THE EFFECT OF LOW-INTENSITY PULSED ULTRASOUND ON ECTOPIC BONE FORMATION INDUCED BY ELECTROPORATIC GENE TRANSFER OF BMP-4

INTRODUCTION:
Long segmental bone loss often results from high-energy trauma, osteomyelitis or wide excision of malignant conditions. Treatment of these long segmental defects remains a difficult clinical problem. Current therapy to treat segmental bone loss involves the use of autogenous and allogeneic bone graft preparations or bone transport surgical techniques. However, these procedures involve considerable donor site morbidity, cause transmission of infectious agents and require a long treatment period. Recently, in vivo electroporation with a plasmid expression vector has been applied to transfer genes into various organs including skeletal muscle. And we have been researching ectopic bone formation in skeletal muscle of mice by electroporative transfer of bone morphogenetic protein-4 (BMP-4) gene. However, the ectopic bone formed by this system was very small in our first report.

Low-intensity pulsed ultrasound (LI{PUS}) is clinically successful in the healing of fresh fractures and established non-unions. Animal studies have shown an acceleration of bone healing and more callus formation by applying LI{PUS}. The objective of this study was to examine in vivo the influence of LI{PUS} on ectopic bone formation induced by electroporative transfer of BMP-4 gene. We hypothesize that LI{PUS} may accelerate the maturation of ectopic bone and may enlarge the size of it.

MATERIALS AND METHODS:
This study was carried out with permission from the committee of animal experimentation, Tohoku University School of Medicine.

Mice: C57BL/6 male mice were used in this study. Mice were nine-week-old at the time of in vivo electroporation.

Plasmid: A 1.6kb mouse BMP-4 cDNA (Gift from Dr. Hogan BLM) was inserted into the multiple cloning site of pCAGGS expression vector (Gift from Dr Miyazaki J). Plasmid was dissolved in 0.9% NaCl saline at 3.0μg/μl.

In vivo electroporation: 150μl of plasmid DNA containing mouse BMP-4 (pcCAGGS-BMP4) solution was injected into the gastrocnemius of mice. Immediately after injection of the DNA, six electric pulses (100 V, 50ms) were applied through paired-needle electrodes inserted percutaneously. Both limbs underwent the same procedures.

Ultrasound stimulation: LI{PUS} (a 200 microsecond burst of sine waves at 1.5MHz, repeated at 1KHz, 30mW/cm²) exposure was performed daily for 20 minutes on the lateral aspect of the gastrocnemius of one limb with ultrasound gel under general anesthesia (LI{PUS} group). The contralateral limb was not exposed to LI{PUS} (control group). Nine animals each were killed at 7, 10, 14 and 21 days after electroporation (6 for quantitative test and 3 for histological evaluation).

Radiographic analysis: Dissected muscles underwent soft x-ray analysis to measure the size of ectopic bone formation.

Quantitative analysis: After pepsin solubilization of the gastrocnemius, total collagen content in the supernatant from this digestion mixture was measured by the Sircol Collagen Assay Kit (Biocolor, Newtown abbey, Northern Ireland, UK). Calcium content in the supernatant was measured by Calcium E-test WAKO (WAKO, Tokyo, Japan).

Histological analysis: Undecalcified frozen axial sections of gastrocnemius were stained with H&E to evaluate general morphology of ectopic bone formation and with Von Kossa to evaluate calcium deposit. BMP-4 and osteopontin were also evaluated immunohistologically.

Statistical analysis: Differences between groups were examined using a Wilcoxon test. Results were significant at p<0.05.

RESULTS:
In the soft X-ray, more osseous tissue was formed in LI{PUS} group at day 10 (Fig. 1). Radiographs measurements showed significantly more bone formation in LI{PUS} group as compared with the controls at day 10 (p<0.031). The size of bone was the maximum in both groups at day 14. However, no difference was detected at day 14 (data not shown).

There was significant increase in the calcium content with LI{PUS} treatment at day 10 (p=0.031) (Fig. 2). Total collagen content with LI{PUS} treatment increased significantly at day 7 (p=0.047) and day 10 (P=0.031) (Fig. 3). However there were not significant differences in both calcium content and total collagen content at day 14.

Von Kossa staining revealed obviously more calcium deposit in the sections from the LI{PUS} group than the control group at day 10. H&E staining and immunohistological examination of BMP-4 and osteopontin demonstrated no marked difference at all time points.

DISCUSSION:
This is the first report of the in vivo influence of LI{PUS} on ectopic bone formation induced by electroporative transfer of BMP gene. According to the results of this experiment, LI{PUS} did not have a beneficial effect on maximum size of ectopic bone. However, LI{PUS} accelerate bone formation in early phase. Moreover, we found that the application of LI{PUS} stimulate collagen synthesis. This is the first report demonstrate that LI{PUS} accelerate the synthesis of collagen in vivo bone formation model.