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Carthamus tinctorius L. Increases BMP-2 Gene Expression during Bone Fracture Healing in Rats

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Carthamus tinctorius L. is known to improve fracture healing, and bone morphogenetic proteins (BMPs) are associated with the formation and healing process of bone. BMP-2 and BMP-7 are two of the most important BMPs during the bone healing process. Human osteosarcoma MG63 cells and rats were used to determine the effects of _Carthamus tinctorius L._ extract (CTE) on BMP-2 gene expression. BMP-2 gene expression by CTE treatment in human osteosarcoma MG63 cells was not different from the control group until 8 hours of incubation, but was significantly higher, by 31%, than that of the control group at 16 hr of incubation. Microscopic findings of the 9th rib 3 weeks after fracture showed typical rimming of the osteoblast and immature bone formation in control and CTE groups. BMP-2 gene expression by _in situ_ hybridization was remarkably increased by a CTE-supplemented diet in the fracture group compared to the control group. In conclusion, _Carthamus tinctorius L._ increased BMP-2 gene expression in human osteosarcoma cells and fractured bone. But further studies would be needed to elucidate the effect of CTE on fracture healing _in vivo_ because our results did not show any evidence of healing improvement histologically 3rd week after fracture.

Key Words: _Carthamus tinctorius L._, Bone fracture, BMP-2 gene expression

INTRODUCTION

The potential use of bone morphogenetic proteins (BMPs) to promote bone healing is of great interest to orthopedic surgeons. The role of BMPs in bone formation and development in bone fracture healing is now well established. In experimental animals, BMPs elicit bone formation in ectopic sites and healing of critical sized segmental bone defects (Bostrom et al, 1995; Khan et al, 2000). Until recently, little was known of the cellular and molecular mechanisms by which BMPs elicit bone formation. In a series of stunning studies over the last several years, molecular biologists working in several laboratories have elucidated some of these mechanisms. When BMPs bind to their cell surface receptors on mesenchymal cells, the BMP signaling cascade is activated. Signals are sent to the cell nucleus via specific proteins. These results in the expression of genes that lead to the synthesis of macromolecules involved in cartilage and bone formation, and the mesenchymal cell becomes a chondrocyte or an osteoblast (Vainio et al, 1993; Bostrom et al, 1995; Barlow & Fracis-West, 1997; Khan et al, 2000; Hanada et al, 2001; Reddi, 2001).

In recent years, *Carthamus tinctorius L.* was found to have a promoting effect for fracture healing in animal ribs (Kim et al, 1998; Seo et al, 2000), and was known as a good agent that promotes bone growth and healing in the field of alternative medicine. *Carthamus tinctorius L.* powder and its water or ethanol extract increased both bone resorption and bone formation during healing of rib fracture (Seo et al, 2000). Many studies have reported that BMPs are associated with bone healing and osteogenesis after fracture (Barlow & Fracis-West, 1997; Khan et al, 2000; Blkhuis et al, 2001; Braddock et al, 2001; Hanada et al, 2001), and BMP-2, BMP-4, and BMP-7, in particular, appear to be the most effective growth factors in terms of osteogenesis and osseous defect repair (Schilephake, 2002). Thus, it is possible that the promoting effect of *Carthamus tinctorius L.* on bone healing is mediated by the increased expression of BMPs. The clinical applications of recombinant BMP-2 and BMP-7 are being studied the most extensively. BMP-2 is a member of the transforming growth factor (TGF) beta superfamily, which is expressed in many regions of the developing embryo. They have been shown to be involved in many developmental processes, including epithelial-mesenchymal interactions during tooth and limb development (Vainio et al, 1993). However, no report of the effect of *Carthamus tinctorius L.* on BMPs during healing of fractured bone has yet been published.

Therefore, this study was conducted to determine the effects of *Carthamus tinctorius L.* on BMP-2 gene expression in human osteosarcoma cells and during bone fracture healing in rats.

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ABBREVIATIONS: BMP, bone morphogenetic protein; CTE, *Carthamus tinctorius L.* extract.

METHODS

In vitro experiment

Cell: Human osteosarcoma MG63 cells (Korean Cell Line Bank) were used for BMP-2 gene expression at various incubation times with *Carthamus tinctorius L.* water extract (CTE).

***Carthamus tinctorius L.* extract:** A CTE yield of approximately 200 g was obtained by rotating evaporation after boiling 1 kg of *Carthamus tinctorius L.* seed powder (Andong, Kyungbook, Republic of Korea) in 20 liters distilled water for 5 hours.

Cell culture: Cells were routinely cultured at 37°C in a 5% CO_2 atmosphere. Human osteosarcoma MG63 cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum. The MG63 cells were dissociated with trypsin-EDTA (Gibco, Grand Island, NY, USA) and plated on tissue culture plates, after which they were cultured for 3 days. To investigate for the effects of CTE on bone morphogenesis, MG63 cells were treated with or without 0.2% CTE in the culture media. After 0, 4, 8, or 16 hours incubation with CTE, RNA was isolated for Northern blot analysis using the RNeasy kit from Qiagen (Valencia, CA, USA) for each of the untreated and treated cells.

Northern blot: Isolated total RNA was fractionated on 1% agarose-formaldehyde gel and transferred onto a nitrocellulose membrane by downward alkaline blotting with 5xSSC/10mM NaOH for 3 hours. The membrane was hybridized overnight at 42°C with formamide hybridization buffer containing cDNA probe for human BMP-2.

After hybridization, the membrane was washed twice in 2xSSPE/0.5% SDS for 15 min at room temperature and then in 2xSSPE/0.5% SDS for 30 min at 55°C. The blot was incubated in alkaline phosphatase-streptavidin for 30 min at room temperature. The blot was then incubated using CDP-Star chemiluminescence and read after exposure to X-ray film.

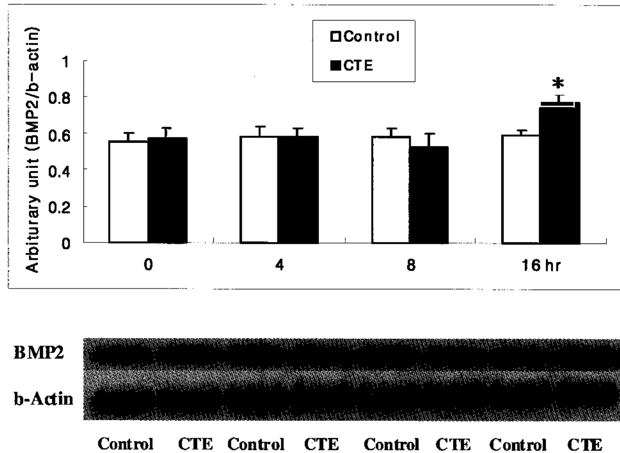

Fig. 1. BMP-2 gene expression according to the duration of incubation with CTE in human osteosarcoma MG63 cells. Values are mean±SE. CTE; Carthamus tinctorius L. extract. *$p<0.01$ vs. control.

In vivo experiment

Animals: Male (~320 g) Sprague-Dawley rats were used in this experiment. Rats were randomly divided into control (n=9), and 1% CTE-supplemented (n=9) groups. All experimental procedures were done in accordance with the guidelines on the use and care of laboratory animals set by the Ethical Committee for the Care and Use of Laboratory Animals of Yeungnam University College of Medicine; these guidelines also satisfy the animal guidelines of National Institutes of Health of the United States.

Diet: Rats were given regular chow or 1% CTE-supplemented chow diets for 3 weeks.

Bone fracture: In the fracture groups, the 9th rib of each rat was fractured using eye scissors while rats were under ether anesthesia, and connective tissues and skin were with 4-0 nylon. The bone healing process was determined after 3 weeks of chow or CTE-supplemented diet.

Histology

***In situ* hybridization:** The bone tissue sections were dewaxed in xylene, followed by dehydration in graded alcohol series. Dried sections were digested by proteinase K(10 μg/ml) for 30 min at 37°C. The sections were postfixed in 4% paraformaldehyde in phosphate buffered saline

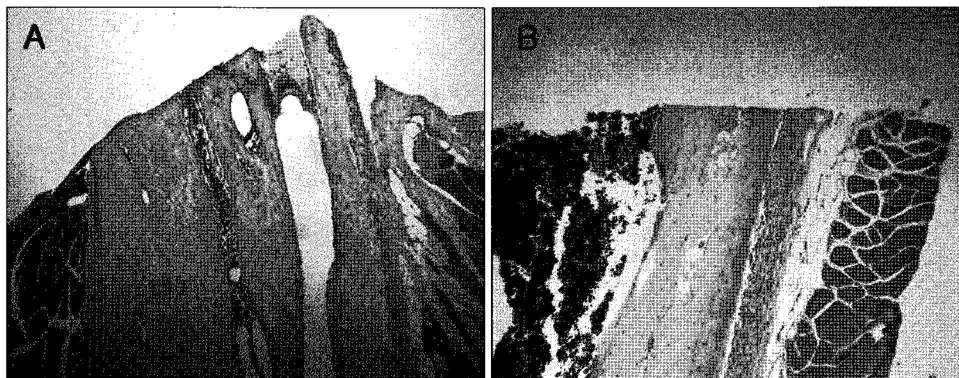

Fig. 2. Microscopic findings (×100) of the normal 9th rib at 3 weeks after fracture in control rats (A) and rats fed a CTE-supplemented diet (B). (A) and (B) show normal bone trabeculae, bone marrow, osteocytes, and periosteum without any pathologic abnormalities.

(PBS) (pH 7.5; 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) for 5 min at 4°C. The sections were incubated with pre-hybridization buffer (4×SSC containing 50% [v/v] deionized formamide) for 10 min at 37°C. The sections were covered with hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1×Denhardt's solution, 4×SSC, 10 mM DTT, 1 mg/ml denatured herring sperm DNA) containing 10 ng of 3'-end-labeled

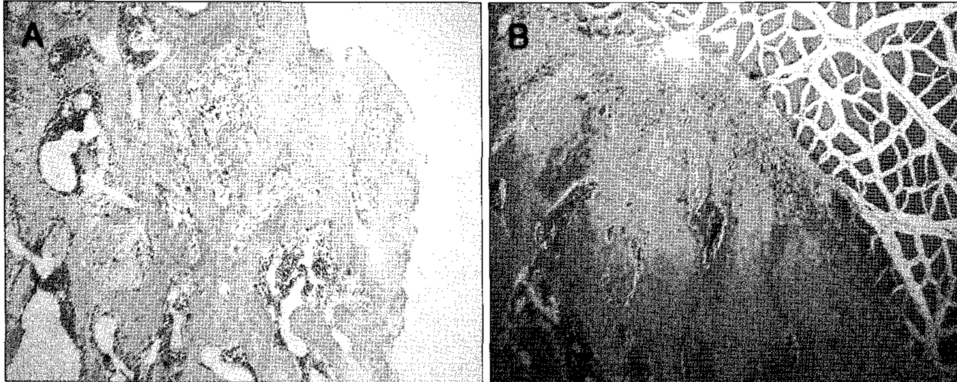

Fig. 3. Microscopic findings (×100) of the fractured 9th rib at 3 weeks after fracture in control rats (A) and rats fed a CTE- supplemented diet (B). (A) showed typical rimming of the osteoblast and immature bone formation, and (B) showed findings similar to those for (A).

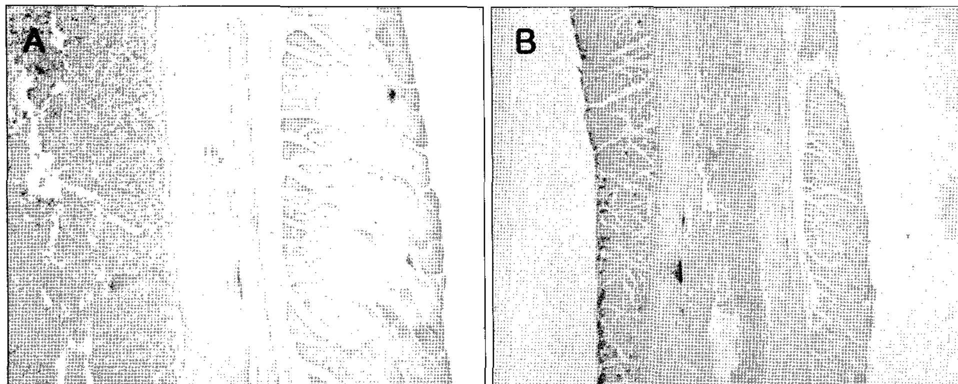

Fig. 4. *In situ* hybridization (×100) of the normal 9th rib at 3 weeks after fracture in control rats (A) and rats fed a CTE-supplemented diet (B). (A) and (B) did not show BMP-2 gene expression. Purple dots represent expression of the BMP-2 gene.

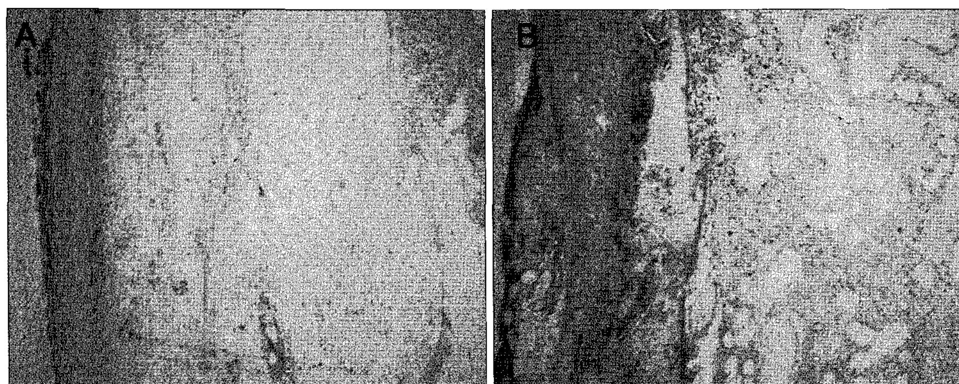

Fig. 5. *In situ* hybridization (×100) of the fractured 9th rib at 3 weeks after fracture in control rats (A) and rats fed a CTE-supplemented diet (B). The BMP-2 gene was expressed in A and was expressed markedly in B. Purple dots represent expression of the BMP-2 gene.

oligonucleotide probe to BMP-2 and incubated at 37°C overnight. The unhybridized probe was washed away by serial washing in 4×SSC, 2×SSC, and 1×SSC (35 min at 37°C), and for a further 15 min in NTE buffer (pH 8.0; 500 mM NaCl, 10 mM Tris, 1 mM EDTA) containing 20 μg/ml RNase for 30 min at 37°C. The sections were then treated with blocking reagent containing 0.1% Triton ×100 and 2% normal sheep serum for 30 min, followed by incubation with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody for 2 hours in a humidified chamber. Excess antibody was removed by washing with 100 mM Tris-HCl (pH 7.5). The antibody was visualized using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium salt (NBT) overnight in a humidified chamber held at room temperature. The sections were treated with 10 mM Tris-HCl (pH 8.1) and 1 mM EDTA in order to stop the color reaction. Finally, the sections were washed with distilled water and mounted with aqueous mounting solution.

Statistical analysis: Values are expressed as means±SE. The significance of difference between two groups was evaluated using Student's t-test.

RESULTS

In vitro experiment

BMP-2 gene expression by Northern blot: BMP-2 gene expression in human osteosarcoma MG63 cells was determined by Northern blot analysis after incubation with CTE for 0, 4, 8, and 16 hours. BMP-2 gene expression induced by CTE treatment was not different from the control group until 8 hours of incubation, and was significantly increased, by 31%, compared to that of the control group at 16 hours of incubation ($p < 0.01$) (Fig. 1). The number of rats in each experimental group was 5.

In vivo experiment

Body weight: Body weight gain (g) in CTE-supplemented rats (67±7.2) did not differ significantly from the control group (84±4.1) when both groups were on the diet for 3 weeks ($p > 0.11$).

Histological changes: Microscopic findings of the non-fractured 9th rib 3 weeks after fracture showed normal bone trabeculae, bone marrow, osteocytes, and periosteum without any pathologic abnormalities in the control or CTE group (Fig. 2). Microscopic findings of the fractured 9th rib 3 weeks after fracture showed typical rimming of the osteoblast and immature bone formation in the control and CTE groups (Fig. 3).

***In situ* hybridization:** BMP-2 gene expression was not expressed in non-fractured ribs of the control or CTE group (Fig. 4). Fractured ribs expressed the BMP-2 gene in the control group, and expression was increased markedly by CTE diet supplementation (Fig. 5).

DISCUSSION

Carthamus tinctorius L. has been reported to improve bone healing after fracture or trauma (Kim et al, 1998; Seo et al, 2000), but its precise mechanism was not elucidated in previous studies.

The process of healing after bone fracture includes inflammation, repair, and remodeling; this process normally restores the injured bone to its original state (Mckibbin, 1978; Simmons, 1985; Hulth, 1989; Sanberg et al, 1993). Inflammation begins immediately after injury and is almost immediately followed by repair. Growth factors and other proteins released by platelets and cells in the fracture hematoma mediate the critical initial events of fracture repair (Nemeth et al, 1988). The bone ends at the fracture site, deprived of their blood supply, become necrotic and are reabsorbed. The cells responsible for this function, the osteoclasts, come from a different cell line than the cells responsible for bone formation (Gothlin & Ericsson, 1976; Buckwalter & Cooper, 1987; Buckwalter, 1994). While the osteoblasts are developed from undifferentiated mesenchymal cells that migrate into the fracture site, some mesenchymal cells at the fracture site proliferate, differentiate, and produce the fracture callus, which consists of fibrous tissue, cartilage, and woven bone. Bone gradually replaces the cartilage through the process of endochondral ossification, which enlarges the hard callus and increases the stability of the fracture fragments (Buckwalter & Cooper, 1987; Buckwalter, 1994; Braddock et al, 2001). In this study, the healing process was determined microscopically 3 weeks after fracture. Thus, at 3 weeks post-fracture, the remodeling process is shown to dominate at the fracture site, as evidenced by the proliferation of osteoblasts and immature bone formation (Fig. 3). The microscopic healing process after fracture did not indicate significant differences between groups in this study. This result was not similar to previous findings reported by other researchers (Kim et al, 1998; Seo et al, 2000), which indicated that *Carthamus tinctorius L.* improved the healing process of fractured bone. This discrepancy might result from different durations of fracture healing and differences in diet supplementation.

The potential use of BMPs to promote bone healing has become of great interest to orthopaedic surgeons in recent years. Although the complex mechanism leading from the local presence of BMP (whether endogenous or exogenous) to bone formation is under increasing scrutiny, limited information is available as to whether endogenous BMPs, their receptors, or other molecules involved in their signal transduction affect the healing process. The osteoconductive capacity of BMPs has been demonstrated in preclinical models, and the efficacy of BMPs for the treatment of orthopaedic patients is now being evaluated in clinical trials.

The results of our *in vitro* experiment showed that CTE supplementation increased BMP-2 gene expression in human osteosarcoma cells at 16 hours of incubation, but not at 0, 4, or 8 hours. This finding suggested that CTE might not stimulate BMP-2 gene expression when exposure is short, or it may indicate that the BMP-2 gene is not an early response gene. In our *in vivo* experiment, BMP-2 gene expression, as evaluated by *in situ* hybridization, was also increased by a CTE-supplemented diet administered to rats for 3 weeks after fracture, as has also been observed in human osteosarcoma cells. It seemed that CTE may act to activate or increase BMP-2 gene expression.

In conclusion, *Carthamus tinctorius L.* increased BMP-2 gene expression in human osteosarcoma cells and fractured bone. But further studies would be needed to elucidate the effect of CTE on fracture healing *in vivo* because our results did not show any evidence of healing improvement histol

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