The Effect of Swimming on Cartilage Formation

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Summary Swimming is a non-weight-bearing exercise. Therefore it has the advantage of maintaining skeletal integrity in aged persons with weakened skeletal structures. Unlike other weight-bearing aerobic exercises, however, it does not appear to exert sufficient stimulus on bone-remodeling activities because the local load-bearing on bone tissues is mild. The purpose of this study was to investigate the effect of swimming on bone remodeling, especially with the use of implanted pellets containing bone morphogenetic protein (BMP) and demineralized bone matrix during the initial stages of the differentiation of mesenchymal cells to cartilage cells. Six-week-old female rats were divided into the swimming group and a control, nonswimming group. Test animals were forced to swim in a water bath for 30 min daily for 2 wk. After the swimming protocol, pellets were implanted and harvested. Messenger RNA isolated from pellets was quantified by means of a reverse transcription-polymerase chain reaction. The expression of RNAs for bone sialoprotein and BMP-6 in pellets from the swimming group was apparently enhanced at 7 d after implantation. These results suggested that systemic hormonal and/or metabolic changes that promote cartilage formation might have occurred after swimming because the effect was observed after the swimming protocol had ended and the pellets were implanted at a non-weight-bearing site.

Key Words bone morphogenetic protein (BMP), exercise, swimming, cartilage, implant

Age-related diseases such as osteoporosis and osteoarthritis have emerged as major public health problems. Exercise may be appropriate because it is a low-cost intervention available to most of the general public.

Swimming is a non-weight-bearing exercise. Therefore it has the advantage of maintaining skeletal integrity in aged persons with weakened skeletal structures. The effectiveness of swimming on bone has been reported (1–3). Unlike other weight-bearing aerobic exercises, however, it does not appear to exert sufficient stimulus on bone remodeling activities because the local load-bearing on bone tissues is mild (4).

In humans, many examinations of the effects of exercise on bone by analyzing bone mineral density have shown the results of slight improvement or of the potential for improvement. The studies in animals have taken a long period, not less than a few months, to evaluate the effectiveness.

Bone morphogenetic protein (BMP), originally identified in the extract of demineralized bone, induces endochondral bone formation in vivo (5). The implants reconstructed with BMP and demineralized bone matrix newly form cartilage and bone within a few weeks (6).

BMP is a bone-inductive protein and can initiate a process that begins with cartilage formation and ends in de novo bone formation. BSP is a sialic acid-rich integrin-binding glycoprotein that is a major noncollagenous protein of bone and other mineralizing connective tissues. The BSP appears to be involved in the initial mineralization of the cartilage matrix before its replacement by bone (7). The cloning of cDNAs for BMPs allowed discoveries of BMP-2 to -8, which belong to the transforming growth factor-beta (TGF-β) superfamily (8). BMP-6 was found to induce ectopic bone formation, and the high levels of expression of BMP-6 were observed in precartilaginous mesenchymal and ectomesenchymal tissues (9). By analyzing the expression of the matrix proteins such as BSP and BMP-6 in the implants, we can determine the maturational stages of chondrocytes.

The purpose of this study was to investigate the effect of swimming on bone remodeling, especially during the initial stages of the differentiation of mesenchymal cells to cartilage cells induced by BMP.

MATERIALS AND METHODS

Purification of BMP and preparation of the implants. BMP was partially purified by the method described previously (10). Bovine cortical bone was frozen with liq-
uid nitrogen, pulverized in a large mill, and sieved to obtain particles 1–2 mm in diameter. Bone powder was demineralized with 0.6 M HCl at 4°C for 12 h and washed with deionized water. Demineralized bone matrix was defatted by several washes with acetone, then extracted in 4 M guanidine hydrochloride (Gdn• HCl). The protein extract was separated into the supernatant and the precipitate by centrifugation. The precipitate was dialyzed against distilled water, lyophilized, and used as the residue. The supernatant was subjected to gel filtration through a precalibrated Sephacryl S-200 column by the use of 4 M Gdn• HCl/50 mM Tris-HCl (pH 7.4). The BMP-containing fraction (1.5 mg of total protein amount) was mixed with 15 mg of the residue obtained from Sprague-Dawley rat bone matrix, reconstituted by ethanol precipitation, and used for the in vivo implantation.

**Swimming protocol and implantation of BMP pellet.** A total of 16 Sprague-Dawley strain female rats (6wk old) were divided into two groups (8 rats each). The average body weight was about 170 g. Animals in the swimming group were allocated for aquatic exercise (swimming) in a water bath for 30 min/d at noon for 2 wk. The water bath was in a stainless steel tank (52×147×60 cm) with a water temperature of 28 ± 1°C. Food and water were allowed ad libitum, and all rats were fed a conventional solid diet containing 1.2% calcium and 0.96% phosphorus. They were housed in individual cages in a room where the temperature (23 ± 1°C) and light-to-dark cycle (12–12 h) were regulated with automatic devices. After the 2-wk exercise protocol, pellets were implanted subcutaneously in the abdominal wall and harvested after 5, 7, or 10 d. The implant was dissected out and the soft tissue carefully removed. The pellets were cut into two parts; half was analyzed for the expression of RNAs and the other half was used for histological examination.

**Reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA from the pellets was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (11). As a template for PCR, single-strand cDNA was prepared from 1 μg with the SuperScript preamplification system (Gibco BRL, Grand Island, NY, USA). For the BSP nucleotide sequence, PCR primers BS1 (nucleotide positions 107–127) and BS2 (nucleotide positions 568–588) were used (12). For the BMP-6, PCR primers B42 (nucleotide positions 870–890) and B43 (nucleotide positions 1146–1166) were used (13). PCR conditions were 5 cycles of 94°C (1 min), 50°C (1 min), and 72°C (1 min), and 25 cycles of 94°C (30 s), 55°C (30 s), and 72°C (30 s), followed by 10 min at 72°C. The amplified sample (10 μl) was analyzed with the use of 5.25% polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained with ethidium bromide solution and observed with UV light.

**Histology.** The histological specimens were fixed in formalin and embedded in paraffin. Six-micrometer sections were stained with hematoxylin-eosin or toluidine blue.

**RESULTS**

**Expression of RNAs from implanted pellets**

Polyacrylamide gel electrophoresis analysis of the PCR products revealed an expression of the mRNAs of BSP, BMP-6, and glyceraldehydephosphate dehydrogenase (GAPDH) (Fig. 1). As shown in Fig. 1, the expression of mRNA for BSP and BMP-6 in pellets from the swimming group was enhanced markedly (Fig. 1, A and B, lane 4). These results suggested that swimming promoted the developmental expression of BSP and BMP-6 mRNAs in the implanted pellets. All bands for the GAPDH mRNA showed essentially the same intensity (452 bp) (Fig. 1, C).

**Histology**

The sequence of histological analysis is shown in Fig. 2, which was obtained from cross sections of the pellets harvested 7 d after implantation. Chondrocytes were observed over a large area in the pellets from the swimming group (Fig. 2, A), but no cartilage formation was observed, and only fibroblast-like cells were present in the pellets from the control group (Fig. 2, B). When the Gdn• HCl-extracted rat demineralized bone residue
Fig. 2. Photomicrographs of pellet cross sections harvested 7 d after implantation. Chondrocytes were observed in the pellet’s bone matrix carrier from the swimming group (A), but only fibroblast-like cells were observed in the sample from the nonswimming (control) group (B).

Without BMP was implanted, no cartilage formation was observed, and only the carrier matrix particles (residue) were present (data not shown).

Therefore we concluded that swimming was effective for cartilage formation in the pellets.

DISCUSSION

Many reports have been made on the effect of physical training to increase bone mass and density and mineralization. Physical activity (inducing weight-bearing loads, such as treadmill or wheel running) is associated with increased bone mass in exercised rats and mice and in humans. We previously reported the effect of voluntary exercise on osteoinductive activity in the bone in rat (14). Sprague-Dawley male and female rats were allowed to exercise freely by running on a treadmill or kept as controls without exercise for 53 d. Decalcified humeral diaphyses from the experimental and control rats were implanted intraperitoneally into host rats and harvested after 33 d. A significant increase in bone formation was confirmed in the implanted bone matrices from the running group in comparison with those from control animals by soft X-ray photography and the determination of alkaline phosphatase activity and mineral content (14). These results suggested that osteoinductive activity in the bone was probably due to increased BMP following voluntary exercise.

Tail suspension in rats, which suppresses limb ground pounding and also reduces the muscular involvement, was shown to induce bone mass loss (15). Although swimming is a non-weight-loading exercise, it seems to be an aerobic and moderate exercise for the whole body. Regarding humans, the conditions provided by the aquatic environment during swimming can be advantageous in exercise programs for populations at risk, such as patients recovering from injuries or nontrained and elderly people. Only a few studies, however, refer to humans. Although swimming stimulates the muscle components and the total physical activities, the mechanism of its effect on the bone formation in vivo remains unclear. For assessing cartilage induction, our examination using implanted BMP-containing pellets is a valuable investigative tool addressing this question.

The strength of the exercise would be an important factor in a study on the prevention of bone loss. It is generally recognized that excessive training is disadvantageous for the protection of human bone. The experimental conditions used in this study (swimming for 30 min daily for 2 wk) can be considered to be moderate (16).

We analyzed BSP and BMP-6 mRNAs as markers for differentiated chondrocytes, and their expressions were enhanced in the pellets harvested from exercised animals within a week after implantation. By histological examinations, we detected cartilage formation in pellets obtained from the swimming group 7 d after implant (Fig. 2, A), but little cartilaginous tissue was detected in pellets from the control group until the 10th day (data not shown). These data suggest that the implants showed cartilage formation being promoted by swimming exercise.

Surprisingly, the exercise effect was observed regardless of the period when the swimming protocol had ended and the pellets were implanted at non-weight-bearing sites. This indicates that systemic hormonal and/or metabolic changes might have occurred after swimming. This is the first report that provides evidence of the maintenance of elevated osteoinductive activity by swimming training.

Le Blanc et al. reported that the exercised rats gained calcium at a significantly greater rate than controls did (17). It has been demonstrated that the intestinal Ca absorption by rats in the aquatic exercise group was significantly higher than by those in nonexercise control group (17). According to these findings, swimming exercise might be effective for increasing intestinal Ca absorption and Ca accumulation. Some other metabolic sequential changes also might have occurred after swimming and improved the bone metabolism in the whole body. The present study clearly demonstrated that swimming is effective in promoting osteoinductive activity, especially during the initial stages of the differentiation of mesenchymal cells to cartilage cells by the use of the implanted BMP-containing pellets, and it suggested that the enhancement might be maintained after the swimming protocol. Future studies on the bone metabolism during and after swimming may reveal the mech-
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