

No effect of growth hormone on bone graft incorporation

Titanium chamber study in the normal rat

Per Aspenberg, Jian-Sheng Wang, Peter Choong and Karl-Göran Thorngren

A recent series of publications reported greatly improved mechanical properties and increased callus size during the late stages of fracture healing in normal (not hypophysectomized) rats after twice daily injections of growth hormone (GH). We tested whether GH could enhance the incorporation of bone allografts in a new experimental model where we have demonstrated an increase in bone allograft incorporation by local application of basic fibroblast growth factor (bFGF). Cylinders of defatted allogeneic cancellous bone were placed as grafts in titanium Bone Conduction Chambers in the tibiae of female rats. Only one end of this chamber is open for tissue ingrowth. This permits us to measure the distance into the graft that new bone penetrates after entering the chamber. We injected 10 rats with 1.5 IU per rat of subcutaneous human re-

combinant GH twice daily for 6 weeks and another 10 rats with similar doses of sterile normal saline. GH caused a constant increase in the rate of weight gain and in the serum concentration of Insulin-like Growth Factor 1 (IGF 1). Tibiae became longer and the ash weight of the second tail vertebra was increased. We also noted an increased joint cartilage thickness. There was no difference in the amount of new bone that had penetrated and replaced parts of the graft in GH-treated or control rats and this was also the case with TcMDP activity of bone samples from both groups. New bone forms in the grafts by membranous (metaplastic) ossification. It appears that the effects of excessive GH stimulation on endochondral and membranous ossification in this model are markedly different.

Department of Orthopedics, University Hospital, S-221 85 Lund, Sweden. Tel +46-46 171500. Fax -46 130732
Submitted 94-01-29. Accepted 94-04-28

We recently demonstrated that local growth factor application increased the incorporation of bone allografts (Wang and Aspenberg, 1993). It would be a clinical advantage if growth hormone (GH) had a similar effect, as GH can be administered systemically. Although GH has striking effects on longitudinal bone growth, its effects on fracture healing are unclear. Many early animal studies reported the effects of GH on fracture healing, but a study by Northmore-Ball et al. (1980) appeared to rule out the possibility that GH could stimulate fracture healing (for review see Bak 1992). All these studies used at most 1 GH injection per day. In contrast, Bak et al. (1990a) showed that due to the natural secretory pattern of GH, repeated daily injections were necessary to obtain growth stimulation in normal (not hypophysectomized) rats. Consequently, a recent series of publications reported greatly improved mechanical properties and increased callus diameters during late stages of fracture healing in normal rats when GH was injected twice daily (Bak et al. 1990a, b, 1991).

These reports stimulated us to examine bone graft incorporation during GH treatment.

Material and methods

The chamber

The Bone Conduction Chamber (Wang and Aspenberg 1993) consists of a threaded titanium cylinder, formed from 2 half cylinders held together by a hexagonal closed screw cap. The overall length is 13 mm, the screw cap is 7 mm, leaving 6 mm of the implant to be screwed into the bone. The bone ingrowth chamber has an inside diameter of 2 mm, and an inside length of 7 mm. The outside diameter is 3 mm. There are 2 bone ingrowth openings, 1 mm in diameter, at the bottom of the chamber (Figure 1).

Graft preparation

The allografts were taken from the proximal tibia of 10 outbred female 200-220 g Sprague-Dawley rats (Møllegaard, Copenhagen, Denmark). This colony

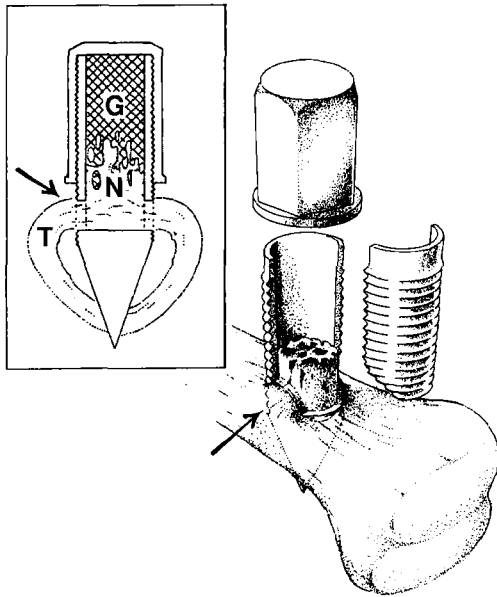


Figure 1. The bone conduction chamber. Implant in position at the proximal tibial metaphysis. The ingrowth entrances (arrow) are situated in cortical bone. Inset shows schema with new bone ingrowth (N) and allograft bone (G). (Reproduced with the permission of European Journal of Experimental Musculoskeletal Research).

expresses 2 haplotypes on RT1A, and a variety of non-MHC cell membrane-associated antigens. A 2×6 mm bone rod was resected in an axial direction from the knee joint with a trephine. The epiphysis was excised. The proximal part of the graft had the densest cancellous bone and was later placed at the ingrowth end of the chamber. Each rat yielded 2 grafts. The pair was kept sterile and frozen at -70°C . Before implantation, the pairs were lipid-extracted in chloroform/methanol overnight, rinsed 3 times in methanol and air-dried. This extraction enhances the graft incorporation rate in another chamber model, by diminishing the immunologic response (Thoren et al. 1993).

Operation procedure

The grafts were implanted in chambers in 20 female rats of the same strain as the donors (230–250 g). We used female rats in order to mimic the conditions of the successful model by Bak et al. (1990a, b, 1991), although we have previously used bigger male rats with the Bone Conduction Chamber. They were kept in our animal facilities for 1 week before experiments started (22°C ; 2 rats in each cage, free access to food pellets and water). The rats were anesthetized with peritoneal injections of 0.6–0.7 mL of a solution containing 1 mL pentobarbital (60

mg/mL) and 2 mL diazepam (5 mg/mL) and 1 mL saline.

Under aseptic conditions, longitudinal incisions were made bilaterally over the anteromedial aspect of the proximal tibial metaphysis. After incising and raising the periosteum, the medial and posterior lateral cortices were pierced with a 1.0 mm spike just anterior to the insertion of the medial collateral ligament. The hole created in the medial cortex was manually enlarged with a 2.7 mm drill. The graft was placed in the chamber, which was then screwed into position such that the bone ingrowth holes were placed at the level of the cortical bone, and the pointed end of the implant was engaged through the opposite cortical bone. The implant was located 1 mm distal to the growth plate. The wound was closed in layers with 5/0 Dexon interrupted fascial sutures and a 4/0 monofilament nylon continuous, subcutaneous stitch.

Hormone treatment

After chamber insertion, the rats were randomized into 2 groups of 10 animals each. One group received intraperitoneal injections of 1.5 IU per rat of human recombinant GH (Genotropin, a gift from Kabi-Pharmacia Peptide Hormones, Stockholm, Sweden), twice daily, and the other received phosphate buffered saline. We started treatment the day after the operation with 2 daily injections administered between 8 and 9 a.m. and 4.30 and 5 p.m.

Evaluation

The rats were weighed once a week and killed at 6 weeks with an overdose of Mebumal. Blood samples were obtained at 3 and 6 weeks and analyzed for IGF 1 by radioimmunoassay, using polyclonal antibodies. The chambers were opened and the contents removed. The second tail vertebra was dissected out. The length of the tibia was measured with a caliper. The patella of the non-operated hind leg was taken out and its distal and proximal thirds were removed. The remainder was fixed in 4% formalin, decalcified and embedded in paraffin. Histological sections were cut parallel to the surface created when the proximal and distal parts were removed, i.e., perpendicular to its long axis. The thickness of the joint cartilage (including its calcified portion) was measured on the center of the medial and lateral patellar facets, respectively, using a grid-counting technique in three separate sections, thus producing 6 measurements for each patella, of which the mean was used for the statistical analysis.

An intravenous injection of 2.3 MBq $^{99\text{m}}\text{Tc}$ -MDP was given 3 hours before the rats were killed. The

scintimetric activity of the tissue harvested from the chambers and of the second tail vertebra was measured in a well counter. The tail vertebra was ashed in a muffle furnace at 800 °C for 24 hours and weighed. The specimens from the chambers were fixed in 4% formalin, decalcified and embedded in paraffin. The specimens were cut parallel to the long axis of the chamber with a microtome and stained with hematoxylin and eosin. 3 sections from the middle of the specimens, each at 300 µm distance from the other, were used for histology and histomorphometry. The area of the new ingrown bone was measured with the Videoplan™ equipment at magnification 125 ×. This area includes marrow cavities and graft bone remnants which had been surrounded by new bone. Sometimes the border between the new bone and the graft was difficult to define. In those cases, a straight line was drawn to connect approximately 7 points, where new bone had reached the farthest distance into the graft.

As we measured an area, but were more interested to know the distance which the new bone had reached in the graft, we calculated the mean ingrowth distance by dividing the new bone area by the distance between the walls of the chamber, i.e., the width of the specimen (Aspenberg and Wang 1993).

All histological measurements were done blindly, so that each specimen was given a code number, and the specimens were investigated in random order. Statistical analysis was done with Student's *t*-test or two-factor Anova.

Results

2 of the GH-treated rats died for unknown reasons, and 1 lost weight during the last 2 weeks and was discarded (although ingrowth distance and scintimetry in this rat did not differ from the others).

Table 1. The effect of growth hormone on body weight and serum levels of IGF 1. Mean SD

Time weeks	Body weight (g)		IGF 1 (ng/mL)	
	GH	Control	GH	Control
0	249 6	245 8	—	—
3	316 9	251 9	940 90	540 70
6	346 10	264 7	720 140	510 70

The GH-treated rats gained weight by 39 percent, whereas controls gained only 8 percent (Table 1). The serum concentration of IGF 1 was higher than controls at both 3 and 6 weeks, but the increase at 6 weeks was less (Table 1). Tibiae on the unoperated side were also longer in the GH-treated groups than controls (Table 2). A similar difference was not detectable between tibiae harvested from the operated side of either group.

The patellar joint cartilage was thicker in the GH-treated rats (Table 2). In 2 control specimens, the histological sections were somewhat oblique to the joint surface, producing a falsely increased thickness and decreasing the difference from the GH group. There was higher ^{99m}TcMDP tracer activity in the second tail vertebra of GH-treated rats in comparison to control animals (Table 2). However, the activity per mg ashed bone was the same for GH-treated and control groups. Unlike data from the tail vertebra, GH treatment did not increase the absolute activity of ^{99m}TcMDP in allograft tissue harvested from the BCC of treated rats.

GH treatment did not increase the ingrowth of new bone into the allograft material. The amount of ingrown bone in GH-treated rats was the same as for the control group (Table 2). There was no apparent qualitative histologic difference between specimens harvested from either treatment group (Figure 2).

Table 2. Local effects of growth hormone. Mean SD

	Control		Growth hormone		P
Tibial length (mm)					
Non-operated side	35	0.5	37	0.5	0.001
Operated side	35	0.8	36	0.9	0.07
Patellar cartilage thickness (mm)	0.27	0.03	0.31	0.06	0.03
Vertebra					
Tc-MDP (cpm·10 ⁻³)	18.0	3.9	22.2	4.0	0.1
Ash Wt (mg)	72	5	85	3	0.001
Tc-MDP Ash Wt (cpm·10 ⁻³ ·mg ⁻¹)	0.25	0.05	0.26	0.05	0.6
Allograft					
Ingrowth distance (mm)	0.6	0.3	0.5	0.3	0.8
Tc-MDP (cpm·10 ⁻³)	12.9	3.5	10.4	2.2	0.1

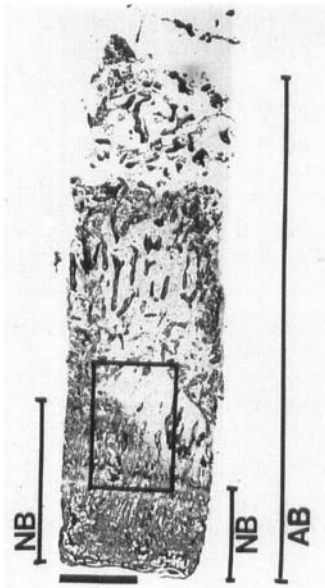


Figure 2a. Histological appearance of tissue from BCC in control rats. Allograft bone (AB) occupies the length of the specimen. In the lower portion of the specimen, corresponding to the entrance of the BCC, new bone (NB) can be seen and the ingrowth distance is clearly visible. (Magnification 18 \times , scale bar represents 1 mm).

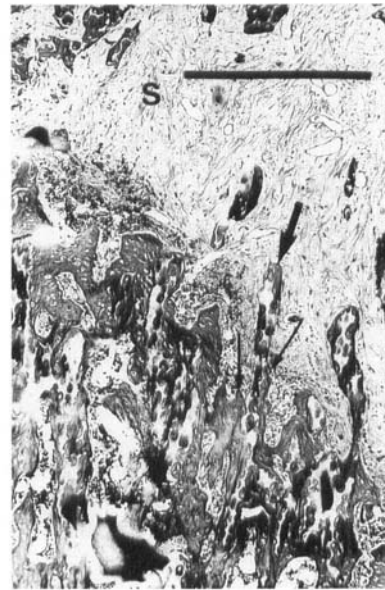


Figure 2b. High power view of host-allograft bone interface from inset area in Figure 2a. New bone (small arrows) is visible adjacent to allograft bone (large arrow) and the vitality of new bone is reflected by the presence of intralacunar nucleated cells. The new bone appears to be insinuating itself between the trabeculae of the lower regions of the allograft bone. The newly formed bone is surrounded by a cellular and vascular stroma (S). The stromal cells are mainly of a spindle-shaped variety. (Magnification 90 \times , scale bar represents 500 μ m).

Discussion

GH stimulates longitudinal bone growth by an effect on chondral precursors in the epiphyseal cartilage (Isaksson et al. 1987) and in-vitro cultures of cartilage progenitor cells (Maor et al. 1989) suggest that this might be a direct differentiation-promoting effect. Identification of GH receptors on osteoblast-like cells (Barnard et al. 1991), as well as the ability of this hormone to stimulate osteoblast proliferation in culture, support the notion that GH may also have a direct effect on cells of the osteoblast lineage (Slootweg et al. 1988). The ability of GH to regulate populations of mononuclear cells representing osteoclast and chondroclast precursors (Lewinson et al. 1993) suggests that GH may also have an important role in bone remodeling. Various studies have implicated insulin-like growth factors (IGFs) as mediators of GH's systemic action (For review, see Mohan and Baylink 1990).

The role of growth hormone in fracture healing is still controversial. Several articles have reported little effect of growth hormone on healing fractures in experimental rats (Harris et al. 1975) and rabbits. In contrast, Bak et al. (1990b) have demonstrated

positive effects of growth hormone therapy on the healing of experimental fractures in rats. Early studies by Simpson et al. (1953) also demonstrated that hypophysectomy prevented the normal healing function of fetal rat calvaria in vivo. Reintroduction of growth hormone was accompanied by full regeneration of the parietal defects. Studies in normal human volunteers (Brixen et al. 1990) have demonstrated that a short course of recombinant human growth hormone not only increased the markers of bone resorption and formation, but was also associated with an elevation of osteocalcin and bone alkaline phosphatase that persisted long afterwards. The authors concluded that rhGH treatment stimulated osteoblasts and activated bone remodeling. The anabolic effects of GH have thus stimulated us to examine its effect on the incorporation of allograft bone, using a previously described model.

The absence of an increased ingrowth distance in GH-treated animals is not inherent in the model. Using these chambers, we have demonstrated a large increase in ingrowth distance into cancellous allografts that were pre-treated with basic fibroblast growth factor (Wang and Aspenberg 1993). The model can also distinguish between different

ingrowth distances into different bone replacement materials (Aspenberg and Wang 1993). Therefore, if GH were to induce an effect on graft incorporation, we believe it would have been evident in this model.

Some of the grafts may not have differed from the host regarding MHC antigens, whereas probably all had antigens creating a weak transplantation barrier. We think this is of minor importance, since the defatting procedure used in this study leads to even less immunologic reactions than those initiated by frozen allogeneic bone (Thoren et al. 1993).

The clear anabolic effect of GH on the experimental rats, as evidenced by increases in weight, elongation of tibiae and increased ash weight of the second tail vertebra, confirmed the efficacy of the substance under the experimental conditions of this study. The increased IGF 1 level at 6 weeks indicates that the exogenous human GH still exerted an effect at that time and had not become inactivated by antibodies. The increased joint cartilage thickness may be a direct or indirect phenomenon, which motivates further study. Surprisingly, regarding the degree of bone graft incorporation the data demonstrated no significant change between the growth hormone-treated and untreated rats, as measured by histomorphometry. This result was in contrast to data on the other effects of growth hormone and raised several questions about the model, the process of bone healing and the role of growth hormone in such a phenomenon.

First, the rat bone ingrowth model which was developed from our experience with similar models in rabbits, has been found to support membranous bone formation with little if any cartilage tissue. The absence of a significant endochondral component in this model may explain the apparent lack of effect of growth hormone as endochondral ossification is thought to be a dominant mechanism in which GH affects the development of bone. Load conditions in this model may be conducive to membranous bone formation, although load data specifically pertinent to the rat chamber model are not available.

In the present study, TcMDP scintimetry of harvested samples from both growth hormone-treated and untreated rats demonstrated activity of the tracer in allograft bone. These findings were consistent with bone formation in the chambers. The results, however, were not different between the treatment groups and this raises doubt about the role of growth hormone in early membranous healing of bone. The process of bone formation is a complex cascade of events beginning with the recruitment of primitive mesenchymal cells and ending with the mineralization of synthesized matrix. During this process, the

development of the osteoblast phenotype has been shown to be under great local paracrine and auto-crine regulation (for review see Martin et al. 1989). One may speculate that these local regulatory mechanisms far outweigh the effects of a systemic hormone, albeit important for post-natal somatic growth. If true, our results highlight the difference between local bone healing and the process of remodeling. Furthermore, similar TcMDP activities per mg ash weight in the vertebral bone of growth hormone-treated and untreated rats suggests no apparent stimulation of osteoblast function by GH.

In conclusion, while confirming the anabolic action of growth hormone on host bone, our results demonstrate little effect of systemic growth hormone on the incorporation of allograft bone. These observations suggest that growth hormone has a greater part to play in the remodeling of bone rather than in the process of bone healing, particularly if the nature of healing is membranous. Clinically, these findings raise doubts upon the ability of GH to increase host-allograft incorporation.

Acknowledgements

We thank Inger Mårtensson and Carina Kristensson for their technical assistance, and Dr Jan Holmberg, Pharmacia Kabi Peptide Hormones, Stockholm, for measuring IGF 1. The study was supported by the Swedish Medical Research Council (2031 and 09509), the Medical Faculty of Lund University, the Crafoord, Österlund, Trygg-Hansa and Tore Nilsson Foundations, and the Royal Australian College of Surgeons.

References

- Aspenberg P, Wang J S. A new bone chamber used for measuring osteoconduction in rats. *Eur J Exp Musculoskel Res* 1993; 2: 69-71.
- Bak B. Fracture healing and growth hormone. A biomechanical study in the rat. Thesis, University of Aarhus, Aarhus, Denmark 1992.
- Bak B, Jørgensen P H, Andreassen T T. Dose response of growth hormone on fracture healing in the rat. *Acta Orthop Scand* 1990a; 61 (1): 54-7.
- Bak B, Jørgensen P H, Andreassen T T. Increased mechanical strength of healing rat tibial fractures treated with biosynthetic human growth hormone. *Bone* 1990b; 11 (4): 233-9.
- Bak B, Jørgensen P H, Andreassen T T. The stimulating effect of growth hormone on fracture healing is dependent on onset and duration of administration. *Clin Orthop* 1991; 264: 295-301.
- Barnard R, Ng K W, Martin T J, Waters M J. Growth hormone (GH) receptors in clonal osteoblast-like cells mediate a mitogenic response to GH. *Endocrinology* 1991; 128 (3): 1459-64.

- Brixen K, Nielsen H K, Mosekilde L, Flyvbjerg A. A short course of recombinant human growth hormone treatment stimulates osteoblasts and activates bone remodeling in normal human volunteers. *J Bone Miner Res* 1990; 5 (6): 609-18.
- Harris J M, Bean D A, Banks H H. Effect of phosphate supplementation, thyrocalcitonin, and growth hormone on strength of fracture healing. *Surg Forum* 1975; 26: 519-21.
- Isaksson O G, Lindahl A, Nilsson A, Isgaard J. Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. *Endocr Rev* 1987; 8 (4): 426-38.
- Lewinson D, Shenzer P, Hochberg Z. Growth hormone involvement in the regulation of tartrate-resistant acid phosphatase-positive cells that are active in cartilage and bone resorption. *Calcif Tissue Int* 1993; 52 (3): 216-21.
- Maor G, Hochberg Z, von der Mark K, Heinegard D, Silbermann M. Human growth hormone enhances chondrogenesis and osteogenesis in a tissue culture system of chondroprogenitor cells. *Endocrinology* 1989; 125 (3): 1239-45.
- Martin T J, Ng K W, Suda T. Bone cell physiology. *Endocrinol Metab Clin North Am* 1989; 18 (4): 833-58.
- Mohan S, Baylink D J. Bone growth factors. *Clin Orthop* 1991; 263: 30-48.
- Northmore-Ball M D, Wood M R, Meggitt B F. A biomechanical study of the effects of growth hormone in experimental fracture healing. *J Bone Joint Surg (Br)* 1980; 62 (3): 391-6.
- Simpson M E, Van Dyke D C, Asling C W, Evans H M. Regeneration of the calvarium in young normal and growth hormone treated hypophysectomised rats. *Anat Rec* 1953; 115: 615-25.
- M C, van Buul Offers S C, Herrmann Erlee M P, van der Meer J M, Duursma S A. Growth hormone is mitogenic for fetal mouse osteoblasts but not for undifferentiated bone cells. *J Endocrinol* 1988; 116 (3): R11-3.
- Thoren K, Aspenberg P, Thorngren K G. Lipid extraction decreases the specific immunologic response to bone allografts in rabbits. *Acta Orthop Scand* 1993; 64 (1): 44-6.
- Wang J S, Aspenberg P. Basic fibroblast growth factor and bone induction in rats. *Acta Orthop Scand* 1993; 64 (5): 557-61.