**PPARγ-2 and BMPR2 Genes Were Differentially Expressed in Peripheral Blood of SLE Patients with Osteonecrosis**

Ching-Sheng Yeh,1 Fu-Yen Chung,2 Chih-Ju Chen,3 Wen-Juun Tsai,4 Hong-Wen Liu,4 Gow-Jaw Wang,5 and Shiu-Ru Lin6

Most researchers believe that the peroxisome proliferative activated receptor gamma (PPARγ-2) and bone morphogenetic protein receptor type II (BMPR2) play important roles in steroid-induced osteonecrosis (ON). However, the molecular mechanism of this process is still unclear. Recent studies indicate that steroid treatments cause adipocyte formation due to differentiation of mesenchymal stem cells, which then prevents osteoblast formation. This study examined PPARγ-2, bone morphogenetic protein 2 (BMP2), and BMPR2 in patients with systemic lupus erythromatosus (SLE) who eventually developed ON after prolonged steroid treatment. The subjects of this experiment included 220 SLE patients who had undergone steroid treatment for at least 2 years. Fifty-five of the 220 patients were ON patients, and 165 were non-ON patients. Real-time PCR was performed to analyze the expression of the PPARγ-2, BMP2, and BMPR2 mRNA in the peripheral blood of these patients. The results indicated that the expression of PPARγ-2 mRNA increased 37% in the ON patients’ peripheral blood, but the expression of BMPR2 mRNA decreased 57%. The average expression of the PPARγ-2 mRNA in the ON patients was significantly higher than that in the non-ON patients \( (p = 0.044) \). Conversely, the expression of BMPR2 mRNA was significantly lower than that in non-ON patients \( (p = 0.036) \), but the expression of BMP2 mRNA did not significantly differ. This study demonstrated that the PPARγ-2 and BMPR2 have important roles in the ON process after prolonged steroid administration in SLE patients; however, the detailed molecular mechanisms of this process require further study.

**Introduction**

In systemic lupus erythematosus (SLE) patients, osteonecrosis (ON) can occur anywhere on the body, but mainly affects the femoral head, distal femur, and humeral head \( (Zizic \textit{et al.}, 1980) \). The prevalence of ON in SLE patients is reportedly 5–40\% \( (Oh \textit{et al.}, 2004) \). Whether the higher incidence is due to inherited susceptibility to steroid requires further study.

Steroid-associated ON was first reported in 1957 after prolonged steroid treatment. Subsequent research on the pathogenesis of ON has focused primarily on mechanisms of the blood supply and cell differentiation within the bone marrow \( (Glueck \textit{et al.}, 2003) \). Mesenchymal stem cells (MSCs) of bones can form osteoblasts via the osteogenesis process; the cells can also transform into adipocytes via the adipogenesis process. Researchers recently indicated that the ON results from adipocyte formation due to inability of MSCs to form the osteoblastic lineage \( (Cui \textit{et al.}, 1997) \). However, the exact cause is still unknown. In a previous study \( (Li \textit{et al.}, 2003) \), dexamethasone was used to stimulate pluripotential mesenchymal cell (D1) in mice. The steroid stimulated cell differentiation, formed adipocytes, and eventually caused fat accumulation in the bone marrow. This process further deteriorated the marrow microenvironment \( (Cui \textit{et al.}, 1997) \). Additionally, further analysis of the relevant genetic interaction of osteogenesis and adipogenesis indicated that expression of the peroxisome proliferative activated receptor

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gamma (PPARγ-2) gene increased, whereas expression of the Cbfα1/Runx2 gene decreased (Li et al., 2005).

PPARγ is a member of the nuclear hormone receptor subfamily of transcription factors (Rosen and Spiegelman, 2001) that forms PPARγ-1 and PPARγ-2. PPARγ-2 protein stimulates adipocyte formation and regulates bone metabolism. Previous studies have shown that increased expression of the PPARγ-2 gene stimulates adipocyte differentiation and suppresses osteoblast formation (Gimble et al., 1996; Kajkenova et al., 1997). Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β family (TGF-β). The major function of the BMP is promoting bone development, formation, and recovery (Hogan, 1996; Urist, 1997; Zou et al., 1997). BMP2 plays a significant functional role in this process (Young et al., 2005). Via the bone morphogenetic protein receptor type II (BMPR2), BMP2 transfers the messages by the Smad signaling pathway to stimulate cell differentiation of the MSCs needed to form osteoblasts (Heldin et al., 1997; Ryoo et al., 2006).

In vitro experiments show that the steroid stimulates the pluripotent D1 cells to differentiate into adipocytes and at the same time suppresses their differentiation into osteoblasts (Yin et al., 2006). During this process, it also triggers overexpression of adipogenesis-related genes such as PPARγ-2 and suppresses osteogenesis-related genes including Cbfα1/Runx2. This study attempted to confirm the findings of these animal studies in a human population of ON patients taking steroid medicines. The primary subjects were SLE patients who had not taken antithrombotic medicine and had no history of alcoholism. The peripheral blood of SLE patients who had not taken antithrombotic medicine, and none had a history of alcoholism did not. All of these patients have taken the corticosteroid medicine for more than 2 years without taking the antithrombosis medicine, and none had a history of alcoholism (Tables 1 and 2). Peripheral blood samples from all subjects were analyzed for expression of PPARγ-2, BMP2, and BMPR2 mRNA.

**Materials and Methods**

**Sample collection**

Whole-blood samples were obtained from the SLE patients under treatment at the Division of Rheumatology, Immunology, and Allergology of our hospital. This study population included 220 SLE patients: 55 had ON, and 165 did not. All of these patients have taken the corticosteroid medication for more than 2 years without taking the antithrombosis medicine, and none had a history of alcoholism (Tables 1 and 2). Peripheral blood samples from all subjects were analyzed for expression of PPARγ-2, BMP2, and BMPR2 mRNA.

### Table 2. Characteristics of Osteonecrosis Observed in 55 SLE Patients

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of SLE patients with osteonecrosis</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hip(s)</td>
<td>41</td>
<td>74</td>
</tr>
<tr>
<td>Shoulder(s)</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Knee(s)</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Ankle(s)</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Jaw(s)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Elbow(s)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Spine(s)</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

*Expressed as patients with osteonecrosis reported at that site.

### Table 3. The Oligonucleotide Primers of BMP2, BMPR2, PPARγ-2, and β-Actin

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP2</td>
<td>Forward: 5'-ACCTGCAACACGCAACTGGAAATT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TCTTGCTGCAAGGGACCTGTATTCC-3'</td>
</tr>
<tr>
<td>BMPR2</td>
<td>Forward:5'-CAGTGAATCCCTTGACCTTGAG-3'</td>
</tr>
<tr>
<td>PPARγ-2</td>
<td>Forward:5'-ACGTGATCTCTGACTTCAAGTCTGTA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TTTCCGACGACTTGATCCAGGAGG-3'</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward:5'-GCCACCGGCTAAACTCTCTCTC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CAGGAGGACAAATGATCTTG-3'</td>
</tr>
</tbody>
</table>

ON, osteonecrosis; Non-ON, nonosteonecrosis.
in total RNA amounts. Sequences of the PCR primers for BMP2, PPARγ-2, and β-actin (Table 3) were designed according to a PCR primer selection program based on primer3 at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www .cgi (Accessed on January 25, 2005). Real-time PCR was performed in a Rotor-Gene 2000 thermocycler (Corbett Research Inc., Sydney, Australia). The reaction mixture contained 2 μL of 20 mM dNTP, 2 μL of 30 mM MgCl, 2 μL of 20× SYBR green, 2 μL of 1 μM forward primer, 2 μL of 1 μM reverse primer, 4 μL of nuclease free water, 2 μL of 80–100 ng/mL cDNA, and 2 μL of 1 U/μL polymerase. The PCR conditions were as follows: 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 74°C for 40 s. The PCR products (i.e., synthesized dsDNA) were quantified by measuring their fluorescent intensity at the end of each amplification cycle. For each sample, real-time PCR analysis was performed in triplicate to ensure the reproducibility of results.

**Statistical analysis**

All data were analyzed using the Statistical Package for the Social Sciences v10.0 software (SPSS, Chicago, IL). Results were expressed as mean ± standard error. The χ²-test and the Student’s t-test were used to compare the clinical–pathologic parameters between the up- and down-expression genes.
A p-value less than 0.05 was considered to be statistically significant.

Results

The data in this study revealed that the PPARγ-2 and BMPR2 have primary roles in ON resulting from prolonged steroid administration in SLE patients. Of these 220 SLE patients, 55 had ON after 4–5 years of steroid use. More than 90% of all patients were female, and less than 10% of the patients were smokers. Peripheral blood was collected for the total RNA extraction and reversed transcription of the cDNA. Figures 1, 2, and 3 show the expression in the individual analysis of the PPARγ-2, BMP2, and BMPR2 genes revealed by the real-time PCR. The mean fold changes of PPARγ-2, BMP2, and BMPR2 mRNA in SLE with ON are 0.83 ± 0.38, 1.15 ± 0.31, and 1.28 ± 0.12, respectively, compared to 0.31 ± 0.06, 1.29 ± 0.12, and 2.23 ± 0.22 in non-ON patients, respectively. The expression of PPARγ-2 mRNA in SLE with ON was significantly higher than that in non-ON (p = 0.044; Fig. 1B). Further, the expression of BMPR2 mRNA was significantly lower than that in non-ON (p = 0.036; Fig. 3B), but the expression BMP2 mRNA did not significantly differ (p = 0.639; Fig. 2B).

Discussion

This study compared the results of steroid treatment in SLE patients with and without ON symptom and compared the expression of PPARγ-2, BMP2, and BMPR2 mRNA in peripheral blood. The analytical results revealed that PPARγ-2 mRNA expression level in the SLE patients with ON increased 37%, but the expression of BMPR2 mRNA decreased 57%. Moreover, expression of BMP2 mRNA did not significantly differ between groups.

MSCs can differentiate into mesenchymal derivatives, including osteocytes, chondrocytes, adipocytes, and myocytes (Bosnakovski et al., 2005). Stromal stem cells are mainly expressed in the bone marrow, but they can also be isolated from adipose tissue, muscle, synovial membranes, vascular elements in the deciduous teeth (Krabbe et al., 2005), peripheral blood (Kuznetsov et al., 2001), and umbilical cord blood (Rosada et al., 2003). Therefore, the expression of relative genes in MSCs can be analyzed by evaluating peripheral blood.

BMP2 is known to induce osteogenic differentiation in bone marrow–derived MSCs (BM-MSCs) and adipose tissue–derived MSCs (AT-MSCs) in vitro and in vivo (Diefenderfer et al., 2003; Dragoo et al., 2003). BMP2 can also induce MSCs to differentiate and form osteoblasts, which are important factors in bone growth and recovery. BMPR2 is a member of the BMPR family of transmembrane serine/threonine kinases. It also accepts signals from BMP2 through the Smad signaling pathway and transmits them to RUNX2 (Shi et al., 1999). RUNX2 is the earliest transcription factor expressed during osteogenic differentiation and can be induced by BMP2 (Ducy et al., 1997; Lee et al., 2000; Miyazono et al., 2005). Previous experiments have shown that after treating multipotential mesenchymal cells (D1) with dexamethasone, the fold change in RUNX2/cbfa1 genes decreases, which is in line with these data.

PPARγ-2 is an adipocyte-specific transcription factor that is important for the differentiation and formation of adipocytes in MSCs (Akune et al., 2004). Embryonic stem cells differentiate and form osteoblasts instead of adipocytes when the lack of PPARγ-2 genes are in pairs; on the other hand, the increase in the PPARγ-2 gene induces MSCs to differentiate and form adipocytes (Tontonoz et al., 1994). Previous studies indicate that dexamethasone could stimulate increased fold changes in the PPARγ-2 gene, and we obtained similar results.

Prior in vivo and in vitro studies demonstrated that dexamethasone stimulates bone marrow mesenchymal cells to differentiate into adipocytes and accumulate fat in the marrow while suppressing differentiation of cells into osteoblasts (Cui et al., 1997). Therefore, steroids stimulating the increased fold changes in the PPARγ-2 gene in MSCs may

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**FIG. 3.** Quantitative PCR of BMPR2 mRNA. (A) The curves show the experimental results of the ON subjects, the non-ON control subjects, and the internal controls (β-actin). (B) Statistical analysis of BMPR2 expression level of ON and non-ON subjects. The mean of the fold changes of BMPR2 mRNA in ON subjects is 1.28 ± 0.12, and in non-ON subjects is 2.23 ± 0.22. BMPR2 mRNA expression in SLE with ON was significantly lower than that in non-ON (p = 0.036).
increase adipogenesis. Moreover, this process may also reduce the activation of BMPR2, which would cause the signal transmission of BMP2 to decline and also influence the activation of RUNX2, which would cause the decline of osteogenesis. Since adipocytes and osteoblasts share a common pool of progenitor cells, differentiation of osteogenesis is restrained when the MSCs preferentially increase adipogenesis. Hence, the bone marrow microenvironment is upset, and the supply of the osteoblasts needed for bone remodeling or repair of the necrotic bone is impaired. Ultimately, ON develops. However, the detailed molecular mechanism of steroid-induced ON is still unclear. We are in the process of identifying the correlation between steroid-induced ON and single-nucleotide polymorphism (SNP) of ON correlation genes. Preliminary results reveal that several polymorphisms of PPARγ-2 and BMPR2 genes in patients with steroid-induced ON are quite different from those in non-ON patients. Ongoing experiments are expected to further clarify the molecular mechanisms of the pathogenesis of steroid-induced ON and to develop the steroid-induced ON susceptibility SNP chip in the near future.

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References


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