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Molecular weight-associated cellular response to silk fibroin fragments demonstrated in MG63 cells

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Abstract

In this study, changes in gene expression after administration of silk fibroin fragments (size ≈ 30 kDa) were evaluated in MG63 cells using a cDNA microarray assay. In addition, the level of alkaline phosphatase (ALP) activity and cellular proliferation in the group administered moderately sized silk fibroin fragments (size ≈ 30 kDa) (MSF) were compared to those in the group administered smaller silk fibroin fragments (size < 1 kDa) (SSF). The results of the cDNA microarray assay show increased expression of genes that are related to the cell cycle and inflammation. ALP, bone morphogenetic protein-7, bone morphogenetic protein receptor type IA, and runt-related transcription factor 2 exhibited significantly lower expression compared to control cells (fold ratio < 0.5). Relative ALP activity of the 100 μg/mL MSF group was significantly lower than that of the SSF group ($P < 0.05$). Thus, the MSF group showed increased expression of genes associated with cellular proliferation and inflammation but decreased expression of genes associated with osteogenesis.

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Introduction

Natural polymers have been studied for tissue engineering and show different host responses from those of synthetic materials (Morris *et al*., 2017). Collagen and silk proteins, which are composed of amino acids, are the most widely studied natural polymers. Although degradation velocity after implantation varies among proteins, most natural polymers composed of protein will eventually undergo proteolysis (Liu *et al*., 2015). Therefore, host interactions with fragmented proteins are important for determining the biological behavior of implanted materials.

Silk fibroin is produced by *Bombyx mori* and is used as a scaffold for tissue engineering (Yoo *et al.*, 2016). Silk fibroin is the degumming product of the natural cocoon of silkworm. As silk fibroin is considered non-toxic, it has been used for blood vessel regeneration (Park *et al*., 2015), sutures (Jo *et al*., 2017), and as a bone regeneration membrane (Seok *et al.*, 2014; Yoo *et al.*, 2016). Silk fibroin has a high molecular weight of over 300 kDa (Cao and Wang, 2009). Two-thirds of silk fibroin is composed of hydrophobic blocks (Cao and Wang, 2009). Silk fibroin fragments with low molecular weight (<1 kDa) show osteogenic potential in MG63 cells (Kim *et al*., 2010). However, to the best of our knowledge,

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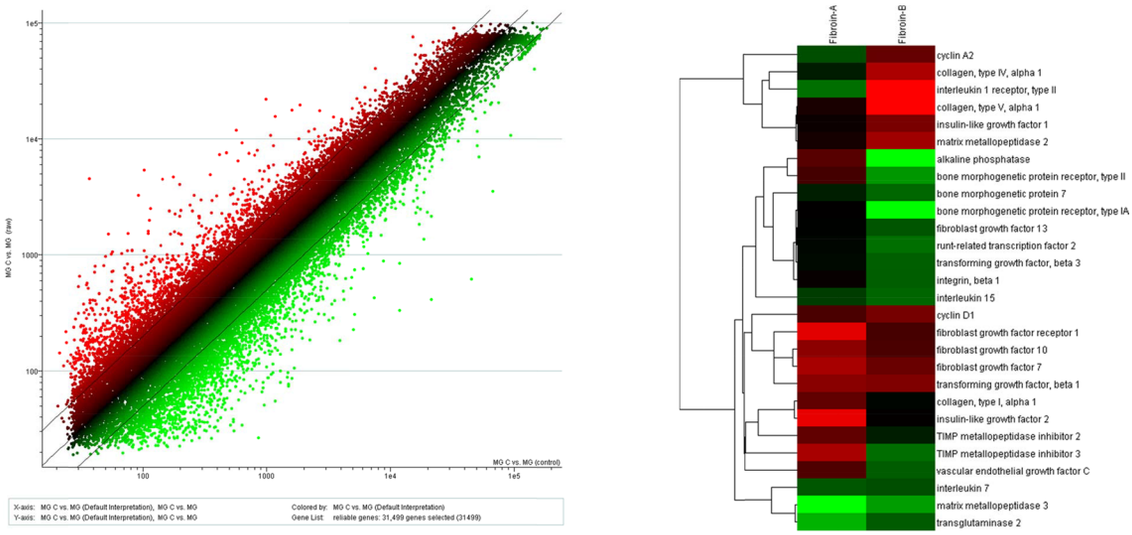

(a) (b)

Fig. 1. A. Scatter plot showing subsets of genes that were up- or down-regulated. The change of gene expression was measured at 2 h after administration of MSF (10 μg/mL). B. Microarray results were compared to a previous report (Kim et al., 2010). Fibroin-A was SSF and fibroin-B was MSF. The genes associated with osteogenesis were depressed in MSF group compared to SSF group. However, insulin-like growth factor 1 and matrix metallopeptidase 2 were highly expressed in MSF group.

the biological effects of differently sized fragments of silk fibroin have not been studied.

Silk fibroin is known to be degraded slowly by proteolysis (Cao and Wang, 2009; Jo *et al*., 2017). Silk fibroin completely degrades in approximately 2 years (Cao and Wang, 2009). During the degradation process, silk fibroin undergoes proteolysis into variously sized fragments (Thurber *et al*., 2015). One study reported that insoluble silk fibroin fragments induced genes related to inflammatory reactions (Panilaitis *et al*., 2003). Micro-array assays are useful for screening changes in the expression of multiple genes simultaneously (Kim *et al*., 2010). Using a micro-array assay, changes in gene expression caused by fragmented silk fibroin can be examined.

In this study, moderately sized silk fibroin fragments (size ≈ 30 kDa) were tested for their impact on gene expression in cells. The purpose of this study was to evaluate changes in gene expression after the administration of silk fibroin fragments in MG63 cells. In addition, the level of alkaline phosphatase activity and cellular proliferation in a group of MG63 cells administered moderately sized silk fibroin fragments (size ≈ 30 kDa) (MSF) was compared to that in a group administered smaller silk fibroin fragments (size < 1 kDa) (SSF).

Materials and Methods

Silk powder

Silk powder composed of fragmented silk fibroin protein was provided by the Rural Development Administration (Wanju, Korea). The source species for the silk fibroin was *Bombyx mori.* Two different sizes of silk fibroin fragments were used: moderately sized silk fibroin fragments (MSF) measuring approximately 30 kDa and smaller silk fibroin fragments measuring <1 kDa (SSF).

Cell culture and cDNA microarray analysis

Cell culture and subsequent cDNA microarray analysis were conducted as described in our previous study (Kim *et al*., 2010). MG63 cells (ATCC, Manassas, VA, USA) were used. For cDNA microarray analysis, MG63 cells were administered 10 μg/mL of MSF. A control group received culture medium without MSF. The culture medium was removed at 2 h after treatment, and total RNA was extracted from the cells. After cDNA synthesis was performed on extracted RNA, microarray analysis was conducted using Genomictree (Seoul, Korea). Briefly, commercially available

microarray chips (G4112A, Agilent Technologies, Santa Clara, CA, USA) were used. To determine which of the 41k chips (41,000 genes) to evaluate, comparative analysis of the combined test pool and averaging of the combined control pool was performed. This approach generated a scatter plot showing subsets of genes that were up- or down-regulated (Fig. 1A).

Real-time RT-PCR

Three genes among 7162 genes showing altered expression by more than 2-fold compared to the untreated control in microarray analysis were selected for real-time reverse transcriptase (RT)-PCR analysis. Transforming growth factor-β1 (TGF-β1), matrix metalloproteinase-2 (MMP-2), and alkaline phosphatase (ALP) were selected. For the purposes of comparison to a previous publication [8], collagen type 1 A1 (COL1A1) was also selected. Real-time RT-PCR was performed using Genomictree. The following primers were used: ALP, F: 5'-ACGAGCTGAACAGGAACAACGT-3' and R: 5'-CACCAGCAAGAAGAAGCCTTTG-3'; COL1A1, F: 5'-GTCGAGGGCCAAGACGAAG-3' and R: 5'-CAGATCACGTCATCGCACAAC-3'; TGF-β1, F: 5'-AGCTCCACGGAGAAGAACTG-3' and R: 5'-AGGACCTTGCTGTACTGCGT-3'; and MMP-2, F: 5'-AGATCTTCTTCTTCAAGGACCGGT-3' and R: 5'-GGCTGGTCAGTGGCTTGGGGTA-3'. Data were normalized by calculating the ratio of the expression of the selected gene to the expression of GAPDH (F: 5'-TGGGCTACACTGAGCACCAG-3' and R: 5'-GGGTGTCGCTGTTGAAGTCA-3'). Relative expression of selected genes was calculated as the gene's expression normalized to GAPDH expression in each group. Measurements were taken three times, and the average value was used for comparison.

ALP assay and MTT assay

For the ALP and MTT assays, MSF and SSF solutions were prepared. The concentration of each solution administered was 1–100 μg/mL. 10^4 MG63 cells were seeded into 24-well-plates with culture medium and incubated for 8 h to allow settling. The prepared solution of MSF or SSF was added to the culture medium. Prepared solution without either type of silk fibroin served as a control. Cells were cultured for 48 h. Next, the medium was removed, and cells were collected for subsequent analysis. A colorimetric ALP assay kit was used for the ALP assay. Subsequent procedures were conducted as described in our previous study (Kim *et al*., 2010).

The experimental conditions for the MTT assay were the same as for the ALP assay. Cells were cultured for 24 h or 48 h. At each measuring point, media was changed. Next, 100 μl of reagent was added and incubated for 2 h. The color change of tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to evaluate cellular proliferation.

Statistical analysis

In the analysis of the microarray results, genes showing a greater than 2-fold change in the Cy3/Cy5 or Cy5/Cy3 values were considered influenced significantly by the silk fibroin fragment administration. Differences between the 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 $p < 0.05$.

Results

The results of the cDNA microarray analysis are shown in Table 1. Comparative analysis with a previous study (Kim *et al*., 2010) is shown as a hierarchical tree (Fig 1B). Genes related to the cell cycle and collagen synthesis showed increased expression. Inflammation-related cytokines such as fibroblast growth factor-7 (FGF-7), insulin-like growth factor-1 (IGF-1), matrix metallopeptidase-2 (MMP-2), and TGF-β1 showed significantly higher expression compared to the control (fold ratio > 2.0). Interestingly, ALP, bone morphogenetic protein-7 (BMP-7), MMP-3 (MMP-3), and vascular endothelial growth factor-C (VEGF-C) showed significantly lower expression compared to the control (fold ratio < 0.5). Real-time RT-PCR results were evaluated for 4 selected genes (Fig. 2: ALP, COL1A1, TGF-β1, and MMP-2). The gene expression levels of ALP, COL1A1, TGF-β1, and MMP-2 in the MSF application group (experimental group) were 0.163 ± 0.004, 0.897 ± 0.022, 1.103 ± 0.024, and 1.164 ± 0.025, respectively. The gene expression levels of ALP, COL1A1, TGF-β1, and MMP-2 in the

Table 1. The results of cDNA microarray

Group	TITLE	GenBank	Chromosome	Fold-ratio
Matrix protein	Collagen, type I, alpha 1	Z74615	17q21.33	0.959
	Collagen, type IV, alpha 1	NM_001845	13q34	4.116
	Collagen, type V, alpha 1	NM_000093	9q34.2-q34.3	21.184
Cell proliferation	Cyclin A2	NM_001237	4q25-q31	2.333
	Cyclin D1	NM_053056	11q13The	2.630
	Fibroblast growth factor-7	NM_002009	15q15-q21.1	2.379
	Fibroblast growth factor-11	NM_004112	17p13.1	2.082
Inflammation	Insulin-like growth factor-1	NM_000618	12q22-q23	2.704
	Interleukin 1 receptor, type II	NM_004633	2q12-q22	10.651
	Matrix metallopeptidase 2	NM_004530	16q13-q21	3.732
	Transforming growth factor, beta 1	NM_000660	19q13.2	2.840
Osteogenesis	Alkaline phosphatase	NM_000478	1p36.1-p34	0.098
	Bone morphogenetic protein 7	NM_001719	20q13	0.429
	Runt-related transcription factor 2	NM_004348	6p21	0.401
	Bone morphogenetic protein receptor, type IA	NM_004329	10q22.3	0.087
	Bone morphogenetic protein receptor, type II	CB052856	2q33-q34	0.289

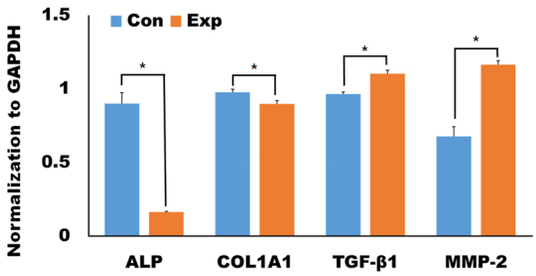

Fig. 2. Quantitative real-time RT-PCR results. Control group (Con) received culture medium without MSF. The group administered MSF (10 μg/mL) was experimental group (Exp). The change of gene expression was measured at 2 h after administration and each gene's expression was normalized to GAPDH expression. The expression levels of alkaline phosphatase (ALP), collagen type I a1 (COL1A1) in Exp group were significantly lower than those in Con group (*P<0.05). The expression levels of transforming growth factor-β1 (TGF-β1) and matrix metalloproteinase-2 (MMP-2) in Exp group were significantly higher than those in Con group (*P<0.05).

control group were 0.900 ± 0.071, 0.975 ± 0.022, 0.962 ± 0.015, and 0.674 ± 0.067, respectively. When the MSF application group is compared to the untreated control group, the difference between the two groups is statistically significant in all compared genes (P<0.05). The results of real-time RT-PCR agreed with the

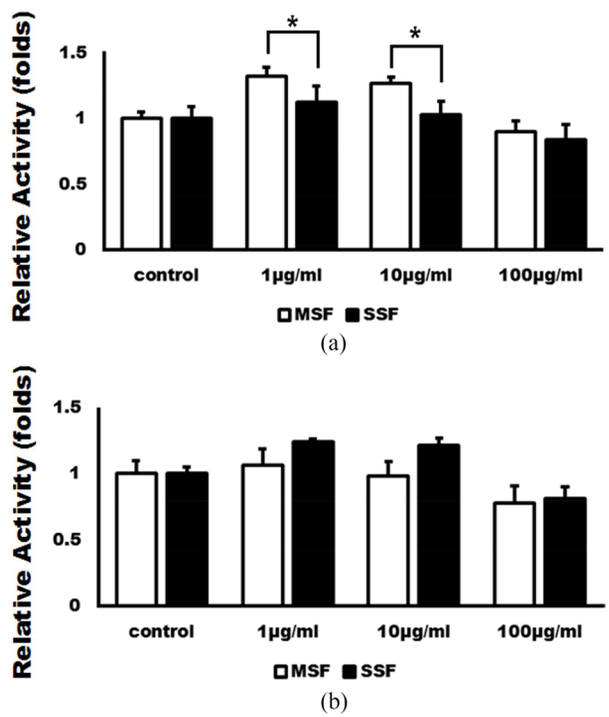

Fig. 3. MTT assay. A. Relative value of MTT assay at 24 h after administration. The difference between groups was significant at concentrations of 1–10 μg/mL (*P < 0.05). B. Relative value of MTT assay at 48 h after administration.

cDNA microarray assay results.

Based on the MTT assay, cell viability was generally higher in the MSF group than in the SSF group 24 h after administration (Fig. 3A). The difference between groups was significant at concentrations of 1–10 μg/mL ($P < 0.05$). However, these trends were reversed at 48 h after administration (Fig. 3B). Cell viability was generally lower in the MSF group than in the SSF group, however, the difference between the two groups was not significant ($P > 0.05$). The relative ALP activity of MSF was similar to that of SSF for solutions at lower concentrations (Fig. 4: 1–10 μg/mL). However, there was a significant difference between solutions at 100 μg/mL concentration ($P<0.05$). The relative ALP activities of MSF and SSF were 1.675 ± 0.003 and 2.022 ± 0.070, respectively.

Discussion

In this study, moderately sized silk fibroin fragments (MW ≈30 kDa) had a different impact on cells than did smaller silk fibroin fragments (MW < 1 kDa) (Fig. 1B). The genes associated with cellular proliferation and inflammation generally showed increased expression after MSF administration (Table 1). Genes associated with osteogenesis showed decreased expression after MSF administration. Cellular proliferation was increased after MSF administration (Fig. 3). Although the enzyme activity of ALP was slightly increased at 48 h after MSF administration, the value was significantly lower than that of SSF (Fig. 4). To the best of our knowledge, this report is the first demonstrating differential cellular response to differently sized fragments of silk fibroin.

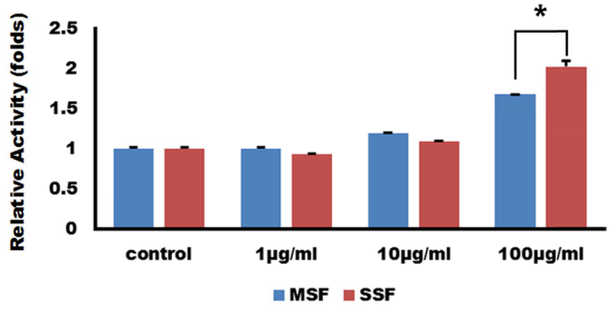

Fig. 4. Alkaline phosphatase (ALP) assay. ALP activity was measured at 48 h after administration. Relative ALP activity of MSF was significantly lower at 100 μg/mL of solution than that of SSF (*$P < 0.05$).

Silk fibroin is a natural macromolecule that slowly degrades in the body (Cao and Wang, 2009; Jo *et al*., 2017). Many types of proteolytic enzymes may be involved in silk fibroin degradation. Among these enzymes, MMPs have been widely studied (Brown *et al*., 2015; Jo *et al*., 2017). MMP-2 can degrade any type of silk fibroin, including silk fibroin films and silk sutures (Brown *et al*., 2015). The products of proteolysis will be considerably smaller than non-degraded silk fibroin protein (MW ≈ 350 kDa). As the proteolytic enzymes are primarily produced by host cells adjacent to the silk fibroin graft, the products of proteolysis are expected to interact with adjacent cells. In our previous study, SSF increased the expression of genes associated with bone regeneration, such as ALP and collagen type I (Kim *et al*., 2010). However, MSF did not increase the expression of genes associated with bone regeneration, such as ALP and collagen type I. Although acute inflammation-related genes such as tumor necrosis factor-α or interleukin-1β were not induced by MSF administration (data not shown), TGF-β1 and MMP-2 showed increased expression.

The elevated expression of TGF-β1 is closely related to allograft rejection with inflammatory cell infiltration (Little *et al*., 1999). MMP-2 has also shown elevated expression in the site of inflammation and tissue destruction (Hadziabdic *et al*., 2016). Antibiotics that can reduce inflammation can also inhibit MMP-2 activity (Bătăiosu *et al*., 2015). However, chronic inflammation is accompanied with not only elevated levels of MMP-2 but also elevated collagen synthesis (Wong *et al*., 2014). In this study, MSF elevated the level of MMP-2 gene expression but did not elevate the level of COL1A1 expression (Table 1). Transient inflammation caused by silk materials is an unavoidable phenomenon, and the exact host response to the silk graft will be associated with the degradation time (Lee *et al*., 2010).

Silk fibroin has been studied as a scaffold for bone tissue engineering (Cao and Wang, 2009). To be an ideal scaffold, silk fibroin must be degraded in a timely manner and replaced by newly regenerated host bone tissue. During the degradation of silk fibroin, its molecular weight is expected to decrease. For bone regeneration, the host response to SSF is acceptable (Lee *et al*., 2010). Based on the results of the microarray assay, the host response to MSF was not desirable for bone regeneration because MSF decreased the expression of ALP and COL1A1. However, the expression of MMP-2 was increased by the administration of MSF (Table 1). Since

MMP-2 can degrade silk fibroin (Brown *et al*., 2015), MSF would be gradually degraded into SSF by MMP-2. In fact, the enzyme activity of ALP was slightly increased at 48 h after MSF administration (Fig. 4) in spite of reduced ALP gene expression at 2 h after MSF administration (Fig. 2). Comparative hierarchical analysis also demonstrated similar results (Fig. 1B). Recently, 4-hexylresorcinol (4HR) has been widely studied as an incorporating drug for silk fibroin scaffolds (Lee *et al*., 2015). Since 4HR can increase the expression of MMPs in macrophages (Jo *et al*., 2017), 4HR would be expected to facilitate the rapid transition from MSF to SSF. Consequently, 4HR-incorporated silk fibroin scaffolds can increase graft degradation and new bone formation (Kweon *et al*., 2014). In addition, 4HR-incorporated silk fibroin scaffolds show decreased formation of foreign body giant cells (Kweon *et al*., 2014).

Conclusions

MSF increased the expression of genes associated with cellular proliferation and inflammation but decreased the expression of genes associated with osteogenesis. However, MSF also increased the expression level of MMP-2, which can degrade MSF and accelerate its transition to SSF. To design scaffolds for bone tissue engineering with silk fibroin, the molecular weight-associated cellular response to silk fibroin fragments should be considered. These preliminary findings indicate that more studies are warranted to generate a detailed characterization of the impact on cells of differently sized silk fibroin fragments.

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