Healing of cortical bone grafts in athymic rats

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We studied healing of allogeneic and syngeneic cortical tibial segment grafts in athymic and normal rats. After 3, 6, and 12 weeks, the weight, circulation, and mineralization rate of the healing segment, and mechanical strength and stiffness of the healing tibia were measured. There were no differences between allogeneic and syngeneic grafts in athymic and normal animals at 3 or 6 weeks. After 12 weeks, the vascularization and mineralization of the grafts, but not of the surrounding callus, were smaller in the allogeneic grafts in the normal recipients than in the other groups. Also after 12 weeks, the stiffness of the healing tibiae was less in allogeneic grafts in normal recipients than in the other groups. The strength of the allogeneic grafts was less than the strength of the syngeneic grafts in both athymic and normal recipients.

This suggests that T-cell-mediated rejection is responsible for decreased vascularization and mineralization of allogeneic bone and that the difference in strength between allogeneic and syngeneic grafts is not due to T-lymphocyte graft rejection.

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Although bone allografts are immunogenic, the nature of the immune response to bone is poorly understood (Burchardt 1987, Goldberg and Stevenson 1987). It has been hypothesized that T-cell response is the most important factor, analogous to the cell-mediated response in skin graft rejection (Horowitz and Friedlaender 1987). Allogeneic bone grafted to athymic rats is a model that can be used to study this problem (Kirkeby et al. 1991).

We have transplanted syngeneic and allogeneic cortical bone segments to athymic and normal rats to study the function of these grafts.

Material and methods

Experimental animals

Donors of allogeneic bone were male Wistar rats, whereas donors of syngeneic bone were male Lewis rats. Recipients were male rats of the Lewis strain inbred with the donor Lewis rats and male Lewis athymic rats inbred with the other Lewis rats, genetically differing from these only by the nude mutation. The Wistar/Lewis combination is a major histocompatibility mismatch (Gill 1978). All the rats weighed between 180 and 200 grams, and were in a growing phase with open epiphyseal lines. The recipients were kept one in each cage postoperatively and were fed water and standard rat pellets ad libitum. 72 recipient rats (36 athymic and 36 normal) were divided into four groups as shown in Table 1.

Technique of grafting

Each donor animal gave bone grafts to one normal and one athymic rat. Donor animals were killed with an overdose of pentobarbitone administered intraperitoneally. All the animals were operated on under aseptic conditions. The grafts were harvested through an anterior longitudinal incision over the tibia. Oblique osteotomies were made with a dental rotating saw under direct vision and continuous saline irrigation. The osteotomies were made 1 mm and 10 mm proximal to the tibiofibular synostosis. The graft was placed in sterile saline in room temperature and transplanted within 30 minutes.

The recipients were operated on under fentanyl/fluanison (Hypnorm, Jansen) and midazolam (Dormicum, Roche) anesthesia. A segment of the left tibia was removed as described for the donors, and a donor segment was inserted into the defect. Care was taken

Table 1. Study design for transplantation of syngeneic and allogeneic segmental tibial grafts to normal and athymic Lewis (L) or Wistar (W) rats. 72 recipient rats with 18 rats in each group and 36 donor rats

Group no.	Recipient (L)	Donor graft
1	Normal	Allogeneic (W)
2	Normal	Syngeneic (L)
3	Athymic	Allogeneic (W)
4	Athymic	Syngeneic (L)

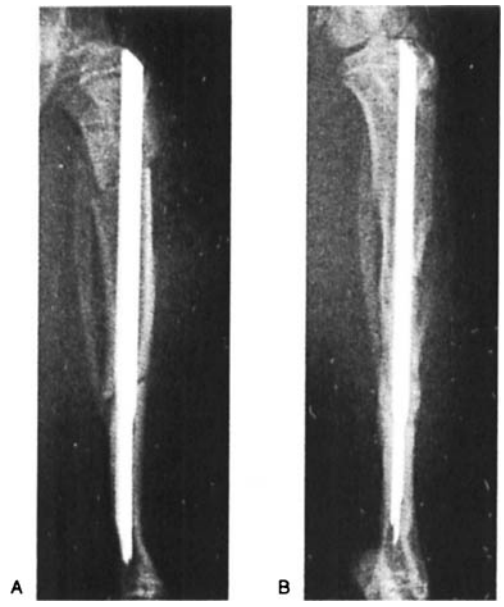

Figure 1. Grafted allogeneic segment in left tibia.
A. Immediately postoperatively. A 9-mm donor segment has been inserted into a similar defect in the recipient tibia.
B. 12 weeks postoperatively. Callus bridges the osteotomy gaps but the graft segment can still be identified.

to leave the fibula intact. The tibia and graft were stabilized with an intramedullary nail consisting of an 18-gauge, a 21-gauge, and a 25-gauge needle (Figure 1). The nail gave axial stability. Rotational stability was provided by oblique osteotomies and an intact fibula. All the tibiae with grafted segments were clinically stable before wound closure. The wound was closed in two layers. The grafting operation described took approximately 20 minutes. The mortality rate was 2.5 percent. Blood loss was insignificant. The animals were fully ambulatory after recovery from anesthesia. They apparently walked on the operated on limb from the first postoperative day.

Three days before the recipients were killed, 1 μCi/100 g body weight of ^{85}Sr was injected intraperitoneally as strontium chloride in 0.5 mL saline (Elves 1974). Before killing 3, 6, and 12 weeks postoperatively, they were anesthetized with fentanyl/fluanison/midazolam. The right common carotid artery was isolated through a longitudinal midline incision and a polyethylene catheter (PE-10, OD 0.63 mm, 2 FG, Portex United, Kent, England) was introduced into the ascending aorta through the right carotid artery. The correct catheter position was confirmed at autopsy. Approximately one million ^{141}Ce-labeled microspheres (NenTrac, New England Nuclear, U.S.A.), 15 μm in diameter, suspended in 1 mL isotonic saline, were injected into the ascending aorta. The syringe was flushed for any remaining microspheres with 1 mL isotonic saline. The animals were killed with an intraarterial overdose of pentobarbitone shortly after the microsphere injection. An operating microscope was also used to observe arteriolar trapping of the microspheres in the iris of each animal.

Sample treatment

Soft tissues and periosteum were removed from both tibiae. Care was taken to leave the transplanted segment, consisting of graft and callus, intact. The nails were removed, and the samples were stored at –20 °C pending mechanical testing. The bones were thawed for two hours at room temperature before the mechanical test. After the test the proximal 5 mm and distal 7 mm of the tibiae were removed to isolate the graft segment with surrounding callus. This segment was weighed to the nearest 0.1 mg (wet weight). The mass of the graft and callus was expressed as the weight on the test side divided by the weight of the corresponding tibial segment on the contralateral side. Total radioactivity of each isotope in the samples was measured by placing the sample in the center of a Packard 5221 Auto-gamma scintillation spectrometer with the windows set over the highest energy peak of each isotope. Individual isotope activity of the samples was calculated with correction for background, cross-talk, and physical decay according to Heymann et al. (1977). The specific activity of strontium (counts per minute per gram) on the test side relative to the intact side was used to express the mineralization rate (Elves 1974). The relative vascularization of the graft and callus segment was calculated in the same manner as ^{141}Ce-microsphere radioactivity of the test segment per gram/ radioactivity of intact segment per gram.

In the animals killed after 12 weeks, the whole segment, with graft and callus, was first counted, then the graft itself was dissected free from the surrounding callus and counted separately. In the groups killed at 3 and 6 weeks, the graft was difficult to separate from the surrounding callus.

The bending tests were performed in a hydraulic testing machine. Intact and healing tibiae were tested 1 mm proximal to the distal tibiofibular synostosis in three-point ventral bending until failure, at a deformation rate of 2.5° per second. The strength of the bones was defined as the ultimate moment, read as the y-coordinate from each load-deformation curve. The corresponding x-coordinate was defined as the ultimate deformation. The stiffness was measured from the slope of the linear elastic part of the curves. The technique and the calculations have been described in

Table 2. Relative mass, vascularization, and mineralization rate of the graft segment, including surrounding callus, and strength and stiffness of the healing tibiae relative to the contralateral intact tibiae. Figures are graft segment values divided by values for the contralateral intact segment. There are 6 rats in each group, and a total of 72 rats. Mean, *SD*

	Normal rats				Athymic rats			
Weeks	Allogeneic grafts		Syngeneic grafts		Allogeneic grafts		Syngeneic grafts	
Mass								
3	4.51	*1.41*	3.82	*1.53*	3.58	*2.40*	4.28	*1.95*
6	2.43	*1.23*	2.62	*1.50*	2.48	*1.00*	2.81	*1.31*
12	1.99	*0.11*	1.89	*0.66*	2.34	*0.76*	2.51	*0.38*
Vascularization								
3	1.41	*1.10*	1.68	*0.90*	1.30	*0.77*	1.80	*1.10*
6	2.38	*0.39*	2.01	*0.41*	2.16	*0.50*	2.29	*0.49*
12	1.74	*0.33*	1.50	*0.38*	1.87	*0.40*	1.85	*0.38*
Mineralization								
3	1.76	*0.70*	1.77	*0.50*	2.10	*0.71*	2.00	*1.00*
6	2.23	*0.63*	2.01	*1.00*	2.16	*0.70*	2.29	*0.51*
12	1.70	*0.37*	1.50	*0.33*	1.97	*0.50*	1.89	*0.51*
Strength								
3	0.10	*0.06*	0.12	*0.05*	0.13	*0.14*	0.09	*0.07*
6	0.25	*0.21*	0.33	*0.28*	0.22	*0.21*	0.18	*0.16*
12	0.46	*0.25*[b]	0.79	*0.23*	0.41	*0.26*[a]	0.76	*0.30*
Stiffness								
3	0.27	*0.07*	0.35	*0.11*	0.40	*0.32*	0.21	*0.15*
6	0.50	*0.35*	0.71	*0.22*	0.60	*0.34*	0.65	*0.45*
12	0.45	*0.30*[b]	1.10	*0.21*	0.71	*0.37*	0.92	*0.35*

[a]$P < 0.05$, [b]$P < 0.025$.

Table 3. Relative vascularization and mineralization rate in the grafts after 12 weeks. The figures are graft values divided by values of the contralateral tibial segment. Mean, *SD*

	Normal rats				Athymic rats			
	Allogeneic grafts		Syngeneic grafts		Allogeneic grafts		Syngeneic grafts	
Vascularization	1.09	*0.94*[a]	2.85	*2.90*	1.62	*0.40*	1.83	*1.05*
Mineralization	0.48	*0.63*[a]	2.38	*2.56*	1.40	*0.68*	1.54	*0.99*

[a]$P < 0.05$.

detail previously by Engesæter et al. (1978) and Ekeland et al. (1982). Biomechanical values form the healing bone were compared with those of the contralateral intact tibia.

Analyses of variance and Student's *t*-test were used for the statistical evaluation.

Results

Relative segment mass is expressed as the ratio between the weight of the graft and callus segment and the weight of intact contralateral bone segment (Table 2). It was greatest at 3 weeks, when the graft segment was approximately four times the mass of the normal segment, and it decreased to approximately twice the normal value at 6 and 12 weeks after transplantation. The relative segment mass did not differ significantly between the four groups at 3, 6, or 12 weeks (Table 2).

The vascularization of the graft and callus segment is shown as counts per minute ^{141}Ce radioactivity per mg bone tissue relative to the contralateral segment (Table 2). It was approximately 1.5–2 times that of the normal bone, highest at 6 weeks, and showed no significant differences between the groups (Table 2).

The vascularization of the graft without the callus at 12 weeks was 62 percent less in the normal animals receiving allogeneic grafts than in those receiving syngeneic grafts ($P < 0.05$). There were no differences

between allogeneic and syngeneic grafts in athymic animals (Table 3).

The mineral accretion rate of the callus and graft is shown as ^{85}Sr radioactivity in the same manner as for the vascularization. It was approximately twice that of normal bone and there were no significant differences between the groups (Table 2).

The mineralization rate of the graft without callus at 12 weeks was 80 percent lower in the normal animals receiving allogeneic grafts than in syngeneic grafts ($P < 0.05$). In the athymic animals, there weres no differences between the two types of grafts (Table 3).

The strength and stiffness of the healing bone increased in time in all four groups. After 12 weeks, the allogeneic grafts were 42 percent weaker than the syngeneic grafts in normal animals ($P < 0.025$) and 46 percent weaker in athymic animals ($P < 0.05$; Table 2). The allogeneic grafts in the normal animals were 59 percent less stiff than the syngeneic grafts at 12 weeks ($P < 0.025$). The allogeneic grafts were 23 percent less stiff than syngeneic grafts in the athymic animals (not significant) (Table 2).

Discussion

The graft-host interface undergoes several stages of healing. Our results show that healing proceeded in a similar manner in the allogeneic and syngeneic grafts until 6 weeks postoperatively. There were no differences in graft segment mass, metabolic properties, or mechanical properties during this period. This suggests that the genetic differences between donor and host are of less importance for the formation and development of the callus in the initial phase of healing. At 12 weeks, however, the mechanical properties of allografts were weaker than those of syngeneic. Our results suggest that the early healing and callus formation is not much affected by the immunologic response provoked by allogeneic bone. In a similar rat model, Pappas and Beisaw (1968) found reduced strength of allogeneic bone grafts already after three weeks. The healing in their model was slower than in ours. This can at least partly be explained by our use of older animals (Ekeland et al. 1982), and different graft fixation and mechanical tests. On a more long term basis, allogeneic grafts have been found to be weaker than their syngeneic counterparts (Halloran et al. 1979), and histologic studies suggest that their incorporation is impaired (Goldberg et al. 1985, Heiple et al. 1963). This is in accordance with our results, which showed that the revascularization and mineralization rate of the graft itself were reduced in allogeneic grafts after 12 weeks. The higher vascularization and mineralization rate of syngeneic grafts could theoretically be expected to result in a structurally weaker graft, because in healing of cortical bone grafts, bone resorption precedes bone formation. Horowitz and Friedlaender (1987) have, however, suggested that immunologic bone rejection causes increased bone resorption due to increased osteoclast activity. This might explain the structural weakness of allogeneic bone grafts as compared with syngeneic grafts.

The reason for the impaired healing and incorporation of allografts has been assumed to be immunologic (Goldberg and Lance 1972, Friedlaender 1983, Burchardt 1987, Goldberg and Stevenson 1987). T-lymphocyte mechanisms have been suggested to be responsible (Horowitz and Friedlaender 1987), as they are for transplant rejection in various other organs (Hall 1987). The athymic rat should be well suited for studying the effect of the T-lymphocyte system on the function of bone grafts. These animals have been used to study the biology of transplantation because they lack the ability to reject foreign tissue (Festing 1981). Their bone structure, metabolism, and bone healing properties are apparently normal (Kirkeby 1991). We have earlier shown that the revascularization, mineralization rate, and resorption of heterotopic cancellous bone grafts are impaired in allografts transplanted to normal rats, but not in allografts transplanted to athymic rats (Kirkeby et al. 1991). In accordance with earlier results, the present study showed a decreased vascularization and mineralization rate of allogeneic cortical grafts in normal, but not in athymic, animals. Surprisingly, however, we also found that the mechanical strength after 12 weeks of healing was less in allogeneic grafts in normal and athymic rats. This observation cannot be explained by the hypothesis that T-lymphocyte transplant rejection mechanisms are responsible for inferior healing of allogeneic bone. The tibiae of the Wistar and the Lewis rats were of the same length and weight and were macroscopically indistinguishable. Structural differences in the skeletal tissues between normal, but different, albino rat strains have not been described. The explanation of this finding is thus not obvious. It may be due to a difference between allogeneic and syngeneic bone which has previously not been described; it may be unrelated to the immune system, such as structural differences in the bone of a genetic nature, or it may be due to immunologic reactions to allogeneic bone unrelated to the T-lymphocyte system. The possibility of coincidence (Type I error) cannot be excluded either, because the significance of these unexpected results is in the region of $0.05 < P < 0.025$. Further experiments are required to clarify this problem, and to elucidate the nature of bone allograft impairment not related to the T-lymphocyte transplant rejection mechanisms.

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