Immunosuppressive effect of tacrolimus (FK-506)

Bone xenografts in rabbits

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We examined the immunomodulatory effect of the macrolide antibiotic FK-506 (tacrolimus) in bone xenograft transplantation. Full-thickness pieces of iliac bone from mongrel dogs were transplanted into the iliac bone of Japanese white rabbits. FK-506 at a dose of 1.6 mg/kg/day was injected into the rabbits for 10 days after transplantation. In the animals treated with FK-506, inflammatory cell infiltration was remarkably reduced and revascularization accompanied by new bone formation occurred in the grafts. At 4 months after the transplantation, the formation of new bone and of mature new bone marrow were observed. In a control group, inflammatory cell infiltration was marked around the graft from 2 weeks after the transplantation. Revascularization from the recipient site to the graft in the control group was poor and only a small amount of new bone had formed at 4 months. Our findings suggest that short-term administration of FK-506 has a beneficial effect on experimental xenograft bone transplantation.

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Tacrolimus is the generic name of FK-506 (code name). It is a macrolide antibiotic which was extracted from a culture strain of *Streptomyces tsukubaensis* in 1984 (Kino et al. 1987). It has a marked immunosuppressive effect in organ allotransplantation and is estimated to have 10–200 times the immunosuppressive effect of cyclosporin, which is now most commonly used in organ transplantation. To our knowledge, there has been no report on the effect of FK-506 on bone xenografts that possess strong antigenicity. We investigated the effect of FK-506 on bone grafts from dogs to rabbits.

Animals and methods

Transplantation

56 randomly bred male Japanese white rabbits, 3–4 months old with mature skeletons, weighing 3–3.5 kg, were recipients and 7 mongrel dogs of both sexes, 8 months–1 year old with mature skeletons, weighing 8–10 kg were donors. The rabbits were anesthetized with an intravenous injection of 25 mg/kg body weight sodium pentobarbital. A 3 cm-long skin incision was made along the right iliac bone and soft tissues around the bone were stripped subperiosteally. After removal of a 5 × 10 mm piece of full-thickness bone from the ilium, using an oscillating saw, a full-thickness xenogeneic iliac bone of 5 × 10 mm extracted from dogs and stored at –80 °C for 2 weeks under aseptic conditions was transplanted into the rabbit bone defect. Two 1 mm Kirschner-wires were used for fixation. The rabbits were divided into an FK-506 group and a control group without medication. Each group consisted of 28 rabbits. In the experimental group, intramuscular injections of FK-506 (Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) in a dose of 1.6 mg/kg/day were given to each rabbit for 10 days after transplantation.

Histologic specimens

At 2, 4, 8, and 16 weeks after the transplantation, perfusion staining was performed on the rabbits. After anesthesia with intravenous sodium pentobarbital, 25 mg/kg body weight, a vertical midline incision was made on the rabbit's abdomen and the peritoneum was opened. The abdominal aorta was cannulated in the distal direction. Before India ink perfusion was started, the circulatory system was cleared by perfusion with 0.5 L of physiological saline, containing 5000 units of heparin. The inferior vena cava was cut to withdraw blood. When the blood from the inferior vena cava became clear, 150 mL of India ink was injected at a pressure of 130 mmHg controlled by a mercury manometer. After the perfusion staining, the ilium was extracted together with the surrounding tissue and fixed in 10% neutral-buffered formalin. 2 transverse sections, about 700 µm in thickness, were cut, using a microsaw from the center of the graft. 2 specimens were decalcified with a mixture of formalin and formic acid and one was made transparent by the modified Spalteholtz technique (Gelberman et al. 1991), so that the stained vessels could be studied in detail to analyze revascularization.

The procedure involved dehydration of the specimen by immersion in serial dilutions of ethanol (50, 70, 95, and 100%) for 2 hours at each concentration, and for an additional 2 hours in 100% ethanol. The specimen was immersed in xylene overnight, and then clarified with 1:1 solution of methylsalicylate and xylene for several hours. The other specimen was sliced 6 μm thick and stained with hematoxylin and eosin, and used to analyze new bone formation.

Analysis of revascularization and new bone formation

Analysis was carried out according to a method which we employed previously at our laboratory (Yano et al. 1993). The transparent 700 μm-thick specimens were used to observe the revascularization rate in the grafts. The original analogue images of the specimens were put into an image processor (NEXUS 600, Kashiwagi Res. Co., Tokyo) through a TV camera connected to a light microscope. The image processor could resolve an image into 512 × 480 pixels and 256 classes of density. The original images of the specimens were processed in 2 values (black and white: black part indicates blood vessels and white part corresponds to the other area) and the areas occupied by blood vessels in grafted bone and recipient bone were calculated to determine the revascularization rate as a percentage:

(Area of blood vessels in grafted bone / Area of blood vessels in recipient bone) × 100.

1 section which consisted of 1 measuring field per animal was analyzed. Analyses were carried out at 2, 4, 8, and 16 weeks after the transplantation, using 7 rabbits in each group. New bone formation rate was calculated as a percentage: (Area of new bone in grafted bone / Area of bone trabeculae in grafted bone) × 100.

The results were analyzed statistically by Mann-Whitney's U-test.

Results

Histology

In patients given FK-506 there was no inflammatory cell infiltration and the granulation tissue contained many blood vessels and appositional new bone had formed around the grafted bone trabeculae at 2 weeks.

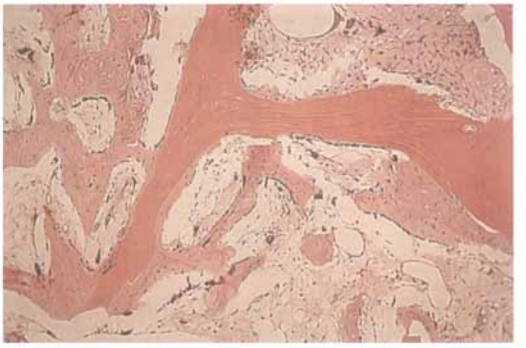

Figure 1. 4 weeks after the transplantation. New bone around the xenogeneic bone trabeculae and no inflammatory infiltration. FK-506(+). (HE, ×150).

Figure 2. At 4 months. Mature bone marrow tissue has formed. FK-506(+). (HE, ×150).

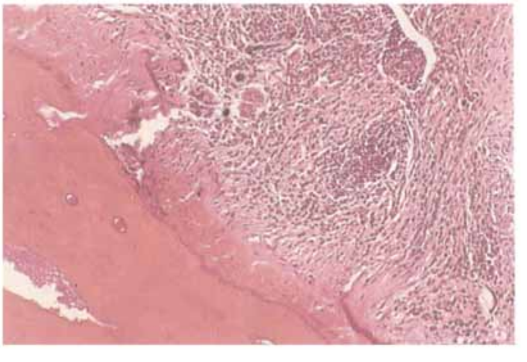

Figure 3. At 4 weeks. Diffuse lymphocyte and eosinophilic cell infiltration with multinucleated cells (foreign body giant cell type and osteoclast type). FK-506(–). (HE, ×300).

From 1 to 4 months after the transplantation, cell infiltration was still absent and the amount of newly formed bone had increased (Figure 1). At 4 months after the transplantation, new bone and new bone marrow had formed in the grafted bone (Figure 2). In the control group, diffuse inflammatory cell infiltration as a sign of an immune reaction was observed around the xenograft at 2 weeks. After 1 month, this

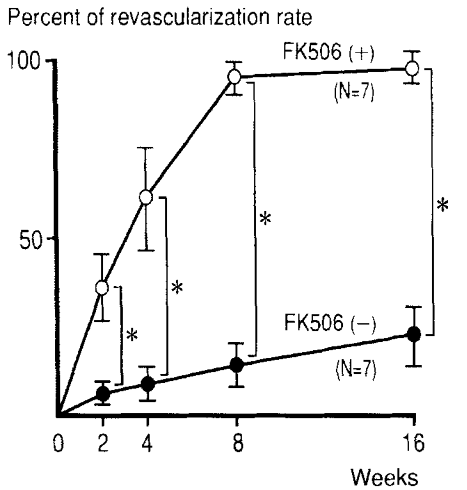

Figure 4. Mean and standard deviations of the revascularization rate in each group.
* p < 0.001.

reaction became distinct (Figure 3). In the grafted bone, new bone formation was almost absent and amorphous necrotic substance was observed. From 2 to 4 months, cell infiltration was still intense and absorption of necrotic bone and fibrosis of bone matrix were observed. Very little new bone formation was seen inside the grafted bone. Cell infiltration around the graft continued at 4 months and fibrosis of bone matrix became distinct, but new bone formation in the grafted bone was almost absent.

Revascularization (Figure 4)

At 2 weeks, the mean revascularization rate was 37% in the experimental group and 7% in the control group. Thereafter, the revascularization rate markedly increased in those given FK-506 (Figure 5), reaching almost the same vascular density as that in the recipient bone at 4 months (mean 99%). In contrast, the revascularization rate over time was poor in the control group (Figures 4 and 6).

Figure 5. Perfusion staining at 4 weeks after the transplantation. FK-506(+). (HE, ×25). Same case as in Figure 1, revascularization rate 53%.

Figure 6. Perfusion staining at 4 weeks after the transplantation. FK-506(–). (HE, ×25). Same case as in Figure 3, revascularization rate 9%.

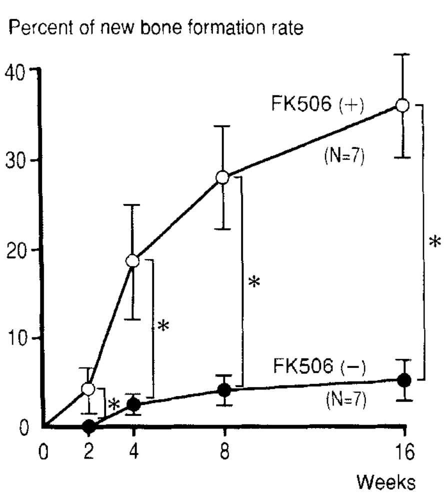

Figure 7. Mean and standard deviations of the new bone formation rate in each group. * p < 0.001.

New bone formation (Figure 7)

New bone formation was seen in the experimental group 2 weeks after the transplantation (mean 4%), but it was totally absent in the control group. From 2 to 4 weeks, bone formation increased rapidly in the experimental group. The formation of new bone in the control group was poor during the study period.

Discussion

We tried to improve the compatibility of bone xenograft by giving the immunosuppressive agent FK-506. Short-term administration of FK-506 resulted in marked revascularization from the recipient site to the grafted bone and new bone formation occurred. There were no signs of a local immunoreaction and donor bone marrow was replaced by new mature bone marrow tissue, suggesting that the xenograft was completely incorporated into the recipient. The immunosuppressive effect of FK-506 is due to its selective inhibition of the activation of T-lymphocytes (Thomson 1990). T-cells play a leading role both in antigen recognition and as sources of cytokines in allotransplantation. Our findings suggest that T-cells might be important actors in xenograft bone transplanttion.

Some previous reports have pointed out that natural killer cells (NK-cells) or antibody-dependent cell-mediated cytotoxicity (ADCC) participate in the immunoreaction in xenotransplantation (Escobar and Sigel 1970, Grant et al. 1971, Harding and MacLennan 1972, Moller and Svehag 1972, Dennert 1974). However, there are no reports stating that the immunosuppressive influence of FK-506 would affect NK-cells and ADCC (Beck and Akiyama 1989, Markus et al. 1991, Wasik et al. 1991).

In our study, 10 days' administration of FK-506 resulted in long-term immunosuppression. Ochiai et al. (1987) reported long-term cardiac rat allograft survival after short-term treatment with FK-506 (Ochiai et al. 1987). They concluded that donor strain-specific suppressor T-cells and humoral factors are induced by FK-506. In bone xenografts, the immunogenicity of xenogeneic bone is present in the donor's bone and bone marrow. However, the newly formed bone and bone marrow tissues originate from the recipient, they have the same antigen of the recipient. Our results suggested that short-term administration of FK-506 has a beneficial effect in experimental xenograft bone transplantation.

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