Gene Expression of Bcl2a1 and Cxcl9 in Soft Tissue Covering Bone Grafting Materials
การแสดงออกของยีน Bcl2a1 และ Cxcl9 ในเนื้อเยื่ออ่อนที่ปกคลุมอยู่บนกระดูกปลูกถ่าย

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ABSTRACT

This study was to assess the expression of Bcl2a1 and Cxcl9 genes in soft tissues covering bone grafted with demineralized freeze-dried bone allograft (DFDBA) and deproteinized bovine bone mineral (DBBM) in comparison with ones covering without grafting. The animals were randomly separated into three groups of treatment. Calvarial defect models were created on parietal bones. Bare defect models without bone graft as a control group. After one and three months, animals were sacrificed. Soft tissues covering the defected area were collected by punch technique. RNA was isolated and proceeded to real-time reverse transcription polymerase chain reaction (RT-PCR). Statistical analysis indicated that, in comparison to control group at 1 month, DFDBA and DBBM up-regulated Bcl2a1 gene in soft tissue covering bone graft at p-value < 0.05. Allograft also up-regulated Cxcl9 gene at p-value < 0.05. At 3 months, both of genes were not statistically significant expressed among treatment groups at p-value > 0.05.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อประเมินการแสดงออกของยีน Bcl2a1 และ Cxcl9 ในเนื้อเยื่ออ่อนที่ปกคลุมกระดูกที่ได้รับการปลูกถ่ายด้วยกระดูกเอกพันธุ์สกัดแร่ธาตุแบบระเหิดและกระดูกวัวสกัดโปรตีนเปรียบเทียบกับเนื้อเยื่อที่ปกคลุมกระดูกที่ไม่ได้รับการปลูกถ่าย สัตว์ทดลองจะได้รับการสุ่มเข้ากลุ่มทดลองสามกลุ่ม แต่ละตัวจะถูกกรอกระดูกเพื่อเตรียมพื้นที่สำหรับปลูกถ่ายกระดูกบนกระดูกข้างกระหม่อมทั้งสองฝั่ง โดยกลุ่มควบคุมจะไม่ได้รับการปลูกถ่ายกระดูก หลังจากปลูกถ่ายกระดูกเป็นเวลา 1 เดือนและ 3 เดือนแล้ว สัตว์ที่อยู่ในกลุ่มทดลองของแต่ละช่วงเวลาจะถูกทำให้เสียชีวิต เนื้อเยื่ออ่อนที่ปกคลุมอยู่บนกระดูกปลูกถ่ายจะถูกเก็บนำมาสกัดอาร์เอ็นเอเพื่อวิเคราะห์ผลการแสดงออกของยีนด้วยวิธีเพิ่มปริมาณสารพันธุกรรมชนิดปฏิกิริยาจริงอัตโนมัติ ผลการวิเคราะห์ทางสถิติพบว่าที่เวลา 1 เดือน เนื้อเยื่ออ่อนจากกลุ่มที่ได้รับการปลูกถ่ายด้วยกระดูกเอกพันธุ์สกัดแร่ธาตุแบบระเหิดและกระดูกวัวสกัดโปรตีนเพิ่มการแสดงออกของยีน Bcl2a1 มากกว่ากลุ่มควบคุมที่ระดับนัยสำคัญทางสถิติน้อยกว่า 0.05 นอกจากนี้ยีน Cxcl9 ในเนื้อเยื่อกลุ่มกระดูกเอกพันธุ์สกัดแร่ธาตุแบบระเหิดยังแสดงออกมากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ที่ระดับนัยสำคัญทางสถิติน้อยกว่า 0.05 เช่นกัน อย่างไรก็ตามเมื่อเปรียบเทียบผลระหว่างกลุ่มทดลองที่เวลา 3 เดือน พบว่ายีนทั้งสองชนิดแสดงออกแตกต่างกันอย่างไม่มีนัยสำคัญทางสถิติ ที่ระดับนัยสำคัญทางสถิติมากกว่า 0.05

Keywords: Gene expression, Inflammation, Bone graft

คำสำคัญ: การแสดงออกของยีน การอักเสบ กระดูกปลูกถ่าย

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Introduction

Most of edentulous patients need a prosthesis to restore masticating function and improve an aesthetic outcome. In some patients, dental implants with a fixed or a removable prosthesis is the first choice of treatment. Correction of the implant placement area with insufficient bone volume, bone augmentation with bone graft needs to be done. Bone augmentation was recommended as a necessary procedure combining with implant placement (Molly et al., 2008; Wood, Mealey, 2012). Guided bone regeneration (GBR) is one of the bone augmentation techniques, which can be performed with bone graft and barrier membrane before or with implant placement (Liu, Kerns, 2014). There are various types of commercially bone grafts such as freeze-dried bone allograft (FDBA), demineralized freeze-dried bone allografts (DFDBA), and deproteinized bovine bone mineral (DBBM). Both FDBA and DFDBA have osteoconductive property and modulate human immune response. Although FDBA has most properties like DFDBA, it has no osteoinductive property (Pagni et al., 2012; Urist, Strates, 1971). DBBM is popularly used owing to its structure as a scaffold to enhance bone formation (Baldini et al., 2011).

In the clinic, we found that using a barrier membrane is difficult to be manipulated, especially in the aesthetic zone. In the case of thin biotype, it is prone to be dehisce. Failures of GBR due to membrane exposure have been reported up to 50%. In one randomized clinical trial, horizontal bone augmentation using DBBM with resorbable or non-resorbable membrane was evaluated. The result shows that both types of membrane undergo high rates of membrane exposures of 64 and 71%, respectively, therefore, membrane cannot be used in some areas. Inconsequence of that, a direct contact of soft tissue (e.g. gingiva) and bone graft will be occurred. Moreover, bone graft as a subtype of dental materials and may play a role as foreign body to promotes inflammation and soft tissue destruction (Offenbacher et al., 2008).

A mammalian calvarium has been used as a study model of a tissue-related biomaterial research, because of its morphology and approximately 85% of genome that resembles human (Batzoglou et al., 2000; Gomes, Fernandes, 2011). Mouse's calvarium is also recommended as a model to examine the tissue response to bone substitution materials.

A previous in vivo study showed the gene expression of inflammatory cytokines in an alveolar bone preservation area (Kumar et al., 2013). However, gene expression profiles in soft tissue covering bone grafted area have not been assessed before. This study aimed to evaluate the expression of inflammatory related genes in soft tissue covering bone grafted with DFDBA and DBBM in a mouse calvarial defect.

Objectives of the study

To study the expression of Bcl2a1 and Cxcl9 genes in soft tissue covering bone grafted with DFDBA and DBBM in comparison with the ones without grafting.

Methodology

Animals

Male mice from the previous study (Kangwannarongkul et al., 2018) strain C57BL/6MLac weight 25-30 gram, age 8 weeks old were used. The experiment was approved by the Animal Care and Use Committee of Chulalongkorn University No. 1432001. These animals were obtained from the National Laboratory Animal Center of Salaya Campus, Mahidol University. The mice were housed in light and temperature-controlled facilities and given food and water *ad libitum*. The animals were randomly separated into two groups: 1 month and 3 months; following the time points of wound healing after operation procedures. Two equal critical size 3 mm. defects were made on both parietal bone of each mouse. In each time point (n = 6, total defects = 12), the defects were randomly assigned to the following 3 types of treatment: (1) bare defect without bone grafted as a control, or (2) DBBM (Bio-Oss®; GeistlichPharma AG, Wolhusen, Switzerland), or (3) DFDBA (OraGRAFT®; LifeNet, Virginia, USA)

Surgical procedures

The procedures were done by one experienced surgeon under aseptic techniques. An animal was sedated using Pentobarbital (Nembutal®). The dilution of Pentobarbital to phosphate buffered saline (PBS) is in the ratio of 1:10. Each animal was received at a concentration of 4 mg/kg or 8µL of dilution/wt (g). The sedative was administered into a peritoneal layer. Hairs above the scalp were removed by blade and scalp was cleaned with alcohol and povidone iodine. An incision of 1.5 mm length was made under 1% Lidocaine with 1:100,000 epinephrine, to visualize the parietal bone. A hand drill trephine bur with normal saline coolant was used for created a cavity size 3 mm in diameter. The cavities were made on both right and left sides of parietal bones of bare defect group and grafting groups. This step was done carefully to avoid dura mater injury. The defects of the grafting groups were randomly filled with 10 mg of OraGRAFT® or Bio-Oss®. Bone graft particles were packed by cotton pellet soaked with normal saline. The scalp was stitched up with primary closure and sutured with nylon 3-0. Animals in each group were sacrificed by cervical dislocation at 1 and 3 months. When surgical procedures had performed, mid-sagittal incision line was made. The center of the grafted area was located. The skin above the center of grafted bone was marked and punched with 4 mm biopsy punch. Labeling of tissues included SC, control group; SB, Bio-Oss® group; and SD, OraGRAFT® group. Tissues were flash-frozen in liquid nitrogen and stored in -80°C.

RNA purifications

All procedures were done under aseptic RNA handling technique. The frozen tissue was transferred into pre-chilled ceramic bead tube (bead size 2.8 mm in diameter). The tissue was homogenized with RLT Plus (Lysis buffer) and β-Mercaptoethanol (β-ME) by PowerLyzer® 24 Homogenizer (Mo Bio Laboratories, QIAGEN N.V., Germany). RNA purifications were done using Rneasy® Plus Mini Kit (QIAGEN N.V., Germany) following the manufacturer protocols. Quantity and quality of RNA were evaluated using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). RNA quantity was determined by its UV absorbance at 260 nm. RNA quality was assessed using A260/A280 ratio. RNA integrity was determined using the Agilent 2100 bioanalyzer and Pico chip kit (Agilent Inc.,

USA). RNA with RNA intregity number (RIN) greater than or equal to 5 was accepted to run the analysis (Ibberson et al., 2009)

Real-time reverse transcription polymerase chain reaction (RT-PCR)

RNA was reverse transcript to cDNA and used as the template in RT-PCR by the reverse transcription kit (Sensiscript, Qiagen, USA). Specific primers were designed by Primer BLAST (https://ncbi.nlm.nih.gov/tools/primer-blast/). Specificity of primer sequences were evaluated by Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primer sequences are shown in Table 1. Bio-Rad CFX96TM RT-PCR system (Bio-Rad laboratories, INC., USA) was utilized to verify the ability of mRNA to observe gene expression. The FastStart SYBR Green Master (FastStart Essential DNA Green Master kit (Roche Diagnostics GmbH Mannheim, Germany) was used for performing the polymerase chain reaction. The RT-PCR was performed in biological replicate. A sample of cDNA that did not contained the target primer was used as a negative control. Rnase-free water with no sample was used as a contaminated monitoring. The negative control and Rnase-free water were included in each run. Protocol was set up according to the manufacturer 's protocol. 18s rRNA was used as a reference gene. Quantitative PCR data analysis was done using qbase+ software, version 3.0 (Biogazelle, Zwijnaarde, Belgium – www.qbaseplus.com). The relative gene expression data was evaluated the difference by one-way analysis of variance (ANOVA). Post hoc Tukey's Honestly test was used for multiple comparison between every two group. The difference between two time points, one and three months after bone grafted, was analyzed with independent t-test. All statistics were performed at the 95% significance level. P-value less than 0.05 was indicated statistically significant difference.

Table 1 Sequences of gene specific primers used for RT-PCR

Gene symbol	Primer	Primer sequences (5' to 3')
Bcl2a1	Forward	CTT CAG TAT GTG CTA CAG GTA CCC G
	Reverse	TGG AAA CTT GTT TGT AAG CAC GTA CAT
Cxcl9	Forward	CAC TTC GCT GCT ATC TAA TTG G
	Reverse	TAG GCA CTG TGG AAG ATT TAG G
18s rRNA	Forward	AGG GGA GAG CGG GTA AGA GA
	Reverse	GGA CAG GAC TAG GCG GAA CA

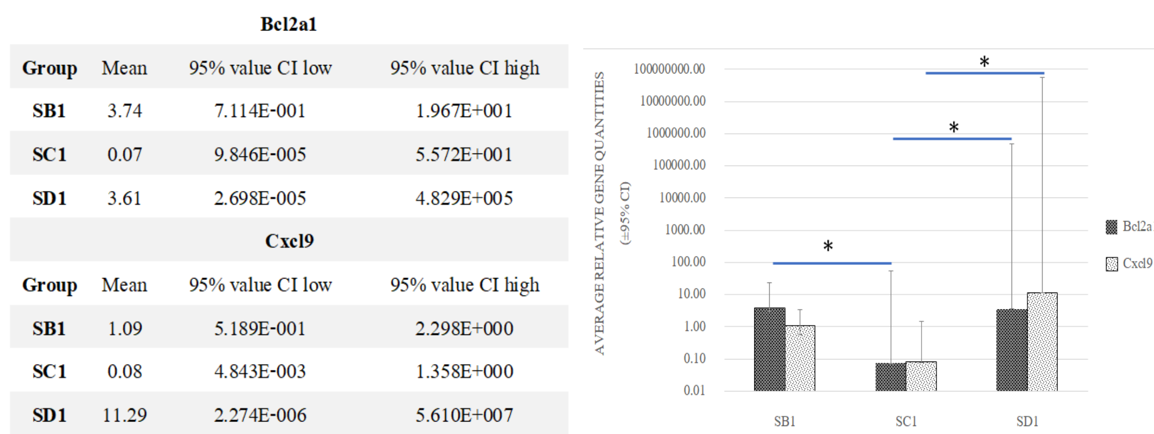
Results

RT-PCR analysis

At 1 month, the relative gene expressions of Bcl2a1 was shown in figure 1. SB group showed greater up-regulation compared to SC group with statistically significant difference (p-value < 0.05). SD group was also statistically significant up-regulated compared to SC group (p-value < 0.05). However, no statistically significant difference between SB and SD groups. The relative gene expression of Cxcl9, SD group was significantly up-regulated

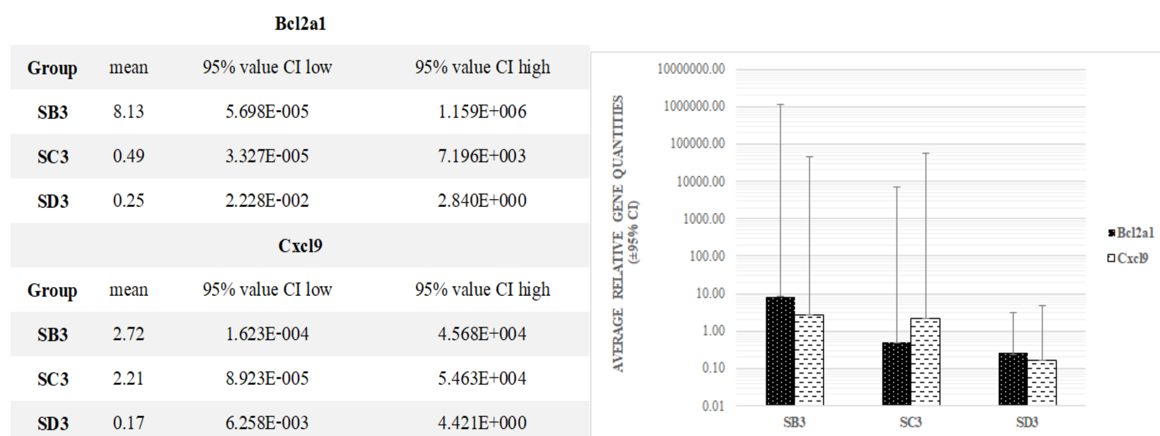
compared to SC group. No significantly difference was founded in SB vs. SC group and SB vs. SD group. At 3 months, the relative gene expression of Bcl2a1 and Cxcl9 were not founded the difference among groups (Figure 2).

The analyzed results of Bcl2a1 and Cxcl9 in treatment groups were shown in Figure 3 and Figure 4, respectively. An independent t-test analysis indicated that there was no statistically significant difference (p-value > 0.05) on the relative gene expression between two time points.



Bcl2a1			
Group	Mean	95% value CI low	95% value CI high
SB1	3.74	7.114E-001	1.967E+001
SC1	0.07	9.846E-005	5.572E+001
SD1	3.61	2.698E-005	4.829E+005
Cxcl9			
Group	Mean	95% value CI low	95% value CI high
SB1	1.09	5.189E-001	2.298E+000
SC1	0.08	4.843E-003	1.358E+000
SD1	11.29	2.274E-006	5.610E+007

Figure 1 RT-PCR validation of Bcl2a1 and Cxcl9 relative expression at 1 month. Each sample was analyzed and 18s rRNA was used as the reference gene. Bars represent logE Mean ± 95%CI, * *p*- value < 0.05.



Bcl2a1			
Group	mean	95% value CI low	95% value CI high
SB3	8.13	5.698E-005	1.159E+006
SC3	0.49	3.327E-005	7.196E+003
SD3	0.25	2.228E-002	2.840E+000
Cxcl9			
Group	mean	95% value CI low	95% value CI high
SB3	2.72	1.623E-004	4.568E+004
SC3	2.21	8.923E-005	5.463E+004
SD3	0.17	6.258E-003	4.421E+000

Figure 2 RT-PCR validation of Bcl2a1 and Cxcl9 relative expression at 3 months. Each sample was analyzed and 18s rRNA was used as the reference gene. Bars represent logE Mean ± 95%CI, * *p*- value < 0.05.

Discussion and Conclusions

The aim of this study was to assess the expression of Bcl2a1 and Cxcl9 genes in soft tissues covering bone grafted with DFDBA and DBBM in comparison with the ones covering bone without grafting. The study used two types of commercially bone graft and bare defect as independent variable groups. Relative gene expression as a dependent variable group. Controlled variables consist of the methodology of sampling animals, surgical procedures, RNA purification technique, and quality of nucleic acid. Bio-Oss® is bovine xenograft collected from difference

species to human. Deproteinization process was done to reduce an immunologic reaction of this graft. OraGRAFT® is derived from the same species of human, but different genotypes. However, the residual graft in the augmented area may stimulate chronic inflammation. RT-PCR result also confirmed our hypothesis by relative expression of Bcl2a1 gene was significantly up-regulated in SB group compared to SC group (p-value < 0.05). SB group also up-regulated Cxcl9 gene compared to SC group but statistically significance was not detected. SD group significantly up-regulated both genes compared to SC group. From the overview of this result, we found Bcl2a1 and Cxcl9 also up-regulated in SB and SD group compared to SC group. OraGraft™ and Bio-Oss® may play a role as the foreign bodies to stimulate an inflammatory response (Offenbacher et al., 2008). At 3 months, Bcl2a1 and Cxcl9 genes were also expressed among groups while the difference of relative expression was not detected.

Bcl2a1

Group	Time (month)	Mean	95% value CI low	95% value CI high	P
SB1	1	0.40	7.684E-002	2.124E+000	0.30
	3	2.48	1.736E-005	3.530E+005	
SC1	1	0.23	3.080E-004	1.743E+002	0.10
	3	4.32	2.935E-004	6.348E+004	
SD1	1	2.26	1.686E-005	3.018E+005	0.32
	3	0.44	3.927E-002	5.005E+000	

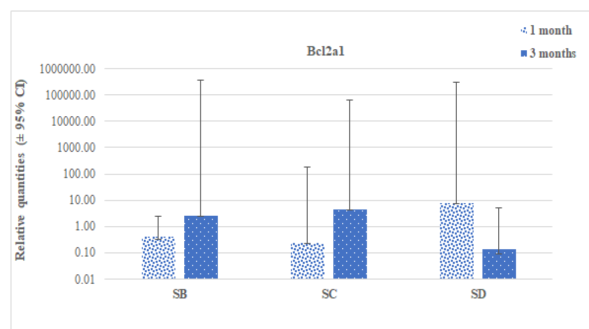


Figure 3 RT-PCR validation of Bcl2a1 relative expression between 1 and 3 months. Each sample was analyzed and 18s rRNA was used as a reference gene. Bars represent logE Mean ± 95%CI, *p-value < 0.05.

Cxcl9

Group	Time (month)	Mean	95% value CI low	95% value CI high	P
SB1	1	0.58	2.760E-001	1.222E+000	0.39
	3	1.72	1.026E-004	2.888E+004	
SC1	1	0.18	1.050E-002	2.943E+000	0.12
	3	5.69	2.300E-004	1.408E+005	
SD1	1	7.56	1.521E-006	3.754E+007	0.17
	3	0.13	4.978E-003	3.517E+000	

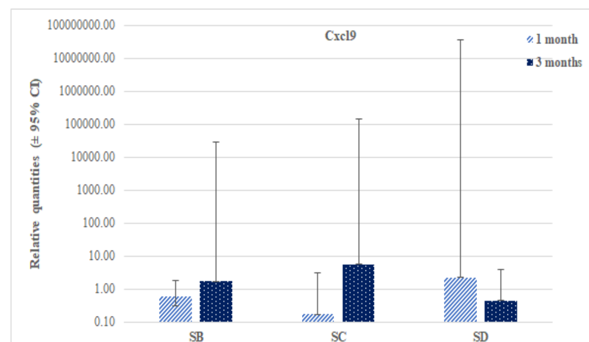


Figure 4 RT-PCR validation of Cxcl9 relative expression between 1 and 3 months. Each sample was analyzed and 18s rRNA was used as a reference gene. Bars represent logE Mean ± 95%CI, *p-value < 0.05.

The relative expression of Bcl2a1 and Cxcl9 was found no statistically significant difference between two time points. Interestingly, the expression of SB and SC group was increased at 3 months compared to 1 month. Bcl2a1 demonstrated the trend of up-regulation like Cxcl9. In contrast, SD group was decreased at 3 months compared to 1 month. Cxcl9 also showed the trend of down-regulation like Bcl2a1.

We chose Bcl2a1 and Cxcl9 as representatives of the inflammatory marker genes because Bcl2a1 expression is positive correlating to the stimulation of leucocytes to protect cells from cell death (Noble et al., 1999). Bcl2a1 is the

gene symbol of B-cell lymphoma 2-related protein A1. This gene mainly expressed in the hematopoietic system, especially in endothelial cells. Previous studies in mouse reported mRNA of Bcl2a1 was produced during lymphocyte development (Su, Rawlings, 2002), and lymphocyte and macrophage activation (Verschelde et al., 2003). Cxcl9 is the chemokine (C-X-C motif) ligand 9 induced by IFN-γ. Cxcl9 mainly functions as the inducer of T-helper 1 cell (Ley, 2008). Cxcl9 is secreted by various immune cell types including T lymphocytes, NK cells, dendritic cells, and macrophages, etc. (Ding et al., 2016). This chemokine is associated with many T-cell-mediated conditions for example organ rejection (Meyer et al., 2001), skin inflammatory such as contact hypersensitivity (Flier et al., 2001). One study suggested that this chemokine was the marker gene of oral inflammatory disease (Marshall et al., 2017). From above mentioned, it is possible to assume that the area with inflammation may increase Bcl2a1 and Cxcl9 expression. Our results (Figure 3 and Figure 4) also demonstrated the trend following this hypothesis.

The limitation of this study is the small sample size. Some of RNA samples were refused to be use because of unsatisfied quality (RIN less than 5, A260/280 ratio less than 1.7 or greater than 2.1). It was due to the enzymatic degradation in the post-mortem tissue from a long period storage (Fordyce et al., 2013). However, Bcl2a1 and Cxcl9 may be used as the candidate genes in a further study.

Conclusion

In comparison to control group at 1 month, DFDBA and DBBM up-regulated Bcl2a1 gene in soft tissue covering bone graft. DFDBA also up-regulated Cxcl9 gene at p-value < 0.05. At 3 months, both of genes were not significantly expressed among treatment groups.

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