Irradiation-sterilization of rat bone matrix gelatin

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Bone matrix gelatin induces bone formation in muscle, and when implanted orthotopically it improves bone repair. Co-60 sterilization of bone gelatin impairs the protein-bound induction mechanisms. Gelatin samples nonirradiated or irradiated by 25 or 50 kGy were implanted into a pouch in the abdominal wall of Sprague-Dawley rats, as well as into a 7-mm calvarial defect. Evaluation was done by histologic studies, histomorphometry of orthotopic implants, and determination of alkaline phosphatase in ectopic implants. Gelatin irradiated with 50 kGy was absorbed in the muscle bed without evidence of any specific host reaction. Irradiation of 25 kGy led to histologically confirmed ectopic bone formation, but the wet weight of the explants was only half that of the nonirradiated control samples. Alkaline phosphatase activity was equal in both of these groups. With orthotopic implantation, neither a histologic nor a morphometric effect was seen with 25 kGy. Loss of osteoinduction with 25-kGy irradiation is apparently masked by osteoconductive mechanisms with orthotopic implantation

Urist and Hernandez (1974) have reported that 25 kGy-irradiated bone gelatin loses 20 percent of its osteoinductive capacity. Loss of capacity to this extent would offset the practical advantages of radiosterilization. We investigated the influence of radiosterilization on bone matrix-gelatin osteoinduction in rats.

Material and methods

Implant materials. Gelatin was obtained from diaphyseal rat bone, which was mechanically cleaned of soft tissue, minced, freed from fat (chloroform/methanol 1+1), decalcified (0.6 N HCl, 24 h), and extracted (2 M $CaCl_2$, 0.5 M EDTA, 8 M LiCl). The lyophilized material was frozen in liquid nitrogen and ground to a particle size of 400–1000 μm (Thielemann et al. 1982) in a micromill (Mikro-Dismembrator, B Braun Melsungen AG, FRG). Samples were implanted without sterilization or following Co-60 irradiation of 25 kGy and 50 kGy, respectively (Table 1).

Experimental animals. The experiments were carried out in 25 male Sprague Dawley rats, which were about 100 days old. The body weight of the animals was 350–450 grams. They were kept in single hutches and fed a standard diet and tap water ad libitum.

Table 1. Irradiation of bone-matrix gelatin. Test protocol

Group	Duration of experiment	Radiation dosage (kGy)	Number of rats	Evaluation
1 Ectopic implantation (50 mg)				
1	21 days	us	6	AP
2	21 days	25	6	AP
3	21 days	25	3	H
4	10 weeks	25	3	H
5	10 days	50	4	H
6	21 days	50	3	H
7	10 weeks	50	3	H
2 Orthotopic implantation (12,6 mg)				
1	26 days	us	7	Hm
2	26 days	25	4	Hm

us unsterile. H histology.
AP alkaline phosphatase. Hm histomorphometry.

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Implantation site. The implantation was ectopic, i.e., into a muscle pouch of the lateral abdominal wall following blunt dissection, or orthotopic into a calvarial defect. For this purpose, the soft tissue was longitudinally incised over 3 cm and the periosteum removed. A 7-mm defect was placed centrally into the calvaria with a crown drill. The dura mater remained intact. The bore hole was rinsed so that no shavings were left. Totally, 12.6 mg BMG was implanted orthotopically and 50 mg BMG ectopically. The wounds were closed with catgut or nonabsorbable sutures.

The operation was done under halothane/oxygen inhalation anesthesia.

Histologic techniques. The calvaria were excised as a whole, fixed (1/4 formalin 37 percent, 3/4 alcohol 80 percent, pH 7.0), and embedded in methyl methacrylate. Horizontal 3-μm sections were stained according to the Kossa and Goldner methods and with toluidine blue. All the sections were evaluated blindly by the pathologists. The muscle specimens were fixed and embedded in paraffin. From each specimen, 4-μm sections were obtained and routinely stained according to the Kossa and Goldner methods.

The implants were evaluated histologically for new bone formation using the presence of the following criteria: osteoid and osteoblasts, woven or lamellar bone structures, bone marrow. Implanted matrix was differentiated from newly synthesized matrix by the fact that a) implanted matrix is cellfree, whereas new bone contains cells, and b) implanted matrix is stained pink by the Goldner method, osteoid red, and bone is stained green.

Determination of alkaline phosphatase. Tissue specimens of about 300 mg were homogenized in 1 ml H_20 for 1 min with a maximum number of revolutions in a tissue homogenizer (Ultra Turrax, Kunkel, Stauffen, FRG) and water was added to produce 2 ml. The supernatant fluid following centrifugation (4,500 rpm 10 min) was diluted 1:5 with H_20 and was processed like a plasma specimen to determine the alkaline phosphatase level on the Hitachi 705 (Kit Boehringer Mannheim, FRG). The calculation was done in International Enzyme Units per gram wet weight (U/g W/W).

Histomorphometry. The orthotopic Goldner-stained specimens were examined. With 40x magnification, a quadratic 64-point grid was randomly positioned twice near the periphery and twice near the center of the defect. The frequency of intersections between bone trabeculae and grid was counted. Volume fraction of newly formed bone related to the defect volume is shown as the mean ± standard error.

Results (Table 2)

Specimens of 25-kGy implants were easily localized by macroscopic evidence in the ectopic bed after 21 days, but they were markedly smaller than the nonirradiated controls. Histologic studies confirmed implant absorption, mesenchymal infiltration, as well as cartilage, bone, and marrow formation, which were not different from the control implants. Twenty-five-kGy implants could not be detected upon dissection 10 weeks after implantation; thus, histologic processing was impossible. The same applied to all ectopically implanted 50-kGy specimens with the exception of a single specimen found 10 days after implantation. In this instance, histologic studies showed implant remnants sheathed in connective tissue. No signs typical of incipient chondroinduction or osteoinduction, as seen regularly in nonirradiated implants, were encountered.

The wet weight of gelatin explants was almost twice as high as the mean wet weight of 25-kGy samples (analysis of variance $P < 0.05$). AP activity was equal in both groups (Table 2).

Twenty-six days after implantation of nonsterile gelatin into the calvarial defect, the border was still well delineated. The bone defect itself was filled with a net of fresh lamellar trabeculae. The trabeculae were surrounded by osteoid seams and osteoblasts. No foreign body reaction was confirmed by histologic studies; no implant remnants were found. Between the trabeculae, there were cell and fat marrow.

Table 2. Comparison of unsterile (U) and 25 kGy radiosterilized BMG (R) bone-matrix gelatin in rats. Values are mean (SD)

	Implant		
	U		R
Wet weight (mg)	342 (38)	$P < 0.05$	199 (57)
n	6		6
Alkaline phosphatase (U/gWW)	51 (8.5)	NS	52 (7.9)
n	6		6
Morphometry	20 (3.0)	NS	26 (4.4)
n	7		4

Descriptive histologic studies did not reveal any obvious effects of irradiation. Morphometry of calvarial defects revealed no measurable difference between the controls and 25 kGy-irradiated gelatin (Table 2).

Discussion

Our experiment showed that 50-kGy irradiation destroys the osteoinductive response, confirming the report by Urist and Hernandez (1974) that 90 to 100 percent induction capacity by 50-kGy irradiation was lost.

It is surprising that there was no difference of alkaline phosphatase activity between the groups with or without 25-kGy irradiation, as was the case with histologic examination. The lower irradiation dosage of 25 kGy reduced the induction capacity as assessed by the explant's wet weight without apparently changing the induction mechanism.

In the orthotopic implantation bed, the inductive effect of biologically active implants cannot be differentiated from osteoconductive mechanisms. In the angiogenic invasion (Trueta 1963) and subsequent replacement of an osseous implant (Dambe et al. 1981), osteoinduction and osteoconduction are inseparably linked (Urist 1986). Cartilage formation during healing of calvarial defects is the only direct evidence of osteoinductive mechanisms (Sato and Urist 1985). The cartilage formation in gelatin-filled calvarial defects in the rat is temporary and ceases in the third postoperative week. After 4 weeks, no cartilage remnants are noted (Schwarz et al. 1987). Because in our series neither histologic nor morphometric differences were demonstrated, we must assume that osteoinduction alone is responsible for defect regeneration only to a limited extent.

From a practical point of view, radiosterilization is the best method for treatment of bone implants. Aside from the sterilization effect, irradiation reduces antigenicity when compared with fresh allografts (Burwell 1985).

Urist (1980) ascribes the 15 to 30 percent rate of unfavorable results obtained with radiosterilized bank bone to the protein-destroying effect of irradiation. The extracellular matrix as a carrier of inductive proteins was damaged by irradiation especially when bone was irradiated in the presence of mineral components (Urist and Hernandez 1974). In this case, a dosage as small as 20 kGy was enough to eliminate the osteoinductive mechanisms, whereas the induction capacity of bone gelatin was only reduced by 20 percent if sterilization was performed after decalcification (Urist and Hernandez 1974). Loss of induction in our series was determined by means of the wet weight and amounted to 60 percent with 25 kGy.

Whereas in small laboratory animals nonsterile implants may be used without any inconvenience, implants must be sterilized if used in larger animals or in the clinical setting. At present, chemosterilization by HCl appears to be the best approach towards providing a biologically active bone replacement material.

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