Comparison of histomorphometry and ^{85}Sr uptake in induced heterotopic bone in rats

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Heterotopic bone formation in the abdominal muscle of 45 male 8-week-old Wistar rats induced by implantation of 5, 10, or 15 mg demineralized bone (DBM) powder was evaluated at 4 weeks by ^{85}Sr uptake of the implants and area histomorphometry of the induced bone. Two indices of ^{85}Sr uptake were calculated: the osteogenic index [(counts/min/mg implant)/(counts/min/mg os ilium)] and an index that we have called the osteoquantum index in which the weight of the implant is disregarded [(counts/min implant)/(counts/min/mg os ilium)]. The osteoquantum index showed a linear relationship to the area of the induced bone with a correlation coefficient (r) of 0.90. Only weak linear relationships were found between the osteogenic index and the area of the bone (r = 0.32) and between the osteogenic index and the osteoquantum index (r = 0.33). The osteoquantum index and the area of the induced bone both increased with increasing mass of implanted DBM, whereas the osteogenic index did not change.

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^{85}Sr uptake analysis has been used to assess osteogenesis by bone grafts in animal models (Elves 1974). However, it can be hypothesized from the formula of the commonly used osteogenic index [(counts/min/mg implant)/(counts/min/mg os ilium)] that this index reflects the calcium content per milligram implant and not necessarily the volume of the new bone, which may be of prime interest.

We investigated the relation between 1) the osteogenic index, 2) another index that we have called the osteoquantum index in which the weight of the implant is disregarded [(counts/min implant)/(counts/min/mg os ilium)], and 3) the area of new bone measured by histomorphometry in demineralized bone-induced heterotopic bone in rats.

Materials and methods

Implants

Demineralized bone matrix (DBM) was prepared by a sterile technique from the femur and tibia of male 8-week-old Wistar rats. Dissected diaphyses were crushed and the marrow was removed. The cortex was cut into chips, demineralized in 0.2 N HCl for 48 hours at 4 °C and flushed in saline (Bang 1973). The demineralized bone was suspended in liquid nitrogen, lyophilized for 22 hours, and pulverized at room temperature to a coarse powder. The particle size was 0.1–2.0 mm^2 as assessed by measurement of random samples on photomicrographs. The DBM was kept at 4 °C and implanted within 48 hours.

Surgical procedure

Forty-five 8-week-old male Wistar rats, mean body weight 194 g (SD 6), were randomized in three groups of 15 rats each. The animals were fed a standard laboratory food and water ad libitum. Anesthesia was induced with 0.15 mL/100 g Hypnorm®-Dormicum® i.m.

The abdominal fascia was exposed through a median incision. One pouch was created between the right oblique abdominal muscles by blunt dissection. DBM was placed in the pouch, and the incision was closed in layers. Five mg, 10 mg, and 15 mg DBM-powder were implanted in groups A, B, and C, respectively.

^{85}Sr uptake

All the animals were killed at 4 weeks after having received 10 microCi/100 g ^{85}Sr as $SrCl_2$ intraperitoneally 4 days earlier. The implant and right os ilium of each rat were dissected free of soft tissue, weighed,

Table 1. Weight and ^{85}Sr uptake by the implant and the os ilium. Mean *SD*

	Implant				Os ilium			
DBM (mg)	Weight (mg)		c.p.m.[a]		Weight (mg)		c.p.m.[a]	
5	24	*9.4*	1.6	*1.0*	444	*58*	71	*32*
10	56	*11*	5.2	*2.7*	444	*43*	74	*31*
15	85	*20*	8.4	*5.6*	440	*45*	63	*39*

[a] Counts per minute in thousands.

Table 2. ^{85}Sr evaluation and histomorphometry. Mean *SD*

DBM (mg)	Osteogenic index		Osteoquantum index		Bone area (mm^2)	
5	0.57	*0.53*	11	*4.2*[a]	1.5	*0.8*[a]
10	0.60	*0.23*	32	*9.5*[a]	3.1	*1.5*[a]
15	0.73	*0.18*	62	*18*[a]	6.8	*1.7*[a]

[a] Significantly different from the other groups ($P < 0.0001$).

and fixed in 4 percent neutral formalin. ^{85}Sr uptake was determined in a Packard gamma counter. Two indices were calculated: the osteogenic index (Elves 1974) [(counts/min/mg implant)/(counts/min/mg os ilium)] and an index we have called the osteoquantum index in which the weight of the implant is disregarded [(counts/min implant)/(counts/min/mg os ilium)].

Histomorphometry

After ^{85}Sr uptake determination, the implants were demineralized in 17 percent formic acid, dehydrated, and embedded in paraffin. Serial sections were cut at 5 microns and stained with Harris' hematoxylin. The implants were placed in the paraffin so that the sections were cut parallel to the greatest dimension of the implant. One random section derived from its greatest dimension was evaluated.

Enlarged photocopies (× 24) of the sections were made. The area of the induced bone was measured on the photocopies with Jandel's Sigma-Scan Measurement System (Jandel Scientific, Corte Madera) including a 12" × 12" electromagnetic digitizing tablet with a cross-hair stylus (resolution 0.025 mm, absolute accuracy 0.25 mm) and Sigma scan software (version 3.90). The corresponding section was studied by light microscopy simultaneously to ensure accurate measurement. The evaluation was done blind; all the identity tags had been covered, and the sections and corresponding photocopies had been arranged in random order.

Statistics

The means of the area of the induced bone, osteoquantum index, and osteogenic index of the different groups were compared. Bartlett's test for the homogeneity of variances showed that the variances of all three parameters were significantly different among the groups, invalidating the use of the overall F-test for one-way analyses of variance. Thus, pairs of means were compared with a two-sample t-test for independent samples with unequal or equal variances. Because comparing multiple groups introduces a greater probability of committing a type I error, the required P-value (0.05) was divided by the number of comparisons (Bonferroni's correction); $P < 0.016$ (0.05/3) was considered significant.

Correlation was used to study the possible association between two variables, and linear regression analysis was used to describe the relation between the values of the two variables. Four sets of two continuous variables were investigated: osteoquantum index and area of induced bone; osteogenic index and area of induced bone; osteogenic index and osteoquantum index; and osteoquantum index and weight of recovered implant.

Results

There were no perioperative or postoperative deaths. The animals gained weight and showed no signs of unhealthiness. All the implants were recovered and analyzed except one (5 mg DBM). The mean weight and ^{85}Sr uptake (counts per minute) of implant and os ilium of the three groups are presented in Table 1.

Both the area of new bone and the osteoquantum index increased with increasing implanted mass of DBM, and the means of the three groups of both parameters were significantly different from each other ($P < 0.0001$; Table 2). The mean osteogenic index was not significantly different between the three groups ($P > 0.07$; Table 2).

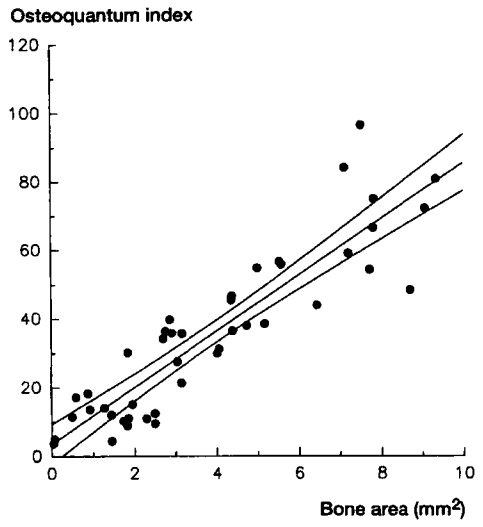

Figure 1. Scatter diagram and regression line with 95 percent confidence interval of osteoquantum index by area-induced bone. Correlation coefficient $r = 0.90$ ($P < 0.0001$), equation of the regression line $y = 3.50 + 8.23x$ ($P < 0.0001$).

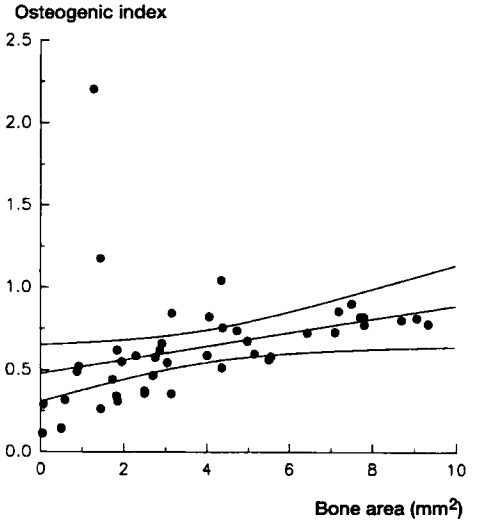

Figure 2. Scatter diagram and regression line with 95 percent confidence interval of osteogenic index by area-induced bone. Correlation coefficient $r = 0.32$ ($P = 0.03$), equation of the regression line $y = 0.48 + 0.04x$ ($P = 0.03$)

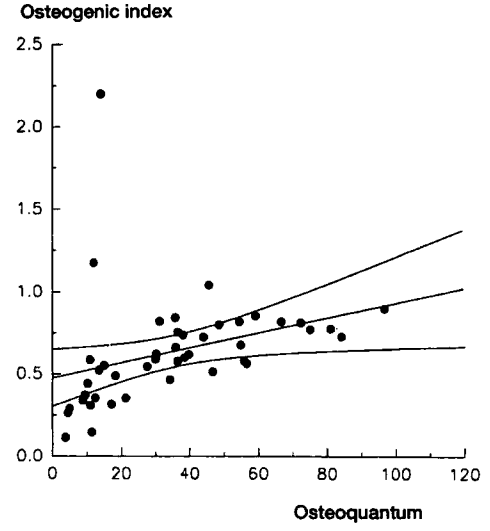

Figure 3. Scatter diagram and regression line with 95 percent confidence interval of osteoquantum index by osteogenic index. Correlation coefficient $r = 0.33$ ($P = 0.03$), equation of the regression line $y = 0.48 + 0.005$ ($P = 0.03$).

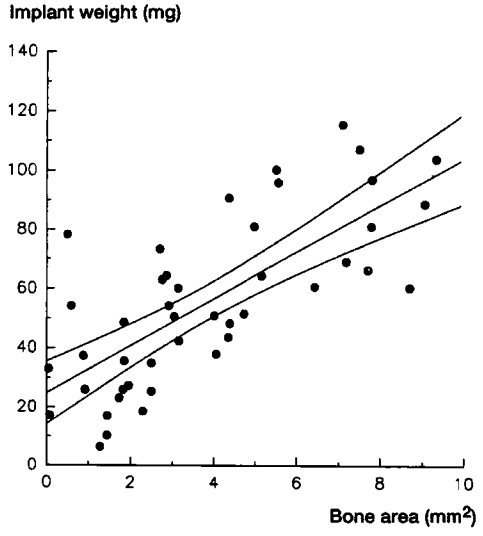

Figure 4. Scatter diagram and regression line with 95 percent confidence interval of weight of recovered implant by area-induced bone. Correlation coefficient $r = 0.73$ ($P < 0.0001$), equation of the regression line $y = 24.8 + 7.95x$ ($P < 0.0001$).

The osteoquantum index showed a strong linear relationship to the area of the induced bone ($r = 0.90$, $P < 0.0001$; Figure 1). Only weak linear relationships were found between the osteogenic index and the area of the bone ($r = 0.32$, $P = 0.03$; Figure 2) and between the osteogenic index and the osteoquantum index ($r = 0.33$, $P = 0.03$; Figure 3). A moderately linear relationship was found between the area of the induced bone and the weight of the recovered implant ($r = 0.73$, $P < 0.0001$; Figure 4).

Discussion

Intramuscular implantation of fresh autogenous bone (Levander 1938), HCl demineralized allogenous bone (Urist 1965, Reddi and Huggins 1972), or purified osteoinductors (Urist et al. 1979) regularly evoke heterotopic bone formation in rodents. Different quantitative methods have been introduced to evaluate such bone formation: viz., ash weight (Urist et al. 1970), calcium content (Urist et al. 1970), alkaline phosphatase activity of the implant (Firschein and Urist 1972), ^{85}Sr uptake analyses (Elves 1974), ^{45}Ca uptake analyses (Reddi 1975), histomorphometry (Hosny and Sharawy 1985, Marinak et al. 1989), and computerized image analyses of the area of induced bone on x-ray films (Kawai and Urist 1988).

DBM-induced osteogenesis consists of chemotaxis of mesenchymal cells, mitosis, differentiation of cartilage, vascular invasion, bone differentiation, and formation of an ossicle filled with bone-marrow elements. We chose 4 weeks as an appropriate time of evaluation, as mature ossicles are formed by this time (Urist 1965, Reddi and Huggins 1972). We analyzed the relationship between Sr uptake and area-induced bone in a random section derived from the greatest dimension of the implants. The two methods reflect two biological events. The former represents the mineralization of osteoid at the time of isotope injection, whereas the latter represents the net amount of bone formation following implantation.

There is no marked difference in skeletal uptake and exchange of Sr and Ca (Bauer et al. 1955, Cohn and Gusmano 1967). The uptake of the two elements occurs by several mechanisms, including simple exchange, diffusion, and active mineralization of osteoid (Bauer et al. 1955, Elves 1974). Remineralization of the implanted demineralized dentin has been shown in guinea pigs, mostly close to the mineralizing osteoid (Nilsen 1980). However, the density, ash weight, and calcium content of the recovered DBM implants from rabbit abdominal muscle are low until Day 15, when they increase steeply coinciding with bone induction histologically (Urist et al. 1970). Thus, the quantity of the remineralization of old bone matrix is probably very little compared with mineralization of newly formed osteoid during osteoinduction. ^{85}Sr has been used for evaluation of bone grafts both as ^{85}Sr uptake in grafts calculated as the osteogenic index (Elves 1974, Elves 1975, Delloye et al. 1985, Munting et al. 1988) and total content (cpm/implant) (Yoshikawa et al. 1988) and loss of ^{85}Sr from prelabeled grafts (Rønningen et al. 1985, Solheim et al. 1986).

Because the osteogenic index (Elves 1974) relates the Sr uptake of the implant to that of the host skeleton, e.g., os ilium, on a weight basis, careful dissection of the implant is important. The uptake of ^{85}Sr in soft tissue is insignificant, but including the weight of the surrounding soft tissue greatly influences the index. When the osteoquantum index is used, the dissection is less important. However, a requirement for using the osteoquantum index for comparing the effect on the induction of different host or implant factors is that the same amount of osteoinductor is used. Because the weight of the implant is disregarded in the osteoquantum index, the index permits evaluation of the effect of biomaterials on osteoinduction in composites without influence of the density and biodegradation of the biomaterial as long as the same amount of osteoinductor is used.

Computerized image analysis of the area of induced bone on x-ray films is positively correlated with ash weight analysis of the implants (Mahy and Urist 1988) and area measurements of induced heterotopic bone on x-ray films or histologic sections have been thought to reflect the volume of induced bone (Kawai and Urist 1988, Kawai and Urist 1989, Marinak et al. 1989). Further, the relationship is indirectly supported in the present study as the area correlated with the mass of the recovered implant (Figure 4).

We found neither qualitative histologic differences nor differences in osteogenic index between ossicles of different bone areas. These results indicate, as hypothesized, that the osteogenic index reflects the relative mineralization ratio of implant and not the volume of the new bone. In contrast, the osteoquantum index was found to have a strong linear relationship to the area of the induced bone. Both the osteoquantum index and the area of the induced bone increased proportionally to the quantity of implanted DBM. These findings agree with those of earlier studies in which the area of induced bone on x-ray film was directly proportional to the weight of implanted osteoinductor, bone morphogenetic protein (Kawai and Urist 1988, Mahy and Urist 1988). We found no qualitative differences of the bone induced by different DBM masses, i.e., 5–15 mg. These results contrast with those of Muthukumaran et al. (1988), who found a threshold for bone induction at 10 mg DBM and an acceleration of induction with increasing amounts of implanted DBM. However, they evaluated osteoinduction at an earlier stage, on Day 11.

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