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Soluble fraction from silk mat induced bone morphogenic protein in RAW264.7 cells

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Abstract

The objective of this study was to evaluate the changes in gene expression after incubation of cells with soluble fraction from different silk mat layers. A silk cocoon from *Bombyx mori* was separated into 4 layers of equal thickness. The layers were numbered from 1 to 4 (from the inner to outer layer). Each silk mat was placed into normal saline and collected soluble fraction. They were administered to RAW264.7 cells, and changes in the expression of genes were evaluated by cDNA microarray analysis. Layer 1 and 4 groups showed significantly higher expression of BMP-2 at 8 h after administration of soluble fraction ($P < 0.05$). Runx2 expression was significantly higher in Layer 4 group at 8h ($P < 0.05$). The silk mat from the innermost and outermost portion of the silkworm cocoon showed a significant change in the expression of genes that are associated with osteoinduction such as BMP-2 and runx2.

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Introduction

Silk mat is produced from silkworm cocoon by eco-friendly method (Kweon *et al*., 2017). As silkworm cocoon is composed of multiple layers, source for silk mat can be from innermost layer to outermost layer (Jo *et al*., 2019). According to previous research, the silk mat from the middle layer has been shown best bone formation ability (Kweon *et al*., 2017; Jo *et al*., 2019). In clinical trials, silk mat from the middle layer has been shown similar performance to high-density polytetrafluoroethylene (dPTFE) membrane (Kim *et al*., 2019a; Kim*et al*., 2019b).

Silk mat is mainly composed of proteins (Zhang *et al*., 2015). Silk fibroin and sericin are main proteins in silk mat. Except for them, many kinds of protease inhibitors and seroin are protein components of silk mat. Detailed composition of these proteins are different to layers (Zhang *et al*., 2015). When silk mat is grafted into the body, water soluble components will be released from silk mat. Among them, sericin is approximately 200 kDa sized protein and it is known to be fragmented when it is in the aqueous solution (Park and Um, 2018; Kaur *et al*., 2013; Mandal *et al*., 2009). Unlike sericin, other proteins have generally lower molecular weight. For example, seroin 1 is 11-13 kDa sized protein (Zhang *et al*., 2015).

When silk mat is grafted into the body, monocytes and macrophages will respond to it (Kim, 2020). As silk mat is foreign material for mammals, inflammatory reaction to silk mat is unavoidable. Tumor necrosis factor-α (TNF-α) is increased its expression level when silk sericin is administered (Jo *et al*., 2017).

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The level of TNF-α may be associated with new bone formation in silk mat application (Jo *et al*., 2017). In addition, angiogenesis is vital component for new bone formation. Silk sericin induces vascular endothelial growth factor (VEGF) in macrophages and endothelial cells (Jo *et al*., 2019). VEGF is an important cytokine for angiogenesis (Kim*et al*., 2020). However, they are supplementary factors for osteogenesis.

The purpose of this study was to investigate cytokines associated with direct bone formation after silk mat application. Silk mats from the different layers were placed into normal saline for extraction of soluble fraction. They were applied to RAW264.7 cells and screened gene expression changes by cDNA microarray.

Materials and Methods

Preparation of a soluble fraction

Silk mat was produced from silkworm, Daesung-jam, *Bombyx mori.* Cocoon was peeled off as 4 layers as Layer 1 through Layer 4 (from the inner to the outer layer). Each layer had the same thickness (approximately 0.1 mm). To collect soluble fraction from silk mat, 2 g of each layer was dipped into 50 mL of normal saline at 37 ºC. Normal saline with silk mat was sonicated for 48 h. Non-soluble part of silk mat was removed. The color of solution was yellow and its protein concentration was measured by spectrophotometry at 280 nm.

Cell cultures and application of soluble fraction

RAW264.7 cells (KCLB No. 40071) were used for screening gene expression profile. RAW264.7 cells were grown in Dulbecco's modified Eagle's medium- high glucose (PAA Laboratories, Linz, Austria) supplemented with 1% of a penicillin/streptomycin solution (100x) and 10% of fetal bovine serum (FBS). When the confluence was reached to 80%, the culture medium was changed to the same composition without FBS. The concentration of each solution was set as 10 μg/mL of the protein. Dilution of soluble fraction to achieve final concentration was done by culture medium. Soluble fraction (750 μL) was added into the culture medium of RAW264.7 cells. Normal saline without soluble fraction was set as a control. Cells were collected at 2 and 8 h after administration. Total RNA from each group was isolated from cellular collection.

cDNA microarray analysis and qRT-PCR

The quality of total RNA was evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Detailed method was in accord to our previous publication (Kim*et al*., 2018). RNA integrity number of all the samples was greater than 7.0 and met the criterion for the subsequent microarray analysis. Commercially available microarray chips for mouse genes (Agilent MouseGE 4 X 44K, Agilent Technologies) were used. By means of Agilent's Low RNA Input Linear Amplification kit plus (Agilent Technologies), extracted total RNA was subjected to amplification and labeling. After unhybridized probes were washed out, chips were scanned, and the scanned images were analyzed in the Feature Extraction Software (Agilent Technologies). Normalization and clustering were performed in GeneSpring software (Agilent Technologies). For comparative purposes, gene expression in the untreated control was also analyzed. The comparison of the expression of genes between each Layer group and the no-treatment control was performed in the generated scatter plot.

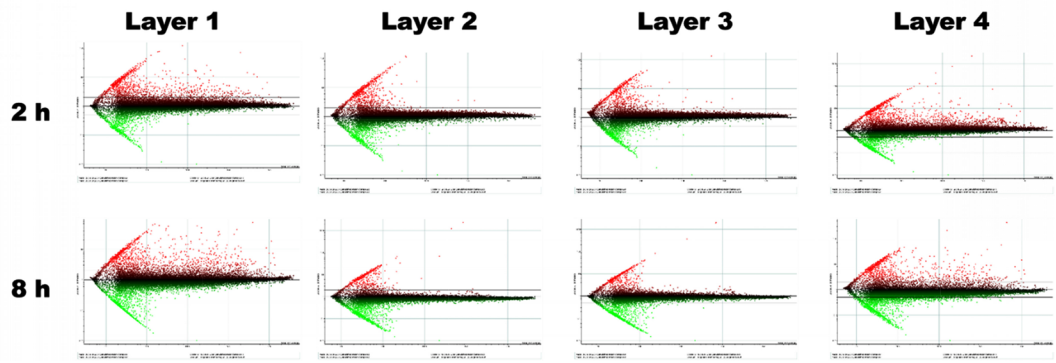

Fig. 1. An M plot for each group. The gene expression ratio between saline control and each silk mat layer is shown as a single spot. Most gene data were collected on the line of 1.0.

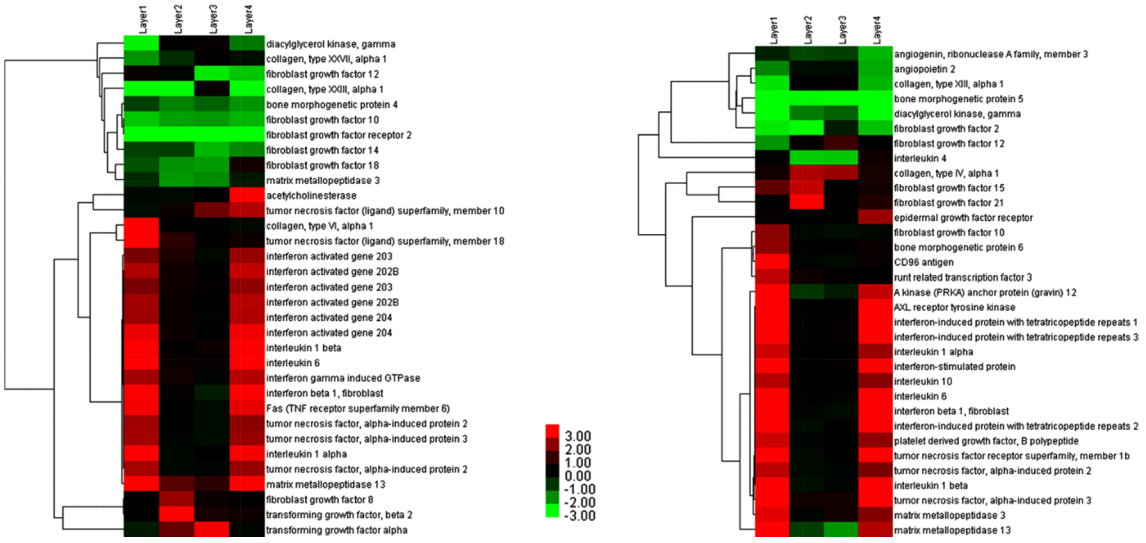

Fig. 2. A gene tree with cluster analysis. The genes associated with inflammation and angiogenesis were highly expressed in Layer 1 and 4 groups at 2 h (left). The elevated expression of some genes persisted until 8 h (right).

Statistical analysis

Cluster analysis was carried out in Gene Cluster 3.0 (Stanford University, CA, USA). Genes were filtered, and data were normalized. For hierarchical analysis, genes were clustered, and uncentered correlation served as a similarity metric. The clustering method was centroid linkage.

Results

Differentially expressed genes profiles were shown as M-plot (Fig. 1). Among genes, genes of interest were displayed as gene tree with cluster analysis (Fig. 2). Most of them were genes related to inflammation and angiogenesis. The relative expression level of BMP-2, BMP-4, BMP-6, and Runt-related transcription factor-2 (runx2) was shown in Table 1. Layer 1 and 4 groups showed significantly higher expression of BMP-2 at 8 h after administration of soluble fraction ($P < 0.05$). In case of BMP-4, all Layer groups showed significantly lower mRNA expression at 2 h ($P < 0.05$). However, it showed significantly higher expression in Layer 1, 3, and 4 groups at 8 h. Layer 1 group showed significantly higher BMP-6 expression at 2 and 8 h ($P < 0.05$). Runx2 expression was significantly higher in Layer 4 group at 8h ($P < 0.05$).

Discussion

Silk mat has been approved for clinical trials and shown

Table 1. Summary of microarray results

Gene	Observation	Layer 1	Layer 2	Layer 3	Layer 4
BMP-2	2 h	-0.167	-0.233	-0.096	-0.014
	8 h	1.820*	-0.124	-0.210	1.917*
BMP-4	2 h	-1.211*	-1.927*	-1.622*	-2.102*
	8 h	1.042*	0.329	1.347*	1.435*
BMP-6	2 h	1.829*	0.842	0.568	0.453
	8 h	2.032*	0.050	0.013	0.243
Runx2	2 h	0.355	-0.415	0.145	0.120
	8 h	-0.050	-0.226	-0.018	0.851*

(*P<0.05, BMP; bone morphogenic protein, Runx2: Runt-related transcription factor-2)

similar performance to commercialized membrane for guided bone regeneration (Kim *et al*., 2019a; Kim *et al*., 2019b). The mechanism of osteogenesis after silk mat application has been suggested as M1 polarizing agent as silk sericin (Kim, 2020). However, there are many other types of proteins in the silk mat (Zhang *et al*., 2015). In this study, the application of soluble fraction from silk mat increased the gene expression of BMP-2, BMP-4, BMP-6, and runx2 (Table 1). All these genes are associated with osteoinduction.

BMP-2 has been found in the ectopic bone formation tissue (Ahmad *et al*., 2020). The application of BMP-2 into the bone defect increases new bone formation (Kim *et al*., 2013; Lee *et al*., 2014). As BMP-2 has low molecular weight and highly hydrophilic, maintenance of therapeutic concentration in bony defect area is an important part in successful bone regeneration after BMP-2 application (Kim *et al*., 2013). If BMP-2 is applied without proper scaffold, it will be washed out and no effect in bone regeneration (Edelmayer *et al*., 2020). Accordingly, fibrotic materials such as acellular collagen, silk fibroin have been suggested as BMP-2 carrier (El Bialy *et al*., 2017). In addition, synthesis of BMP-2 is expensive procedure. It can be produced from micro-organism or cell as recombinant protein (Medikeri *et al*., 2019). Therefore, chimeric protein of BMP-2 also has been developed for increasing its effect and reducing its production cost (Kim *et al*., 2013).

In this study, certain component from silk mat increased BMP-2 gene expression in RAW264.7 cells (Table 1). It was water soluble component of silk mat. There are many hydrophilic protein components in silk mat. They are silk sericin, seroin, and protease inhibitors (Zhang *et al*., 2015). Lipopolysaccharide from micro-organism also increase BMP-2 expression in immune cells (Zhou *et al*., 2019). However, silk mat used in this study was grown in germ-free environment and sterilized. In case of BMP-2, it was highly expressed in Layer 1 and 4 (Table 1). Layer 4 showed highest expression of runx2. Therefore, some proteins abundant in Layer 1 and 4 might be responsible for BMP-2 inducing. Actually, each protein of silk mat has different protein composition from inner to outer layer. The identification of BMP-inducing protein in the silk mat would be interesting topic in following research.

The limitations of this research were as follows. (1) The results in this study was preliminary. As cDNA micro-array is not highly reliable, it should be considered as screening. Confirmative study should be followed. (2) The mechanism of BMP-induction was unclear. According to previous reports (Kweon *et al.*, 2017; Jo *et al*., 2017), middle layer showed best bone formation compared to inner or outer layer. However, this study showed opposite result. Even after the identification of BMP-inducing protein from silk mat, some proteins from silk mat might have opposite response in bone regeneration. In this case, development of extraction technology will be important step. Through this technology, unnecessary protein should be removed from silk mat.

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