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Bone formation enhanced by induction

Bone growth in titanium implants in rats

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Bone-inducing materials have been investigated for the purpose of augmenting bone formation in implants made of porous fiber titanium. The bone-inducing materials used were: (1) Bone from the iliac crest of inbred rats (isografts), (2) Antigen-extracted, autolyzed, demineralized bone from outbred rats (AAA bone a.m. Urist), and (3) AAA bone combined with bone marrow from inbred rats. Tubes of fiber titanium were packed with bone-inducing materials and implanted in the back musculature of inbred rats. Bone formation was assessed by labelling with ^{3}H-proline (collagen synthesis) and ^{47}Ca (mineral deposit) and by content of calcium of the harvested implants.

Isografts and AAA bone with marrow yielded a substantial amount of new bone. Without the marrow, AAA bone yielded very small amounts of new bone.

Bone ingrowth in porous materials is a promising method for fixation of weight-bearing implants to bone (Galante & Rostoker 1973, Andersson et al. 1978, 1982, Hench & Ethridge 1982, Kuo et al. 1983, Chen et al. 1983, Ronningen et al. 1983) secure fixation requires that the host bone can produce new bone that invades the pores. Among remedies that can possibly augment this osteogenesis are autogeneic bone and other bone-derived materials.

The idea of a bone-inducing factor, introduced decades ago and comprehensively reviewed by Burwell (1966), remained a theoretical concept until the recent discovery of chemical substances that can promote bone formation (Farley & Baylink 1982. Urist et al. 1983). Urist (1980) has developed a procedure for chemosterilisation, autolysis and antigen extraction of allogeneic bone (AAA bone) that preserves a bone morphogenic protein (BMP), and sufficient mechanical strength as well.

We have packed tubes of porous fiber titanium with bone-inducing materials and implanted them in the muscles of rats for assessment of their osteogenic potential.

Material and methods

Surgical procedure

Twenty-four male, inbred rats (Wistar Kyo/Nih/Mol/SPF) 299–319 g were used. During anaesthesia a pouch was prepared in the erector spinae musculature on both sides 2 cm proximal to the iliac crests. The muscle fascia was incised longitudinally and the pouch created by gentle stump dissection.

We used three kinds of bone-inducing materials, isografts, and AAA bone with or without bone marrow. Each recipient carried two implants; the allocation of implant type was randomized for each pouch. Forty-eight implants, 16 of each kind, were installed.

Fluanisone (Hypnorm Vet®, LEO, Denmark) 5 mg/kg s.c. was used for anaesthesia.

Fiber titanium implants

Cylindrical tubes of porous fiber metal 5.1 mm outer diameter by 5.2 mm length, and with an inner diameter of 2.0 mm were used. The implants (Zimmer, USA) had been made from 0.25 mm thick wire of pure titanium to a relative density of 50 per cent (Rostoker et al. 1974).

Bone-inducing materials

(1) Isografts. Sixteen inbred male rats (Wistar Kyo/Nih/Mol/SPF) weighing 39–61 g received 7.4×10^4 Bq ^{85}Sr intraperitoneally every second day for 42 days (body weight 150–211 g) as a mineral tracer.

At the time of implantation the animals were anaesthetized and both iliac crests removed. The crests from one donor served for one implant. The radioactivities of the crests were immediately measured in a Packard Auto-Gamma Scintillation Spectrometer. The right crests were thereafter minced to 1–2 mm particles that were packed into the implants

scheduled to have isografts. The surplus minced bone was recounted to obtain the radioactivity of the graft. The left iliac crests (reference bone) were stored for analysis together with the implants.

(2) AAA bone. The femurs of 32 male rats (outbred Wistar/Af/Mol/SPF), weighing 280–300 g, were used for preparation of chemosterilized, autolyzed, antigen-extracted allogeneic (AAA) bone. Six-millimeter lengths of the mid-diaphyses were removed and prepared according to Urist (1980). Before freeze-drying, the segments of the right femurs were split longitudinally in 8 nails. At implantation as many as possible of the nails were inserted into the implant. The weights of the 8 nail portions before implantation and surplus nails after, and of the left femur segments (references) were recorded.

(3) Bone marrow. Half of the implants with AAA bone were to have bone marrow as an additional bone-inducing material. Sixteen inbred female rats (Wistar Kyo/Nih/Mol/SPF) 250–300 g were used as donors. During anaesthesia the marrow from the left femur was removed and put into the implants before the nails of AAA bone were entered. After the implants had been placed in the muscle pouch, the marrow from the right femur was placed at the ends of the implants. Meticulous care was taken to obtain marrow without any bone particles from the donor.

Analyses

Twelve animals were killed 12 days after implantation and 12–25 days after implantation. Twenty-four hours before sacrifice the animals had received 7.4×10^5 Bq/kg ^{47}Ca (Amersham Int., Ltd., England) and 3.7×10^7 Bq/kg L-(5-^{3}H)-proline (New England Nuclear, USA), administered intraperitoneally, for labelling of bone mineral and collagen. After sacrifice, the implants and right tibiae were removed and counted for activity of ^{47}Ca and ^{85}Sr (^{85}Sr relevant for the implants with isografts only).

The tissue was extracted from the implants as described by Rønningen et al. (1984). The extracts, right tibias, reference iliac crests and reference AAA bone were hydrolyzed and analyzed for hydroxyproline, as described by Firschein (1969). The hydrolysates of the tibiae and of the tissue from the implants were passed through a Dowex column to separate proline and hydroxyproline (Firschein 1969). The radioactivity of the hydroxyproline fraction was measured in a liquid scintillation counter (Mark II, Nuclear Chicago, USA). The method of channels ratio with external standard was applied for correction of quenching. The content of calcium in all hydrolysates was measured by atomic absorption spectrophotometry (Instrumentation Lab. Inc., USA).

Computation and statistics

The collagen contents at the isografts and AAA bone implants were computed by combining their data with the radioactivities (isografts), dry weights (AAA bone) and collagen contents of the reference samples.

The specific activities of the tibias were expressed as radioactivity per amount of substance. The results from the implants were expressed as the ratios between the total radioactivities of the implants and the relevant specific activities of the tibiae. The ratios were referred to as collagen and mineral indexes. The mineral indexes were also calculated as described by Elves (1974), with the modification that we replaced dry weights by the calcium contents in molar units.

The net bone formation in the isograft containing implants was assessed by their total calcium content minus the amount of calcium that stemmed from the donors. The latter was computed by combining the ^{85}Sr activities of the isografts after harvest with the ^{85}Sr activities (corrected for radioactive decay) and calcium content of the reference iliac crests.

If not otherwise stated, a nonparametric procedure for simultaneous multiple comparisons (Sokal & Rohlf 1969) was applied to test differences. This precaution was taken because heterosedastic variances invalidated parametric tests. Averages are expressed as arithmetic means and dispersion as ± 1 standard deviation.

Results

There were no postoperative complications. At sacrifice the animals weighed 313–336 g at 12 days and 348–372 g at 25 days.

The hydroxyproline content of the bone-inducing materials was 5.78 ± 1.15 μmol. Tested by analysis of variance, there was no difference between the groups. None of the AAA bone references contained any calcium.

At 12 days there were no significant differences in collagen synthesis (Table 1). The isograft maintained its collagen synthesis at the longer interval, while the AAA bone and AAA bone with marrow both had significant declines. AAA bone without marrow had lower collagen synthesis at 25 days than any other group at any interval.

At both time intervals the AAA bone without marrow had lower mineral incorporation than the other types (Table 2). At 25 days the AAA bone with marrow had a mineral incorporation higher than the isografts. The isografts had an increase in mineral deposit from 12 to 25 days. None of the mineral indexes calculated a.m. Elves (1974) was lower than 1.0. Both isografts and AAA bone with marrow had a substantial increase in bone formation from 12 to 25 days (Table 3). At both intervals the AAA bone without marrow yielded only about 4 per cent of the amount of bone yield of AAA bone with marrow.

Discussion

Three kinds of bone-inducing materials have been assessed for their osteogenic capacity after implantation in the back musculature of rats. Demineralized, antigen-extracted autolyzed allogeneic bone (AAA bone) combined with isogeneic bone marrow had an osteogenic potential similar to isogeneic bone. AAA bone without marrow induced a vivid collagen synthesis at the early interval, but had otherwise a very small osteogenic potential.

Table 1. Collagen synthesis rate within implants expressed as indexes by dividing the total radioactivity of hydroxyproline in the implants by the specific radioactivity of hydroxyproline of the right tibia (Mean ± SD)

Graft	12 days		25 days
Isograft	31.2± 8.5		31.3±4.8 *
AAA bone	43.9±32.4	*	15.8**±3.4 *
AAA bone marrow	40.9± 7.0	*	26.5±4.8

* Statistically different, $p < 0.05$.
** Statistically different from all other groups, $p < 0.05$.

Table 2. Mineral deposit rate within implants expressed as indexes by dividing total radioactivity of ^{47}Ca in the implants by the specific radioactivity of the right tibia (Mean ± SD)

Graft	12 days	25 days
Isograft	199±68.5 *	331±50.7 *
AAA bone	13.2±12.0 *	21.9±17.6 *
AAA bone w/marrow	390±289	473±59.9

* Statistically different, $p < 0.05$.

Table 3. Bone formed within implants assessed by the amounts of calcium deposition during the experiment (mol × 10^{-6}; Mean ± SD).

Graft	12 days	25 days
Isograft	29.7±10.5 *	70.6±11.3 *
AAA bone	1.5± 1.9 *	2.8± 2.8 *
AAA bone w/marrow	35.1±20.3	74.2± 7.9

* Statistically different, $p < 0.05$.

Content of calcium is clearly a valid measure of net bone formation in the AAA bone containing implants, since the AAA bone had been completely demineralized before implantation. The results for the isografts rest on some assumptions. Retention or release of radioactive constituents has been widely applied for the purpose of studying bone formation and resorption both *in vivo* and *in vitro* (Cohn & Gusmano 1967, Firschein & Alcock 1969, Klein et al. 1983, McDonald et al. 1983, Reeve et al. 1976). In a pilot study we measured the radioactivity of transplants massively labelled with ^{85}Sr as compared to unlabelled transplants placed in rat musculature. At 14 days the labelled transplants had lost about 30 per cent of their radioactivities. The radioactivities of the unlabelled transplants and tibiae of the host animal did not exceed background activity. Thus ^{85}Sr release due to resorption seems not to cause any detectable reincorporation in locations known to have a rapid bone formation. Incorporation of ^{47}Ca due to passive exchange with the calcium in the dead donor bone might introduce an erratic overestimation in the mineral indexes for the isografts (Elves 1974, Craig Gray & Elves 1979, Mellonig et al. 1981). However, as the indexes calculated a.m. Elves stayed far above 0.30 (Elves 1974), this contribution is only minor. The collagen indexes are free of this objection.

There have been numerous publications in which the authors have addressed the osteogenic capacity of various skeletal tissues and their combinations, or specially treated tissue derivatives (Burwell 1966, Newman & Boyne 1971, Urist et al. 1975, Nade 1977, Oikarinen

& Korhonen 1979, Craig Gray & Elves 1979, Lindholm & Urist 1980, Iwata et al. 1981, Urist & Dawson 1981, Lindholm et al. 1982, Volpon et al. 1982, Gupta & Tuli 1982, Cummine et al. 1983, Wittbjer et al. 1983). McDavid et al. (1979) found that porous ceramic implants impregnated with bone mattow had bone formation within the pores. Vandersteenhoven & Spector (1983) were able to augment bone formation within porous polysulfone implants by inserting particles of demineralized bone into the superficial pores before implantation in the subcutis of rats.

Demineralized bone combined with bone marrow has unanimously been found to have a high osteogenic potential, that typically matches that of autogeneic bone, or is even better. Lindholm & Urist (1980) found that the source of AAA bone was essential for the bone yield, diaphyseal bone being the best, and took this as indirect evidence that AAA bone plays an active role when combined with bone marrow. While the existence and effect of a bone morphogenic protein are well documented (Urist et al. 1983), the mechanism advocated that decalcified matrix releases BMP which stimulates precursor cells to differentiate to bone-producing cells, has been questioned by other authors. Nade (1977) and Cummine et al. (1983) argue that the bone marrow is the crucial factor for osteogenesis while the matrix attains a passive "scaffolding-like" role. Craig Gray & Elves (1979) assessed the osteogenic potential for bone fractions separately. They concluded that the endosteal and intrahaversian osteoblasts account for 60 per cent of the osteogenesis, the stromal cells of marrow and periosteal cells for only 30 per cent. This finding was reconciled with those of other authors by a proposal that the real osteogenic tissue of a "gently removed marrow" resides in removed endosteal osteoblasts.

Our results support the hypothesis that the bone marrow is the crucial factor for bone formation. The contribution of bone marrow without AAA bone is being investigated in an ongoing study.

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References

Andersson, G. B. J., Gaechter, A., Galante, J. O. & Rostoker, W. (1978) Segmental replacement of long bones in baboons using a fiber titanium implant. *J. Bone Joint Surg.* **60**-A, 31–40.

Andersson, G. B. J., Lereim, P., Galante, J. O. & Rostoker, W. (1982) Segmental replacement of the femur in baboons with fiber metal implants and autologous bone grafts of different particle size. *Acta Orthop. Scand.* **53**, 349–354.

Burwell, G. (1966) Studies in the transplantation of bone. *J. Bone Joint Surg.* **48**-B, 532–566.

Chen, P. Q., Turner, T. M., Rønningen, H., Galante, J. O., Urban, R. M. & Rostoker, W. (1983) A canine cementless total hip prosthesis model. *Clin. Orthop.* **176**, 24–33.

Cohn, S. H. & Gusmano, E. A. (1967) Kinetics of strontium and calcium skeletal metabolism in the rat. *Proc. Soc. Exp. Biol. Med.* **126**, 79–83.

Craig Gray, J. & Elves, M. W. (1979) Early osteogenesis in compact bone isografts: A quantitative study of the contributions of the different graft cells. *Calcif. Tiss. Int.* **29**, 225–237.

Cummine, J., Armstrong, L. & Nade, S. (1983) Osteogenesis after bone and bone marrow transplantation. *Acta Orthop. Scand.* **54**, 235–241.

Elves, M. W. (1974) An evaluation of the strontium-85 for the assessment of experimental bone grafts. *Acta Orthop. Scand.* **45**, 641–651.

Farley, J. R. & Baylink, D. J. (1982) Purification of a skeletal growth factor from human bone. *Biochemistry* **21**, 3502–3507.

Firschein, H. E. (1969) Collagen and mineral dynamics in bone. *Clin. Orthop.* **66**, 212–225.

Firschein, H. E. & Alcock, N. W. (1969) Rate of removal of collagen and mineral from bone and cartilage. *Metabolism* **18**, 115–119.

Galante, J. O. & Rostoker, W. (1973) Fiber metal composites in the fixation of skeletal prosthesis. *J. Biomed. Mater. Res.* **4**, 43–61.

Gupta, D. & Tuli, S. M. (1982) Osteoinductivity of partially decalcified alloimplants in healing of large osteoperiosteal defects. *Acta Orthop. Scand.* **53**, 857–865.

Hench, L. L. & Ethridge, E. C. (1982) Orthopedic implants. In: *Biomaterials. An interfacial approach.* (Ed. Noordergraaf, A.), pp. 225–252. Academic Press, New York.

Iwata, H., Hanamura, H., Kaneko, M., Yasuhara, N., Terashima, Y., Kajino, G., Ida, K., Mabuchi, Y. & Nagakava, M. (1981) Chemosterilized autolyzed antigen-extracted allogeneic (AAA) bone matrix gelatin for repair of defects from excision of benign bone tumors. A preliminary report. *Clin. Orthop.* **154**, 150–155.

Klein, L., Goldberg, V. M., Zica, J. M., Powell, A. E. & Heiple, K. G. (1983) Effect of avascularity on bone resorption and formation in prelabelled rats. In: *Transactions of the 29th annual meeting of the Orthopaedic Research Society,* Anaheim, CA, (Ed. Radin, E. L.), Vol. 8, p. 118. Chicago,

Kuo, K. N., Gitelis, S., Sim, F. H., Pritchard, D., Chao, E., Rostoker, W., Galante, J. O. & Mc Donald, P. (1983) Segmental replacement of long bones using titanium fiber metal composite following tumor resection. *Clin. Orthop.* **176**, 108–114.

Lindholm, T. S., Nilsson, O. S. & Lindholm, T. C. (1982) Extraskeletal and intraskeletal new bone formation induced by demineralized bone matrix combined with bone marrow cells. *Clin. Orthop.* **171**, 251–255.

Lindholm, T. S. & Urist, M. R. (1980) A quantitative analysis of new bone formation by induction in compositive grafts of bone marrow and bone matrix. *Clin. Orthop.* **150**, 288–300.

McDavid, P. T., Boone, M. E., Kafrway, H. A. H. & Mitchell, D. F. (1979) Effect of autogenous marrow and calcitonin on reactions to ceramic. *J. Dent. Res.* **58**, 1478–1483.

McDonald, D. F., Scoefield, B. H., Humphrey, R. L. & Frondoza, C. G. (1983) Direct bone resorption by multiple myeloma cells. In: *Transactions of the 29th annual meeting of the Orthopaedic Research Society,* Anaheim, CA, (Ed. Radin, E. L.), Vol 8. p. 191. Chicago.

Mellonig, J. T., Bowers, G. M. & Clifton Bailey, R. (1981) Comparison of bone graft materials. Part I. New bone formation with autografts and allografts determined by Strontium-85. *J. Periodontol.* **52**, 291–296.

Nade, S. (1977) Osteogenesis after bone and bone marrow transplantation. *Acta Orthop. Scand.* **48**, 572–579.

Newman, M. G. & Boyne, P. J. (1971) The effect of calcified bone matrix on the osteogenic potential of hematopoietic marrow. *Oral Surg.* **32**, 506–512.

Oikarinen, J. & Korhonen, L. K. (1979) The bone inductive capacity of various bone transplanting materials used for treatment of experimental bone defects. *Clin. Orthop.* **140**, 208–215.

Reeve, J., Hesp, R. & Wootton, R. (1976) A new tracer method for the calculation of rates of bone formation and breakdown in osteoporosis and other genralized skeletal disorders. *Calcif. Tiss. Res.* **22**, 191–206.

Ronningen, H., Lereim, P., Galante, J. O., Rostoker, W., Turner, T. & Urban, R. M. (1983) Total surface hip arthroplasty in dogs using a fiber metal composite as a fixation method. *J. Biomed. Mater. Res.* **17**, 643–653.

Rønningen, H., Solheim, L. F. & Langeland, N. (1984) Invasion of bone into porous fiber titanium implants in cats. *Acta Orthop. Scand.* **55**, 352–358.

Rostoker, W., Galante, J. O. & Shen, G. (1974) Some mechanical properties of sintered fiber metal composites. *J. Test. Eval.* **2**, 107–112.

Sokal, R. R. & Rohlf, F. J. (1969) *Biometry.* W. H. Freeman and Company, San Francisco.

Urist, M. R. (1980) Bone transplants and implants. In: *Fundamental and clinical bone physiology.* (Ed. Urist, M. R.), pp. 331–368, Lippincott, Philadelphia.

Urist, M. R. & Dawson, E. (1981) Intertransverse process fusion with the aid of chemosterilized autolyzed antigen-extracted allogeneic (AAA) bone. *Clin. Orthop.* **154**, 97–113.

Urist, M. R., De Lange, R. J. & Finerman, G. A. M. (1983) Bone cell differentiation and growth factors. *Science* **220**, 680–686.

Urist, M. R., Mikulski, A. & Boyd, S. D. (1975) A chemosterilized antigen-extracted autodigested alloimplant for bone banks. *Arch. Surg.* **110**, 416–428.

Vandersteenhoven, J. J. & Spector, M. (1983) Osteoinduction within porous polysulfone implants at extraosseous sites using demineralized allogeneic bone matrix. *J. Biomed. Mater. Res.* **17**, 793–806.

Volpon, J. B., Xavier, C. A. M. & Concalves, R. P. (1982) The use of dedalcified granulated homologous cortical bone matrix in the correction of diaphyseal bone defect. *Arch. Orthop. Traumat. Surg.* **99**, 199–207.

Wittbjer, J., Palmer, B., Rohlin, M. & Thorngren, K. (1983) Osteogenic activity in composite grafts of demineralized compact bone and marrow. *Clin. Orthop.* **173**, 229–238.