Quantitative assessment of angiogenesis and osteogenesis after transplantation of bone

Comparison of isograft and allograft bone in mice

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We performed a vital microscopic study in mice bearing dorsal skinfold chambers to characterize microvascular perfusion and leukocyte/endothelium interaction and their effects on elongation and mineralization of neonatal isograft and allograft bone. Isograft (C57/BL to C57/BL) and allograft bone (C57/BL to BALB/C) revascularized simultaneously. However, vascular perfusion and density were lower in allograft bone than in isograft bone. Leukocyte/endothelium interaction was the same in isograft and allograft bones. Revascularization was not detected in allograft bone transplanted to presensitized recipients. Moreover, in preexisting vessels at the transplantation site, leukocyte/endothelium interaction was altered in allograft bone of presensitized recipients, despite a normal systemic leukocyte count. Femoral growth resulting from thickening of both epiphyses did not differ between experimental groups, however, mineralization occurred in isograft bone only. Isograft bone was histologically intact, allograft bone hypovital and allograft bone in presensitized recipients necrotic 12 days after implantation. Our findings suggest that graft incorporation or rejection is mediated by the microvasculature and that presensitizing of recipients accelerates rejection of allograft bone.

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Experimental and clinical evidence indicate that immediate or early revascularized bone grafts remain viable and contribute directly to union with the recipient bone. In contrast to the biological and mechanical superiority of rapidly revascularized autograft bone, revascularization of allograft bone may act rather detrimentally since recipient-derived immune cells invade the graft through the vasculature, potentially triggering an immune response.

Revascularization of autograft and isograft bones has been assessed using orthotopic (Albrektsson 1980, Albrektsson et al. 1984, Winet and Albrektsson 1988) and heterotopic (Sandison 1928, Sudmann 1975, Leunig et al. 1994) models of bone transplantation by means of vital microscopy. Only sparse information exists on early revascularization (Sabet et al. 1961) and no information on intravascular events after transplantation of allograft bone. To characterize microvascular perfusion and leukocyte/endothelium interaction during bone graft incorporation or rejection, we have used a vital microscopic model and assessed their effect on elongation and mineralization of isograft and allograft bones.

Material and methods

Dorsal skinfold chamber

Immunocompetent male mice (n 75) ranging between 2 and 3 months of age (25–30 g) and differing in both classes of their MHC (C57/BL (H2-b) and BALB/C (H2-d)) were fitted with dorsal skinfold chambers. This microcirculatory preparation (Leunig et al. 1994) served as the site for implantation of femora of neonatal C57/BL mice. Following a recovery period of 24–48 h, only chambers meeting the criteria of intact microcirculation (Sewell 1966) were utilized as sites for implantation of bone.

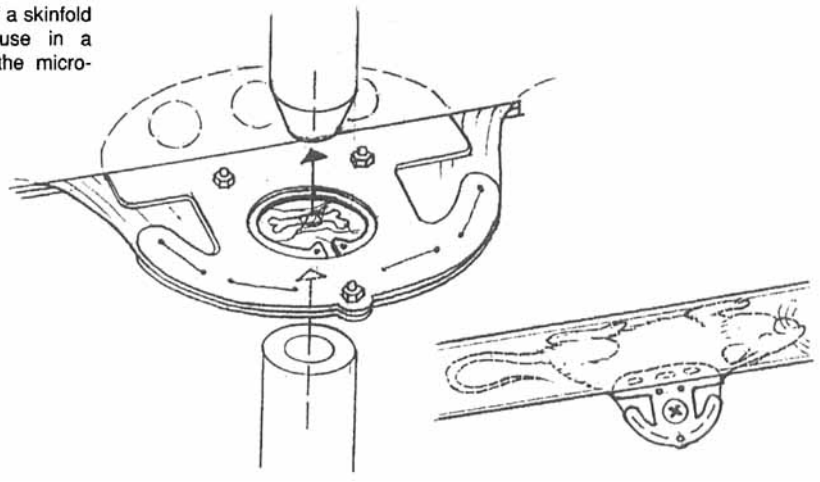

Figure 1. Positioning of a skinfold chamber bearing mouse in a plexiglas tube under the microscope.

Preparation of bone grafts

After decapitation, neonatal C57/BL mice were kept for 5 min in 70% ethanol. Femora were bluntly dissected in 10 mL Dulbecco's modified Eagle's medium (Seromed, Bichson, Berlin, Germany) at room temperature whereby soft tissues were meticulously removed under a dissection microscope. Thereafter, the bones were transplanted into dorsal skinfold chambers.

Experimental groups

In 3 experimental groups, isograft bone (C57/BL to C57/BL), allograft bone (C57/BL to BALB/C) or allograft bone transplantation to presensitized recipient (C57/BL to SenBALB/C) was carried out. To presensitize allograft bone recipients (SenBALB/C), BALB/C mice were exposed to 10^6 antigen-presenting cells (i.p. injection) of C57/BL mice 4 days before bone transplantation (Austyn and Gordon 1981). These cells were obtained by peritoneal lavage of adult C57/BL mice.

Vital microscopy

For vital microscopy, the mice were positioned in a plexiglas tube and mounted under the microscope, as shown (Figure 1). During the first 4 days after implantation, measurements were performed in an unanesthetized animal every 8 hours, thereafter once a day for a period of 12 days using a ×1.25, ×2.5, ×5, ×10, and ×20 objective. For in vivo observations, a green filter was used to enhance black/white contrast. By day 12, oxytetracycline (OTC, 100 mg/kg b.w. in 0.9% NaCl) was injected i.p. 6 hours later. When OTC was readily cleared from uncalcified tissues, OTC-fluorescence was examined by incident light using a mercury lamp, an excitation filter (356–418 nm), a dichroic mirror (450 nm) and a barrier filter (495 nm). For analysis of intravascular events on days 6 and 12, a 1 mL bolus of fluorescein isothiocyanate-labeled dextran (FITC-Dextran, 5 mg/100 µL of NaCl 0.9%, Sigma Chemical Co., St. Louis, MO) and rhodamine 6G (Rh 6G, 50 mg/100 mL, Sigma Chemical Co.) were injected 5 min before the microscopic observation i.v. FITC fluorescence was obtained with an excitation filter (485–505 nm), dichroic mirror (510 nm) and barrier filter (530 nm), Rh 6G fluorescence with an excitation filter (530–560 nm), dichroic mirror (580 nm) and barrier filter (580 nm).

Real-time coded images were obtained by means of a highly sensitive TV-camera and recorded on video tapes and digitized by the use of a frame-grabber board.

Image analysis was performed off-line on a computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available from Internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from the National Technical Information Service, Springfield, Virginia, part number PB95-500195GEI).

Angiogenesis. To evaluate angiogenesis, we determined: (i) the onset and amount of bleeding in the vicinity of the implants, (ii) the onset of recirculation of preexisting blood vessels of the femoral diaphysis, (iii) the appearance of newly formed blood vessels and the first flow in these vessels

and (iv) the functional vascular density of newly formed blood vessels.

(i) The extent of bleeding was determined by morphometric measurements and is given in mm^2. (ii) Recirculation was quantified by densitometric measurements of the intensity of transmitted light absorbed by the femur diaphysis, as reported earlier, and is expressed as the ratio I(v)/I(d) (Leunig et al. 1994). (iii) The first appearance of newly formed blood vessels and the first flow in these vessels are given in hours after implantation. (iv) By day 12, the vascular density of perfused and non-perfused newly formed blood vessels covering the surface of the implant (cm/cm^2) was quantified by image analysis, using Leunig's method (Leunig et al. 1992).

Leukocyte/endothelium interaction. We assessed the interaction of leukocytes with the inner vessel surface (observation period 30 s) of preexisting blood vessels of the graft periphery and newly formed blood vessels, covering the implant at days 6 and 12 after implantation of femora and determined it quantitatively. Floaters (F_{WBC}) are the number of leukocytes passing a defined cross-section of the vessel per unit time (10^3 cells/mm^2/s). Rollers are defined as cells with a velocity of < 50% of the centerline velocity of the blood stream (v_{center}) and are expressed as the fraction of rolling cells = rolling cells/(nonadherent cells + rolling cells) (%). The centerline velocity (v_{center}) was determined by measuring the velocity of leukocytes representing the centerline blood stream (mm/s). Stickers are defined as cells adherent to the inner vessel surface and are given as the number of leukocytes per vessel surface: number of cells/(length of observed vessel segment • π • D) (10 cells/mm^2). The vessel diameter (D) is given in μm. The apparent wall shear rate (γ) is calculated using the Poiseuille law for Newtonian fluids: $\gamma = (v_{center}/D) \cdot 8$ (10^4/s)(Perry et al. 1991). In each animal at least three vessel segments were analyzed and the resulting measurements were averaged.

Elongation and mineralization. Elongation was defined as the increase in length of the femur. Mineralization is reflected by the increase of the OTC-stained femur fraction. Based on previous studies, growth rates were calculated assuming a linear femoral elongation in vivo (Leunig et al. 1994).

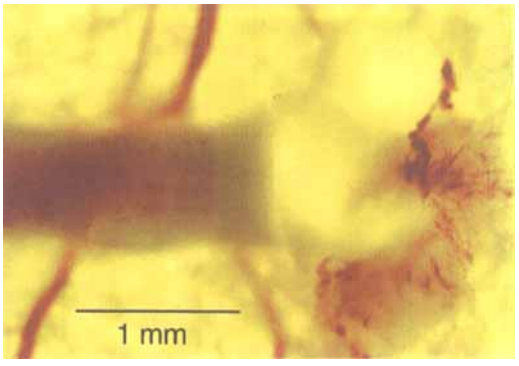

Figure 2. Overview photograph of an isograft femur on day 2 after implantation into a dorsal skinfold chamber. Onset of revascularization is reflected by vessels (red) containing red blood cells at the proximal cartilage (right side) and partly in the diaphysis (left side).

Systemic leukocyte count

Blood samples of 200 μL were obtained on days 6 and 12 from the retrobulbal plexus and overall leukocyte counts were performed by multiparameter hematological analysis.

Histology

After termination of in vivo experiments on day 12, the femora were fixed in formaldehyde, dehydrated in alcohol, cleared in xylene and embedded in methyl methacrylate. Longitudinal sections having a thickness of 6 μm were stained with the van Kossa McNeal, and differentially stained with a modified Movat's tetrachrome stain.

Statistics

Data are presented as the mean (SD). Non-parametric tests (Kruskal-Wallis and/or Mann-Whitney U-test) were used for statistical comparisons.

Results

Vital microscopy

Angiogenesis. Isograft and allograft bones in recipients that had not been presensitized elicited a significant microvascular response (Figure 2), while allograft bone in presensitized recipients did not induce detectable vascular reactions (bleeding, vessel formation and perfusion). The first bleeding in the vicinity of transplanted femo-

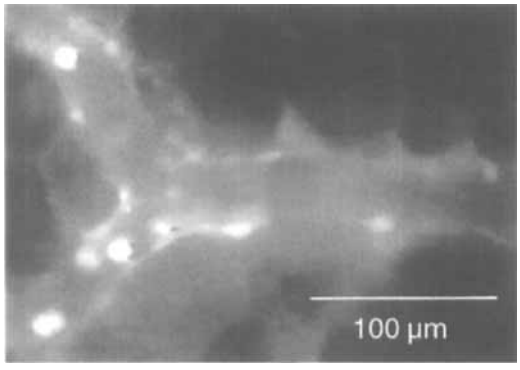

Figure 3. Leukocytes stained by rhodamine 6G in a post-capillary venule of an allograft bone.

ra occurred between 24 and 48 h in isograft and allograft bones, approaching peak sizes between days 3 and 5 after transplantation and being cleared thereafter. Preexisting blood vessels in the femoral diaphysis reperfused in isograft and allograft bones on days 2–3 after transplantation. The time course and extent of hemorrhages and reperfusion did not differ among isograft and allograft bones (data not shown). Newly formed blood vessels appeared after 57 (11) h in isograft bone (n 8) and after 80 (7) h in allograft bone (n 5), revealing a first perfusion after 87 (26) h and delayed after 119 (7) h, respectively ($p = 0.005$). The appearance of newly formed blood vessels preceded their perfusion in isograft ($p = 0.001$) and allograft bones ($p = 0.02$). On day 12, total vessel density was 464 (216) cm/cm^2 with a perfusion density of 427 (230) cm/cm^2 in isograft bone (n 6). In allograft bone (n 8), total vessel density (215 (236) cm/cm^2) and density of perfused vessels (85 (88) cm/cm^2) also did not differ. However, both were reduced as compared to isograft bone ($p = 0.05$ and $p = 0.005$).

Leukocyte/endothelium interaction. The interaction between leukocytes and the vessel wall was analyzed in vessels ranging between 20 and 40 µm (Figure 3). On days 6 and 12, floater, roller and sticker did not differ in graft and preexisting vessels of isograft and allograft bones (data not shown). In contrast, in preexisting vessels of allograft bone of presensitized recipients, floater was decreased as compared to isograft or allograft bone (Figure 4). Moreover, from days 6 to 12, sticker decreased in this group, while floater and roller remained constant.

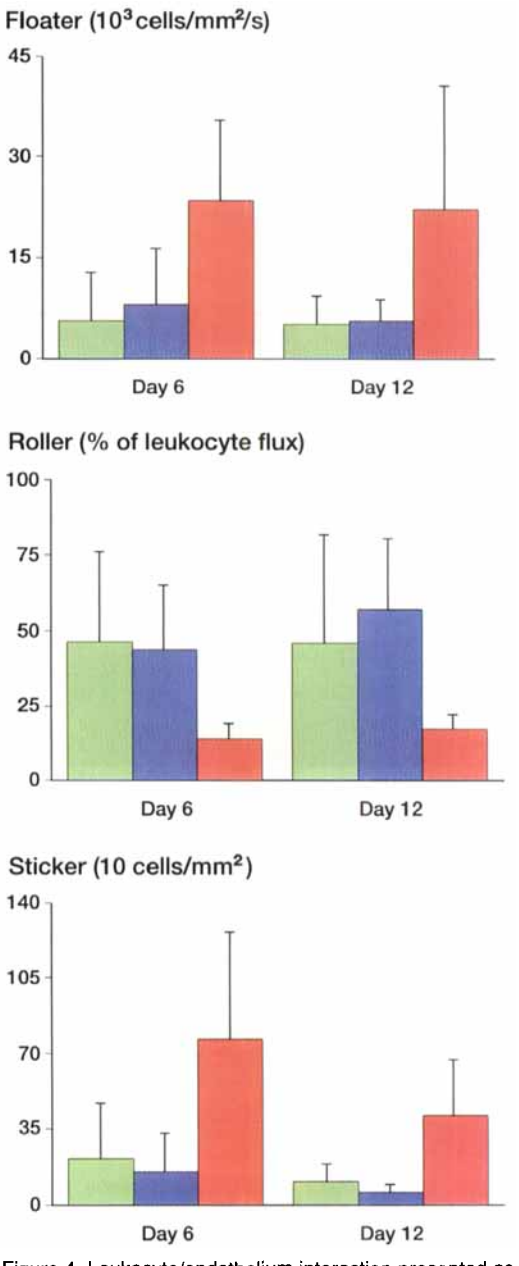

Figure 4. Leukocyte/endothelium interaction presented as floater, roller, and sticker in preexisting vessels of isograft bone (green bar; iso) (day 6: n 8; day 12: n 5), allograft bone (blue bar; allo) (day 6: n 11; day 12: n 9), and allograft bone of presensitized recipients (red bar; pr) (day 6: n 20; day 12: n 11).

P-values:

Floater day 6:	Iso vs pr	0.0007,	allo vs pr	0.0007.
Floater day 12	Iso vs pr	0.01,	allo vs pr	0.001.
Roller day 6:	Iso vs pr	0.0003,	allo vs pr	0.0002.
Roller day 12:	Iso vs pr	0.06,	allo vs pr	0.0007.
Sticker day 6:	Iso vs pr	0.003,	allo vs pr	0.0002.
Sticker day 12:	Iso vs pr	0.02,	allo vs pr	0.0002.

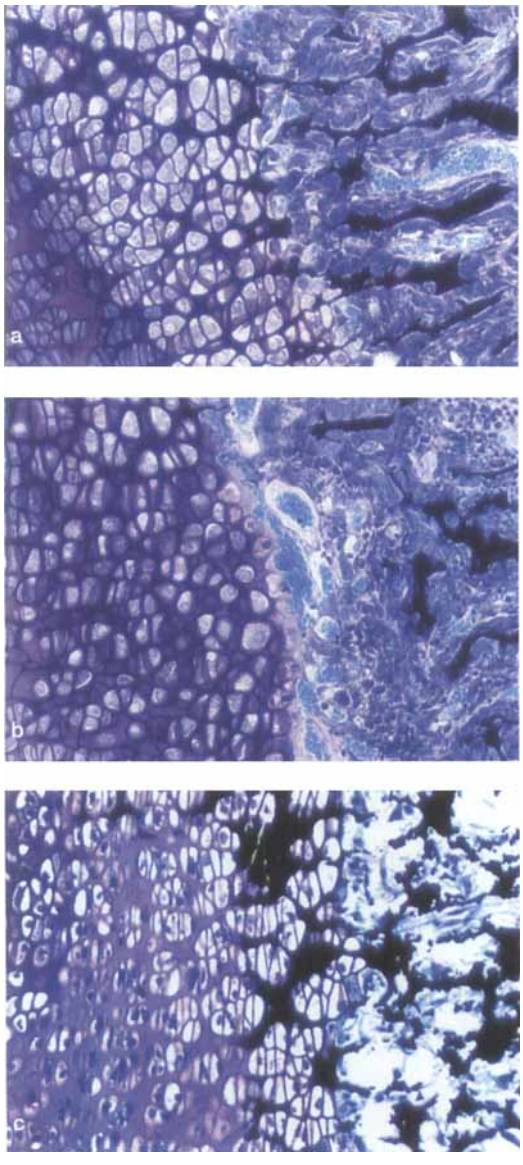

Figure 5. Van Kossa McNeal-stained tissue sections through the epiphysis show intact blood vessels invading the cartilage in isograft bone (a), saccular sprouts incapable of invading the cartilage in allograft bone (b) and no blood vessels in allograft bone in presensitized recipients (c). (This technique stains calcified substances black and red blood cells light green.)

Elongation and mineralization. Femora grew longitudinally (baseline length: approx. 4.0 mm) within the observation period of 12 days, revealing similar growth rates in all experimental groups (isograft bone (n 19): 70 (58) μm/day, allograft bone (n 24): 65 (56) μm/day, and allograft bone in presensitized recipients (n 25): 71 (46) μm/day). In contrast, there was a significant difference in the mineralization between isograft bone (n 5; 113 (111) μm/day) ($p = 0.004$), allograft bone (n 4; 6 (3) μm/day; $p = 0.01$ vs. isograft bone) or allograft bone in presensitized recipients (n 9; 10 (8) μm/day; $p = 0.003$ vs. isograft bone). We found no significant difference between allograft bone and allograft bone in presensitized recipients.

Systemic leukocyte count

Systemic leukocyte count (range: 5–6 × $10^9 L^{-1}$) and leukocyte differentiation, quantified in 6 animals each on days 6 and 12, did not differ among the experimental groups.

Histology (Figure 5)

Isograft bone revealed endosteal, periosteal and perivascular formation of osteoid. In the epiphysis, the chondrocytes formed columns. The central mineralization and epiphyseal calcification were physiologic. Many erythrocytes and only a sparse number of leukocytes were present in the medullary cavity. Allograft bone did not mineralize centrally and the calcification of the epiphysis was impaired. The medullary cavity revealed saccular sprouts rather than normal capillaries, that did not invade the cartilage. Allograft bone of presensitized recipients did not mineralize. The myeloid cavity showed no capillaries and revealed necrotic bone marrow.

Discussion

To evaluate the incorporation or rejection of bone grafts, neonatal and embryonic bone grafts have been used because they can undergo various stages of endochondral ossification (Sandberg et al. 1993) and are able to induce an immune response (Billingham et al. 1956). The neonatal femora used for this study are vascularized at the time of transplantation (first center of ossification is present on day 15 of gestation) (Johnson 1933) and they contain bone marrow participating in hemato- and leukopoiesis (Bannerman 1983). Using vital microscopy, early microvascular events were quantified, revealing significant differences after transplantation of neonatal femora into adult re-

cipients with different immunologic backgrounds. The early onset of revascularization in isograft and allograft bones confirms earlier reports using embryonic bone grafts in mice (Sabet et al. 1961) and reflects the ability of bone to establish end-to-end anastomoses between preexisting graft and recipient vessels (Albrektsson 1980). The stimulation of this process (angiogenesis) is mediated via growth factors synthesized by cells in the bone (Hauschka et al. 1986). The decreased vascular density and perfusion in allograft bone of recipients that have not been presensitized (first set or acute rejection) in the later course and the absent vascularization in allograft bone of presensitized recipients (second set or accelerated rejection) probably result from the immune response after tissue allorecognition has occurred (Czitrom 1996). Despite the decrease in microvascular parameters during acute rejection of allograft bone, there was no change in the leukocyte/endothelium interaction, as compared to isograft bone. This might be explained by local inflammation (wound healing) (Bennett and Schultz 1993) induced by isograft bone, whereby recruited and activated leukocytes serve a beneficial rather than detrimental function, as opposed to allograft bone. The increased recruitment (floater) and activation of leukocytes (sticker physically plugging and/or biochemically damaging microvessels) of allograft bone in presensitized recipients are similar to that described after xenograft pancreatic islet transplantation (Beger and Menger 1997) and reflects accelerated rejection. The lack of epiphyseal mineralization of allograft bone is caused by the insufficient vasculature, revealing saccular sprouts incapable of invading the epiphyseal cartilage in allograft bone of non-presensitized recipients and absent vasculature in allograft bone of presensitized recipients, as evidenced by our histological findings (Figure 4). Blood vessels and osteoblasts responsible for bone formation are the primary targets of allograft bone rejection, while epiphyseal chondrocytes (no difference found for femoral elongation) have been said to maintain their function in small grafts (Gotfried et al. 1987). In contrast, the intact medullary and periosteal vascularization and osteoblast survival in isograft bone reflect tissue viability, enabling the graft bone to participate actively in bone healing (Berggren et al. 1982). The early revascularization and small size of neonatal femora allowing implant survival by diffusion (Maroudas 1976) create an experimental situation representing vascularized rather than non-vascularized transplantation of large grafts. Since vascularized free bone transplantation has received increasing clinical attention (Weiland 1981), information about this work seems to be of clinical interest. The finding that early revascularized allograft bone can be rejected, such as heart and kidney allografts, is not new but again shows the need for a donor-recipient matching or the development of strategies to reduce graft immunogenicity. Moreover, the accelerated allograft rejection in presensitized recipients shows the importance of a thorough clinical history in free vascularized bone allograft transplantation.

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