Improved long-term bone-implant integration

Experiments in transgenic mice overexpressing bovine growth hormone

Per H Morberg[1,2], Olle G P Isaksson[3], Carina B Johansson[1], Jonas Sandstedt[4] and Jan F Törnell[4]

Several recent studies have investigated the effects of growth hormone (GH) on the healing of fractures and bone ingrowth, but with conflicting results. The negative results may be due to antibody formation against injected GH or because some experimental models are able to prove only positive GH effects. In this study, we wanted to investigate the effect of GH on implant integration in bone. To avoid potential formation of antibodies against injected GH, we used a model with transgenic mice overexpressing bovine GH (bGH).

Titanium implants were inserted in the forehead of the mice. 4 months after insertion, the implants were cut out en bloc with the surrounding bone. The calcified specimens were cut and ground to a thickness of approximately 10 μm. Histomorphometry demonstrated significantly more direct bone-to-metal contact in the transgenic mice than in the nontransgenic littermates. Our findings indicate that systemic administration of GH in humans may improve implant integration in bone.

[1]Biomaterials group, Dept. of Handicap Research, Medicinaregatan 8, S-413 90, Gothenburg, Sweden. Tel +46 31 773-29 50. Fax -29 41, [2]Dept. of Orthopaedics, East Hospital, Inst. for Surgical Sciences, [3]Dept of Internal Medicine, RCEM, Sahlgrens Hospital, [4]Dept. of Physiology, University of Gothenburg, Gothenburg, Sweden
Submitted 95-10-21. Accepted 97-03-08

Recently several in vitro studies have demonstrated enhancement of osteoblast-like cell metabolism and proliferation during stimulation with growth hormone (GH) (Chenu et al. 1990, Scheven et al. 1991, Kassem et al. 1993). Positive effects on bone have also been demonstrated in vivo, using GH-loaded polymethylmethacrylate (PMMA) bone cement and bioactive ceramics (Downes et al. 1990, 1991a,b).

Improved fracture-healing or bone formation has been observed in animal experiments after administration of GH (Nielsen et al. 1991, Ehrnberg et al. 1993, Mosekilde and Bak 1993). In contrast, other studies have found no effect on fracture healing after systemic administration of GH (Carpenter et al. 1992) or an effect only of GH administered early in the fracture healing process (Bak et al. 1991). It has been demonstrated that the effect of injected GH depends on the time of its evaluation (Bak and Andreassen 1991). One explanation of the different results regarding experimental fracture-healing in animals may be antibody formation against injected non-species-specific GH. This has been shown as early as eight days after administration (Groesbeck and Parlow 1987). Recently Aspenberg et al. (1994) observed increased IGF-1 levels during prolonged systemic GH treatment, indicating that the injected GH had an effect throughout the entire treatment period of 6 weeks. However, they found no increased bone graft incorporation in their rat chamber model.

We wanted to investigate the effect of high systemic levels of GH on implant integration. To avoid the possible negative effects of antibody formation against non-species-specific GH, we used transgenic animals with endogenous high levels of bovine GH (bGH). In a previous study (Morberg et al. 1995), we have demonstrated improved implant integration in the early healing phase. The aim of this study is to evaluate whether the positive effects observed after short-term follow-up persist in the long term.

Animals and methods

12 transgenic mice and a control group of 22 nontransgenic littermates were used in the study. The animals were 16–26 weeks old at implant insertion.

A transgenic mouse has normal mouse DNA. However, by using genetic manipulation an extra gene has been added to the DNA, in this case a gene expressing bGH, resulting in high endogenous production of bGH. With this specific regulatory sequence used for expressing bGH, the hormone is produced in several parts of the body, but mainly in the liver. As the hor-

Figure 1. Undecalcified ground section with the implant in situ. (Total length of the implant 2.0 mm)

mone is not produced locally in the bone, it has to reach the bone through the circulation and this model may therefore be compared to other models using injections or continuous infusion.

Generation of transgenic animals

A BstEII-EcoRI fragment was isolated from the plasmid Mt-bGH 2016 (kindly provided by Dr. Richard Palmiter) and used for pronuclear injection. This DNA fragment contains the mouse metallothionein I promoter, linked to a genomic sequence encoding bGH. The generation of transgenic mice was performed as described earlier (Hogan et al. 1986). Mice that integrated the bGH gene were identified with polymer chain reaction (PCR) analysis of DNA from tail biopsy specimens obtained three weeks after birth of the animals. One primer hybridizing to the metallothionein promoter and another primer hybridizing to the bGH gene were used.

Measurements of bGH

The concentration of bGH in serum was determined by a radioimmunoassay (RIA) with antisera, kindly provided by AF Parlow (Pituitary Hormones and Antisera Center, Torrance, CA, USA). Serum was collected from the animal tails at the time of implant insertion and analyzed in triplicate samples. The assay was carried out in 200 µL PBS (pH 7.4) containing 0.05% bovine serum albumin (RIA grade, Sigma Chemicals Co, St Louis, MO, USA), 1.25 µL mouse serum antibGH-antiserum (1/400,000) and ^{125}I-labeled bGH (5000 cpm). The bGH standard (0.1–50 ng/tube) contained 1.25 µL normal mouse serum. After overnight incubation (4 °C), the bound hormone was precipitated by adding 1.4 mL of a mixture of polyethylene glycol (16% w/v, final concentration), bovine gamma globulin (2 mg/mL; Cohn fraction II,III Sigma) and triton X-100 (0.02%) in 0.05 M TRIS-HCl buffer (pH 8.5). The samples were further incubated for 30 minutes at +4 °C, centrifuged, and the supernantants were aspirated. The pellets were counted for gamma-radioactivity and the results were expressed as ng bGH/mL.

Implants and surgical technique

Threaded implants with an outer diameter of 1.4 mm and a length of 2 mm were manufactured from commercially pure titanium. A curved skin incision was made on the forehead of the mice. Gentle surgical technique and only well-sharpened instruments were used. After removal of the soft tissue, a 1.2 mm wide hole was drilled through the bone and the implant was screwed into the bone. The bottom of the implant was thus placed in the nasal cavity, while the top of the implant projected one or two threads above the cortical level (Figure 1).

4 months after implant insertion, the animals were killed and the implants were cut out en bloc with the surrounding bone.

Histological procedures

The specimens were dehydrated and embedded in polymethylmethacrylate plastic. Using the procedure described by Donath (1988), sections were cut through the implants and the surrounding calcified bone. From each implant 1 section was ground to a thickness of approximately 10 micrometers. The sections were stained in 1% toluidine blue in a 1% borax solution mixed 4:1 with 1% pyronin G solution.

Histomorphometry

The amount of bone apposed to the surface and the percentage of the total area inside the threads with bone (Figure 2) were calculated for the best thread as well as for all the threads of the implants, using a Leitz Aristoplan light microscope with objectives 1.6×–50×, and a zoom of 2.5×.

By delineating with an on-screen pointing device, the total interface length and the area inside each thread were measured. To calculate the percentage of bone-metal contact (BMC), the interface without bone apposed to the surface was excluded from the total length of the interface. When calculating the percentages of bone inside the threads (AREA), the areas with soft tissue inside the threads were excluded from the total area inside the threads. Bone thickness was measured as close as possible to the implants, but always lateral to the region with endosteal or periosteal callus, usually 100–300 µm lateral to the implants.

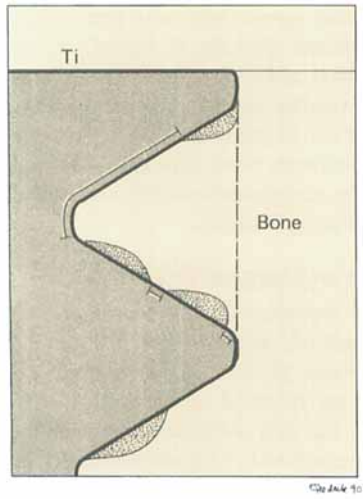

Figure 2. The percentage of bone directly apposed to the implant and percentage of bone area inside the threads were calculated using a computer-based morphometric assessment.

The thickness was measured on both sides of the implants and the average of the 2 values was given in the results.

The light microscope with a Leitz Microvid unit and a semiautomatic computer-based morphometry package were used for blinded evaluation of the sections.

Statistics

Statistical differences were calculated using a nonparametric test (Wilcoxon rank-sum test). $P < 0.05$ was considered significant.

Table. The amount of bone apposed to the surface, the percentage of the total area inside the threads with bone for the best thread as well as for all the threads of the implants and bone thickness at the implant site

Animal no.	A	B	C	D	E
bGH transgenic animals					
1	34	88	38	83	520
2	33	62	46	77	641
3	24	56	41	90	561
4	39	85	45	82	721
5	12	41	34	51	844
6	22	52	47	87	563
7	44	73	64	87	637
8	39	75	48	90	933
9	23	50	44	62	707
10	39	56	53	85	524
Mean	31	64	46	80	665
Controls (bGH-negative littermate)					
11	19	55	41	62	522
12	21	37	43	75	847
13	0	0	0	0	522
14	17	39	53	73	615
15	24	31	47	75	654
16	28	74	49	78	630
17	24	54	55	83	750
18	22	50	39	81	732
19	18	38	48	85	552
20	10	17	36	65	916
21	28	72	59	90	850
22	13	31	47	84	685
23	10	14	60	86	842
24	16	32	42	81	673
25	12	41	44	80	765
26	0	0	12	26	617
27	46	69	51	70	655
28	48	62	46	65	765
29	46	67	64	92	706
Mean	21	41	44	71	700

A percentage of direct bone-to-metal contact (BMC) for all threads, B percentage of direct bone-to-metal contact (BMC) for the best thread, C percentage of bone in all threads (AREA), D percentage of bone inside the best thread (AREA), E bone thickness (μm) at site of implantation.

Results

5 animals, 2 transgenic and 3 nontransgenic littermates, died during or shortly after surgery.

There were no macroscopic observations of adverse tissue reactions at the time of killing the animals. Microscopic investigation revealed soft tissue and bone in the threads and in contact with the implants. Extensive endosteal bone regeneration was observed in most of the sections with growth of bone down the part of the implants located in the nasal cavity. As a result, most implants were located with one full thread plus part of a thread on each side in the original dense bone with newly formed endosteal bone on the part of the implant in the nasal cavity. The thread on each implant entirely in original dense bone is called the "best thread" in the Table. Little, of any, periosteal callus was seen near the skin. We observed no major differences between the groups regarding periosteal callus formation.

Numerous macrophages and fibroblasts were noted at the interface in areas without direct bone-to-metal contact. Giant cells were occasionally seen. At the implantation site, the bone was not composed of two clearly distinguishable cortical shells with intervening spongios bone, but appeared more like dense bone penetrated by air-filled cavities.

The mean levels of bGH in the transgenic mice were 1124 ng/mL, on average ten times higher than the peak levels of mouse-GH (Sinha et al. 1977) in normal mice. Normal littermates had no detectable levels of bGH.

The bGH-transgenic mice had significantly higher percentages of direct bone-to-metal contact (BMC)

than controls, especially in the best thread ($p = 0.008$), but this was also true of the entire implant ($p = 0.03$). The transgenic animals demonstrated more bone in the best thread than the control group, although this difference was not statistically significant. The transgenic animals were larger than normal animals, having an average weight of 55 versus 41 grams. However, no statistical difference was observed in bone thickness at the implantation site (mean 665 µm for the transgenic versus mean 700 µm for the control group).

The percentage BMC, the amounts of bone in the threads and bone thickness at the implant site are shown in the Table.

Discussion

The different results in the literature after systemic administration of GH are confusing. Besides the demonstrated antibody formation against the injected non-species-specific GH (Groesbeck and Parlow 1987), it is also possible that only certain experimental models can prove positive GH effects, because of the complex effect of GH. However, in our previous study we demonstrated that transgenic mice with high endogenous levels of bGH have a better early implant incorporation capacity than non-transgenic littermates (Morberg et al. 1995). We have now shown that this effect persists after a long follow-up. When evaluating the amount of bone inside the threads, we found no significant difference between the groups, but there was a small difference between the best threads in the bGH-transgenic mice and those in the control group. One possible explanation is that there may be a "true" difference between the amounts of bone inside the threads of the two groups, but the groups are too small to demonstrate this statistically, resulting in a type 2 error. However, another explanation that can not be ruled out is that the transgenic mice may have a specific capacity to tolerate implants better, resulting in a higher BMC but no major changes in the AREA. When evaluating biocompatibility of different materials, we usually found more significant differences in the BMC than in the AREA between the materials. Often there are significant differences in the BMC, but not in the AREA (Johansson 1991, Morberg 1991).

4 months of follow-up may not seem to be "long-term", but the bGH-transgenic mice have a normal lifespan of 1–1.5 years and normal animals 2 years. As the transgenic animals are heavier than the normal mice, one might expect that the primary fixation would be better, due to thicker bone. However, no significant difference in bone thickness was observed at the site of implantation. We therefore conclude that the high levels of bGH are the main reason for the improved long-term implant integration in the bGH-transgenic animals. To our knowledge, this is the first demonstration that high systemic levels of bGH may improve the long-term implant incorporation in bone. These results are encouraging and indicate that systemically administered GH may improve implant/prosthesis incorporation.

Acknowledgements

This study has been supported by grants from the Medical Research Council (MFR), Greta and Einar Asker Foundation, Hjalmar Svensson Research Foundation, Gothenburg Medical Society, Swedish Medical Society, Eivin and Elsa K:son Sylvan Foundation, Wilhelm and Martina Lundgren Research Foundation, Royal Academy of Science and Literature and Loo and Hans Osterman Foundation.

References

Aspenberg P, Wang J-S, Choong P, Thorngren K-G. No effect of growth hormone on bone graft incorporation. Acta Orthop Scand 1994; 65 (4): 456-61.

Bak B, Andreassen T T. The effect of growth hormone on fracture healing in old rats. Bone 1991; 12 (3): 151-4.

Bak B, Jörgenssen P H, Andreassen T T. The stimulatiing effect of growth hormone on fracture healing is dependent on the onset and duration of administration. Clin Orthop 1991; 264: 295-301.

Carpenter J E, Hipp J A, Gerhart T N, Rudman C G, Hayes W C, Trippel S B. Failure of growth hormone to alter the biomechanics of the fracture-healing in a rabbit model. J Bone Joint Surg (Am) 1992; 74 (3): 359-67.

Chenu C, Valentin-Opran A, Chavassieux P, Saez S, Meunier P J, and Delmas P D. Insulin-like growth factor I hormonal regulation by growth hormone and by 1,25(OH)2D3 and activity on human osteoblast-like cells in short-term cultures. Bone 1990; 11: 81-6.

Donath K. Die Trenn-Dünnschliff-Technik zur Herstellung histologischer Präparaten von nicht schneidbaren Gewerben und Materialen. Der Präparator 1988; 34 (1): 197-206.

Downes S, Wood D J, Malcolm A J, Ali S Y. Growth hormone in polymethylmethacrylate cement. Clin Orthop 1990; 252: 294-8.

Downes S, Di Silvo L, Klein C P A T, Kayser M V. Growth-hormone-loaded bioactive ceramics. J Mat Sci: Materials in Medicine 1991a; 2: 176-80.

Downes S, Kayser M V, Blunn G, Ali S Y. An electron microscopic study of the interaction of bone with growth hormone-loaded bone cement. Cells and Materials 1991b; 1 (2): 171-6.

Ehrnberg A, Brosjö O, Låftman P, Nilsson O, Strömberg L. Enhancement of bone formation in rabbit by recombinant human growth hormone. Acta Orthop Scand 1993; 64 (5): 562-6.

Groesbeck M D, Parlow A F. Highly improved precision of the hypophysectomized female rat body weight gain bioassay for growth hormone by increased frequency of injections, avoidance of antibody formation, and other simple modifications. Endocrinology 1987; 120 (6): 2582-90.

Hogan B, Costatini F, Lacy B. In: Manipulating the mouse embryos. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 1986.

Johansson C B. On tissue reaction to metal implants. Thesis, University of Gothenburg, Gothenburg, Sweden 1991.

Kassem M, Blum W, Ristelli J, Moskilde L, Eriksen E F. Growth hormone stimulates proliferation of normal human osteoblast-like cells in vitro. Calcif Tissue Int 1993; 52: 222-6.

Morberg P. On bone tissue reaction to acrylic cement. Thesis, University of Gothenburg, Gothenburg, Sweden 1991.

Morberg P, Isaksson O, Johansson C B, Sandstedt J, Törnell J. Improved early bone-implant integration in transgenic mice overexpressing bovine growth hormone. J Mat Sci: Materials in Medicine 1995; 6: 541-4.

Mosekilde L, Bak B. The effect of growth hormone on secondary fracture healing in rats- a histological description. Acta Orthop Scand (Suppl 253) 1993; 64: 29.

Nielsen H M, Bak B, Jörgenssen P H, Andreassen T T. Growth hormone promotes healing of tibial fractures in the rat. Acta Orthop Scand 1991; 62 (3): 244-7.

Scheven B A A, Hamilton N J, Fakkeldij T M V, Duursma S A. Effect of recombinant human insulin-like growth factors I and II (IGF-I/II) and growth hormone of normal adult human osteoclast-like cells and human osteogenic sarcoma cells. Growth Regul 1991; 1: 160-7.

Sinha Y N, Salocks C B, Wickes M A, Vanderlaan W P. Serum and pituitary concentrations of prolactin and growth hormone in mice for a twenty-four-hour period. Endocrinology 1977; 100 (3): 786-91.