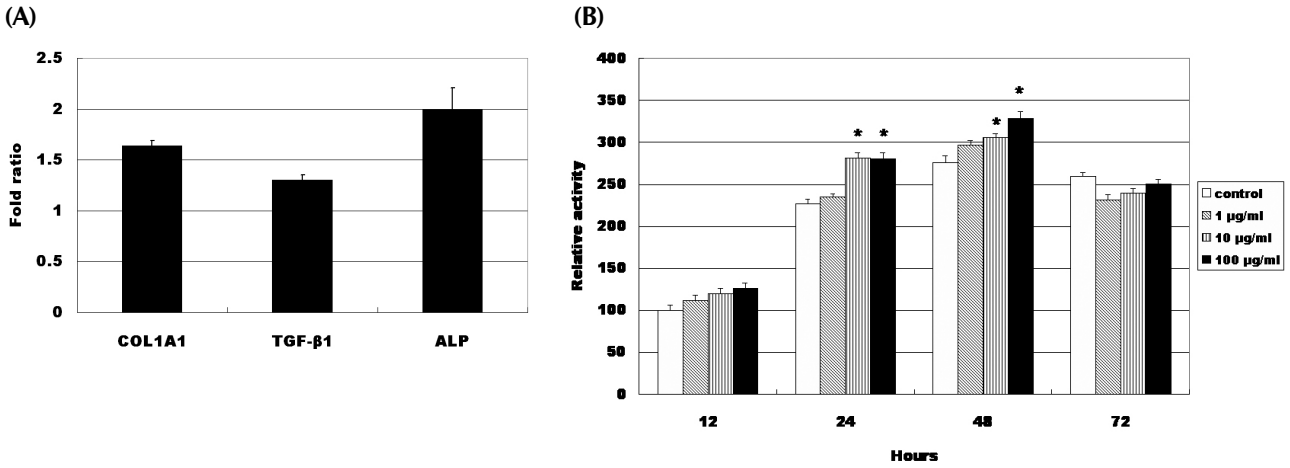

Fig. 1. (A) Real-time RT-PCR results. The fold ratios are values relative to the control value. The expressions of *type I collagen a1 (COL1A1), transforming growth factor-β1 (TGF-β1), and alkaline phosphatase (ALP)* were significantly higher in MG-63 cells treated with fragmented silk fibroin compared to the untreated control ($P < 0.001$ for COL1A1 and ALP; $p=0.002$ for TGF-β1). (B) Results of the ALP assays. ALP activity in MG-63 cells treated with 1, 10, and 100 μg/ml concentrations of silk fibroin is compared with untreated control cells. Treated cells exhibited increases in ALP activity up to 48 hours after application, with significant increases at 24 and 48 hours (*$P < 0.05$).

treated with 1, 10, and 100 μg/ml concentrations of degraded silk fibroin exhibited increases in ALP activity up to 48 hours after application (Fig. 1B). In particular, the 10 and 100 μg/ml silk fibroin application increased ALP activity significantly at 24 and 48 hours after application when compared to their respective controls ($P < 0.05$) (Fig. 1B). However, there was no significant difference 72 hours after the application ($P > 0.05$) (Fig. 1B). Therefore, the degraded silk fibroin fragments appear to result in an increase in the expression of ALP. Cai *et al*. (13) have reported that a poly (D,L-lactic acid) surface modified with 30 kDa of silk fibroin showed more ALP activity than an untreated poly (D,L-lactic acid) surface. In addition, MC3T3-E1 cells cultured on a silk fibroin membrane were reported to show ALP activity levels comparable to those on a surface-treated culture dish (14). Other researchers have demonstrated that when silk fibroin is cultured with the bone cells or stem cells, bone tissue growth *in vitro* is induced (9, 10). Since ALP is an early osteogenic marker, and is related to the mineralized product, its increased expression following the addition of a small fragment of silk fibroin indicates that silk fibroin may have osteogenic potential. Col1A1 expression also increased following the addition of silk fibroin fragments (Table 1, Fig. 1A). Along with hydroxyapatite, type I collagen is a main component of bone. The increases in the expression of both ALP and type I collagen by silk fibroin fragments suggest that degraded fragments may be helpful in new bone formation.

However, it is unclear whether macromolecular silk fibroin in its undigested form can increase ALP activity. If the macromolecular silk fibroin in its undigested form cannot increase ALP activity, then silk fibroin used for scaffolding in bony defects should be designed to degrade in a timely manner. The molecular weight of heavy chain silk fibroin is 325 kDa, while that of

light chain silk fibroin is 25 kDa (1), and the structure of silk fibroin may be a random-coil with amorphous regions or an anti-parallel β-sheet (15). The latter structure is hydrophobic and soluble under acidic conditions. The rate of silk fibroin degradation can be altered by varying the porosity and molecular weight distribution of the silk fibroin, with low molecular weight and less-compactly structured silk fibroin resulting in a more rapid degradation (1, 11). The molecular weight of our hydrochloric acid degraded product was between 0.5 and 1.0 kDa (data not shown).

In this study, the expression of *fibronectin,* which is involved in cell adhesion (16-18), increased in the silk fibroin treated cells (Table 1, Fig. 2). Fibronectin also plays a major role in cell adhesion, growth, migration, and differentiation (16, 17), and it is important in processes such as wound healing and embryonic development (18). Altered fibronectin expression, degradation, and organization have been associated with a number of pathologies, including cancer and fibrosis (19). Previous experiments have indicated that silk fibroin appears to promote wound healing by increasing the expression of genes that are related to tissue healing (6, 7). Furthermore, silk fibroin is reported to be non-cytotoxic and able to promote cell adhesion and proliferation in an immortalized fibroblast cell line (20).

TGF-β1 expression also increased following the application of fragmented silk fibroin (Table 1, Fig. 2). Osteoarthritis (OA) is a leading cause of morbidity in aging populations and is characterized by degradation and loss of cartilage, inflammation of the synovium, formation of osteophytes, and bone sclerosis (21). Type I collagen levels are elevated in the trabecular bone within the femoral heads of patients with OA, which can lead to an increase in mineralization (22). Osteoblasts from OA patients are reported to show elevated levels of *TGF-β1* (23), and the expression of *TGF-β1* is higher in OA bone than in normal bone tissue (24). However, the expression of TGF-β1 has also been reported to increase with inflammation (25). Therefore, increased expression of TGF-β1 following the addition of silk fibroin should be cautiously interpreted.

Silk fibroin is useful as a biocompatible scaffold, and has been mixed with hydroxy apatite (26) or BMP-2 (9) to form bone graft materials. Such pre-mineralization of highly porous silk fibroin protein scaffolds has been reported to enhance the outcome of bone tissue engineering (27). We speculate that a highly porous structure might not only provide for improved cellular growth, but also may induce easy breakdown of the scaffold. Our animal research involving silk fibroin scaffolds with small pore sizes showed that new bone formation was only observed after the breakdown of the silk scaffold (manuscript in preparation).

In conclusion, small fragments of degraded silk fibroin induced increases in ALP, type I collagen, and fibronectin levels. Therefore, timely degradation of a silk fibroin scaffold may be useful in the promotion of osteogenesis.

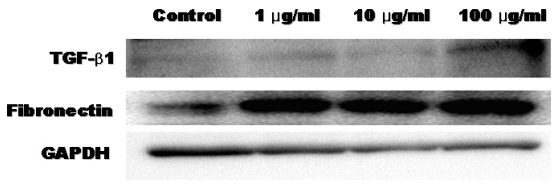

Fig. 2. The results of western blotting. The expression levels of fibronectin and TGF-β1 were increased in MG-63 cells treated with fragmented silk fibroin.

MATERIALS AND METHODS

Silk powder

Raw silk obtained from the Rural Development Administration (Suwon, Korea) was degummed twice using a Marseilles soap (0.5% of weight of fiber) and sodium carbonate (0.3% of weight of fiber) solution at 100°C for 1 hour. The resulting sericin-free silk was then washed with distilled water. Subsequently, the silk fibroin was dissolved in 6 N hydrochloric acid for 5 hours. The acid degradation was stopped by adding sodium hydroxide. An electrodialysis system (Acilyzer-02, Astom, Tokyo, Japan) was used to remove the salt residues. The molecular weight of the thus obtained degraded silk fibroin was between 0.5 and 1.0 kDa.

Cell culture

In vitro tests were performed using MG-63 osteoblast-like cells (ATCC, Manassas, VA, USA). The cells were grown to 80% of confluence in Dulbecco's modified Eagle's medium-high glucose (PAA Laboratories, Linz, Austria), containing 1% penicillin/streptomycin (100X), supplemented with 10% fetal bovine serum (PAA Laboratories, Etobicoke, ON, Canada) and kept at 37°C in an atmosphere of 5% CO_2 and 99% relative humidity. Media were changed every three days.

DNA microarray analysis

The culture medium was removed from cultured MG-63 cells at 2 hours after 10 μg/ml concentration of degraded silk fibroin application, and the total RNA was extracted using easy-BLUE (iNtRON Biotechnology, Sungnam, Korea), according to the manufacturer's protocol. The concentration of total RNA was measured using a spectrophotometer (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). The synthesis of cDNA was done from the extracted total RNA using a reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA). The reactions were primed with 3 μl of oligo (dT) and 1 μl of 10 mM dNTP mixture. Diethyl pyrocarbonate-treated tertiary distilled water was added to raise the total volume to 40 μl. The reaction was performed at 65°C for 5 minutes; subsequently, the temperature was slowly lowered to room temperature. Next, 2 μl of 10x first-strand buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, 1 μl RNased block ribonucleotide inhibitor (40 units/ml), and 1μl RTase were added. The total volume thus became 50 μl and the reaction was done at 42°C for 1 hour.

Commercially available microarray chips (G4112A, Agilent

Technologies) were used and the microarray procedure accorded with a previously-published procedure (28). The chips comprised approximately 41,000 (60-mer) oligonucleotide probes, which spanned conserved exons across the transcripts of the targeted full-length genes. To stratify which of the 41k chips (41,000 genes) to evaluate, a comparative analysis of the combined test pool and the average of the combined control pool was performed. This generated a scatter plot (supplementary Fig. 1) showing subsets of genes that were up- or down-regulated.

Real-time RT-PCR

Three genes of 4422 genes that showed significant up- or down-regulation during microarray analysis were randomly selected, and real time RT-PCR was performed to confirm the microarray results. Real-time RT-PCR was performed for relative quantification of mRNA levels for the genes of interest using a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primers were designed using Primer Express software (Applied Biosystems); see supplementary Table 1. Total RNA (2 μg) was reverse-transcribed and 200 ng of cDNA was used as template in each PCR. SYBR Green PCR Master Mix (25 μl) and 1 μl of 10 pmol of the specific primers were combined with the template, and distilled water added to create a total volume of 50 μl. A negative control with no template was also included in each assay. PCR was performed at 95°C for 10 min (denaturation) and then 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. The cycle threshold (Ct) values, corresponding to the PCR cycle number at which fluorescence emission in real time reached a threshold above the baseline emission, were determined. The Ct value assigned to a particular well thus reflects the reaction time in which the number of amplicons that indicate a statistically significant increase above the baseline accumulate in that well. Relative expression was calculated as a ratio of the selected gene's expression compared to the expression of glyceraldehydes 3-phosphate dehydrogenase (GAPDH). Measurements were taken three times and the average value was used for comparison. Measured values were compared to the corresponding value in the microarray.

Alkaline phosphatase assay and western blot analysis

ALP was evaluated from the transformation of p-nitriphenylphosphate into p-nitrophenol at 37°C and pH 10.2 using appropriate reactive (Sigma, St. Louis, MO, USA) while the specific activity of ALP was calculated with regard to the protein concentration of lysates determined by means of a commercially available colorimetric assay (#71230, AnaSpec, Freemont, CA, USA).

Samples for western blotting were mixed with reducing sodium dodecyl sulfate buffer, heated, and electrophoresed on 10% polyacrylamide gels according to the method of Laemmli (29). Gels were then electroblotted onto a polyvinylidene difluoride membrane for immunoblot analysis. After blocking for 1 hour with 5% nonfat dry milk in phosphate buffered saline with 0.1% Tween 20 (PBST; Bio-Rad Laboratories, Hercules, CA, USA), blots were probed with primary antibody diluted in 0.5% milk in PBST (for 1.5 hour at 25°C) and then horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, diluted to 1 : 50,000. Sources and specifications of the primary antibodies are as follows; fibronectin (ab6328, Abcam, Cambridge, UK), TGF-β1 (sc-146, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and GAPDH (ab9385-200, Abcam). The antibody dilution ratio was 1 : 500. Signals were detected by chemiluminescence using an ECL kit (RTN2106, GE health care, Buckinghamshire, UK).

Statistical analysis

For the analysis of microarray results, the genes that showed more than 2-fold changes in the Cy3/Cy5 or the Cy5/Cy3 values were regarded as genes that were influenced significantly by the application of the silk fibroin fragments. The differences between mean values of experimental and control samples in the real-time RT-PCR and ALP assays were evaluated by independent sample t-tests. The level of significance was set as $P < 0.05$.

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