No effect of immunosuppression with cyclosporin A detected on bone ingrowth into cancellous allo- and xenografts in the rat

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We studied the effects of the immunosuppressant cyclosporin A on bone ingrowth into allo- and xenografts in the rat using titanium bone chambers. The bone chambers were implanted bilaterally in the tibia. Each rat had one allograft and one xenograft. At 6 weeks the distance of soft tissue and bone ingrowth was measured on histological slides. In xenografts, soft-tissue ingrowth was only slightly less than in allografts, but the ingrown tissue became much less ossified. These differences were unaffected by cyclosporin A treatment, suggesting that the lesser amount of bone formation in xenografts is only weakly associated with T-cell-dependent immune reactions.

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Large corticocancellous allografts, used to reconstruct defects after trauma or tumors, are incorporated at a slow rate, and revascularization and bone ingrowth extend only a few millimeters into the graft (Enneking and Mindell 1991, Hooten et al. 1996). Furthermore, in one third of cases, complications such as infection, fracture, resorption of the graft or non-union develop within the first few years (Mankin 1983, Mankin et al. 1983, Berrey et al. 1990).

Frozen allografts contain no living cells, and the incorporation of a graft is dependent on osteoconduction and perhaps osteoinduction. Osteoinduction is the process in which undifferentiated mesenchymal cells are induced by local factors to proliferate and differentiate into cartilage- and bone-forming cells (Urist 1965). Osteoconduction is the process of bone growing into or onto a graft or implant. The bone is preceded by less differentiated mesenchymal tissue (Aspenberg and Wang 1993, 1995). Allo- and xenografts are capable of stimulating a specific T-cell response, and these immune reactions may affect both osteoinduction and osteoconduction (Hallén 1966, Salama 1983, Horowitz and Friedlander 1991, Thoren et al. 1993, Fukunaga et al. 1995). Earlier studies have shown that bone induction in xenogeneic demineralized bone matrix is inhibited by immune reactions and normalized by immunosuppressive treatment (Ekelund and Nilsson 1992, Ekelund et al. 1997). Cyclosporin A acts primarily by inhibiting the production of cytokines involved in the regulation of T-cell activation (Russel et al. 1992, Borel et al. 1996). Allografts in humans are osteoconductive rather than osteoinductive (Enneking and Mindell 1991).

We evaluated the importance of immune reactions on bone ingrowth (osteoconduction) into cancellous allo- and xenografts and analyzed the effects of immunosuppressive treatment with cyclosporin A on the osteoconductive process.

Material and methods

Preparation of the grafts

24 male donor Sprague-Dawley rats (200 g) were killed by carbon dioxide inhalation. The tibias were fractured through the proximal growth plates. Under aseptic conditions, the growth cartilage was removed, and cylindrical dowels of metaphyseal cancellous bone were taken from each tibia in the distal direction with a manual hole-cutter. The bone cylinders to be used as

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grafts were 2 mm wide and 5–6 mm in length. The grafts were lipid-extracted in 1/1 chloroform/methanol overnight, rinsed 3 times in methanol, 3 times in distilled water and air dried. Each rat yielded 2 grafts. The grafts were kept sterile and frozen at –70 °C.

The humeri, tibias and femora from 4 rabbits (New Zealand, 3–4 kg bodyweight) were used for the preparation of xenografts. After removal of the periosteum and cortical bone layer, metaphyseal cancellous bone grafts were taken from these long bones with a hole-cutter. 2 grafts were taken from each location and 12 pairs of grafts were prepared. The bone cylinders to be used as grafts were 2 mm wide and 6–7 mm in length. These grafts were lipid-extracted and treated similarly to the rat grafts.

Bone conduction chamber

The bone conduction chamber consists of a threaded titanium cylinder formed from 2 half-cylinders held together by a closed screw cap (Aspenberg and Wang 1993, 1995). The chamber is screwed into the bone. The bone ingrowth chamber has an inside diameter of 2 mm and an inside length of 7 mm. There are 2 openings, each 1 mm in diameter, at one end of this chamber. It is designed to allow bone ingrowth from that end into a long cylinder.

Treatment with cyclosporin A

Cyclosporin A was dissolved in 0.5 mL of 95% ethanol, and diluted in castor oil to the appropriate concentration (4 mg/mL). The rats were randomly divided into two groups. One group was treated with cyclosporin A (Sandoz AG, Basel, Switzerland) 2 mg/kg bodyweight/day, and the other group with castor oil for only 6 weeks. The rats were given daily intraperitoneal injections of the solution, starting on the day of implantation. The serum level of cyclosporin A in whole venous blood was determined in the first 6 rats at 6 weeks using an RIA test (CYCLO-Trac SP®-Whole Blood RIA kit, Incstar Corp, Stillwater, MN, USA).

Surgery

24 male Sprauge-Dawley rats from a breeder other than the graft donors, with a mean weight of 349 ± 7 g, were anesthetized with intraperitoneal injections of 0.6–0.7 mL of a solution containing 15 mg pentobarbital and 2.5 mg diazepam per mL. Under aseptic conditions, bilateral incisions were made over the anteromedial aspect of the proximal tibial metaphyses. The medial and posterior lateral cortices were pierced with a 1.0 mm spike just anterior to the insertion of the medial collateral ligament. The hole created in the medial cortex was manually enlarged with a 2.7 mm drill. The grafts were placed in the chambers. The chambers were screwed into position so that the bone ingrowth holes were placed at the level of the cortical bone, and the pointed end of the titanium implant was engaged through the opposite cortical bone. The skin was sutured. Each rat received 2 chambers, one with an allograft (rat) and one with a xenograft (rabbit). The grafts in the bone chambers of the two groups of animals had an identical origin, since all grafts from the same donor or site were kept in pairs, and the pairs were split between cyclosporin A and control.

Analyses

The rats were killed after 6 weeks by an overdose of pentobarbital. 3 hours previously they had received an intravenous injection of approximately 4.5 MBq of ^{99m}Tc-MDP. The chambers were removed, and the specimens removed. The scintimetric activity of the graft specimens was measured in a well counter. The specimens were fixed in 4% formalin, decalcified, and embedded in paraffin. They were cut parallel to the long axis of the chamber with a microtome. 3 sections that were to be used for histology and histomorphometry, each at a distance of 300 µm from the others, were taken from the middle of the specimens and stained with hematoxylin and eosin. The identity tags were covered, and each slide was numbered at random. The area of newly ingrown bone was measured in a microscope with a video monitor and drawing on a digital table (Videoplan, Kontron Bildanalyse GmbH, Esching, Germany) at magnification ×40. Areas of marrow cavities and graft bone remnants in new bone areas were included. The ingrowth distance was calculated by dividing the new bone area by the width of the specimens. The non-parametric Mann-Whitney test and Wilcoxon signed rank test were used for

Table 1. Soft-tissue and bone ingrowth and scintimetric activity of allo- and xenogeneic cancellous bone grafts regardless of treatment

Type of graft	Bone ingrowth mm	Soft-tissue ingrowth mm	Scintimetric activity 10^3 cpm
Allograft (rat)	1.2 (0–3.4)	3.7 (2.6–5.1)	5.0 (1.8–9.3)
Xenograft (rabbit)	0.22 (0–1.2)	2.7 (1.2–5.2)	2.4 (0.34–6.7)
	$p < 0.001$	$p = 0.007$	$p < 0.001$

Bone and soft-tissue ingrowth measured in mm (median (range)) and scintimetric activity 10^3 counts/min/graft (median (range)). N 23, with 1 allo- and 1 xenograft in each animal. The non-parametric Wilcoxon signed rank test was used for the analyses.

Table 2. Bone and soft-tissue ingrowth and scintimetric activity of allogeneic and xenogeneic cancellous bone graft in control (C) and cyclosporin A (CsA)-treated rats

Type of graft	Bone ingrowth mm	Soft-tissue ingrowth mm	Scintimetric activity 10^3 cpm
Allograft (C)	1.2 (0.3–2.7)	3.8 (2.6–5.1)	4.8 (1.8–8.3)
Allograft (CsA)	1.2 (0–3.4)	3.7 (2.6–4.7)	5.5 (3.0–9.3)
	$p = 0.9$	$p = 1$	$p = 0.1$
Xenograft (C)	0.1 (0–0.8)	2.7 (1.2–5.2)	1.6 (0.34–6.3)
Xenograft (CsA)	0.4 (0.02–1.2)	3.1 (1.9–4.1)	2.8 (0.47–6.7)
	$p = 0.2$	$p = 0.8$	$p = 0.2$

Bone and soft-tissue ingrowth measured in mm (median (range)) and scintimetric activity 10^3 counts/min/graft (median (range)). N 11 in the control group, N 12 in the CsA group, with 1 allograft and 1 xenograft in each animal. The Mann-Whitney non-parametric test was used for the analyses.

statistical analyses. P-values < 0.05 were regarded as significant.

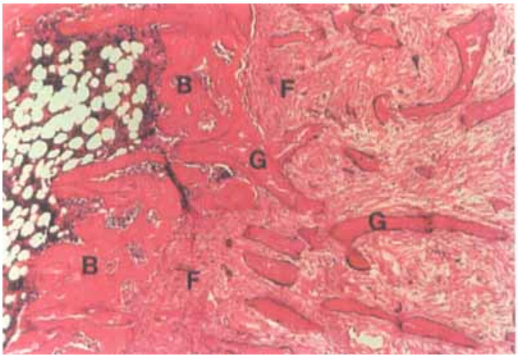
Results

All rats withstood the operation and the medication. There was no statistically significant difference in weight between the groups at 6 weeks (474, SD 41 g vs 450, SD 47 g). One rat from the control group was excluded because one of the chambers was found to be loose at 6 weeks.

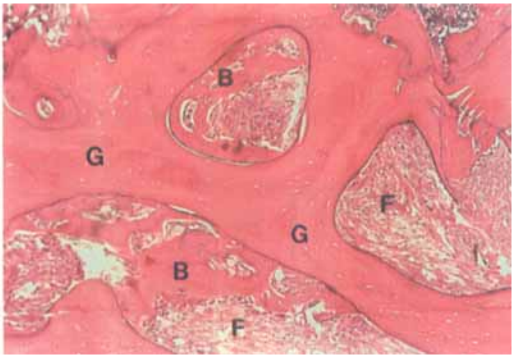
The total tissue ingrowth distance was slightly less in xenografts, but bone ingrowth was only minute, less than 0.5 mm, both with or without cyclosporin A. In allograft controls, bone ingrowth was 1.2 mm. When comparing all allografts with all xenografts regardless of treatment, allografts showed more bone ingrowth, total-tissue ingrowth and scintimetric activity than xenografts (Table 1). In both graft types, these differences were unaffected by cyclosporin A treatment (Table 2). The ratio of bone ingrowth to total ingrowth was higher in allografts than in xenografts (0.35, SD 0.18 and 0.11, SD 0.09; $p < 0.001$). The 95% confidence interval for the effect of cyclosporin A on bone ingrowth into allografts ranges from an increase of 0.79 mm to a decrease of 0.62 mm, and on bone ingrowth into xenografts it ranges from an increase of 0.5 mm to a decrease of 0.12 mm.

The mean cyclosporin A concentration was 398, SD 77 ng/mL. One glass test tube was broken at the time of the analysis, and the sample was therefore excluded.

Qualitative histology consistently revealed bone ingrowth in the proximal region (close to the ingrowth openings) with a zone of mature remod-



Allograft with old graft (G) and newly ingrown bone (B) and fibrous tissue (F), HE ×100.



Xenograft with old graft (G) and newly ingrown bone (B) and fibrous tissue (F), HE ×100.

eled bone. More distally, there was a zone of woven bone followed by an area of fibrous tissue. There were thicker bone trabeculae and larger "pores" in the xenografts than in the allografts (Figure). There were only minimal signs of inflammatory reactions in the xenografts. Cyclosporin A treatment did not alter the histologic picture. Thus, there were no signs of strong immune reactions within the grafts.

Discussion

There was a larger amount of total-tissue ingrowth, bone ingrowth and scintimetric activity in the allografts than in the xenografts. These differences were unaffected by cyclosporin A treatment.

The bone conduction chamber is designed to study bone conductive properties of different grafts or materials (Aspenberg and Wang 1993, 1995). To measure osteoconduction specifically, it is important that the surrounding soft tissues should be shielded and that bone ingrowth does not extend to the distal end of the grafts. Earlier studies with the bone conduction chamber have shown that a graft placed in the chamber increases the fibrous tissue ingrowth (Aspenberg and Wang 1993, 1995). The fibrous mesenchymal tissue becomes metaplastically ossified (membraneous ossification). It was concluded that an osteoconductive material is in reality often "mesenchymal tissue conductive", and that the ossification process partly depends on other factors such as mechanical load (Aspenberg and Wang 1995). Thus, to analyze the importance of immune reactions, the effects on total tissue ingrowth and ossification must be evaluated separately.

In our study, there was less soft tissue ingrowth into xenografts than into allografts. However, the mean total tissue ingrowth into allografts (3.8 mm) and xenografts (3.0 mm), respectively, was better than the mean ingrowth into empty chambers (1.8, SD 0.31mm: unpublished data). This indicates that both allo- and xenografts have soft-tissue conductive properties, but these are less favorable in xenografts than in allografts. The soft tissue is then metaplastically ossified and the amount of bone ingrowth into the grafts is an indicator of the efficacy of this process. In our study, one third of the total ingrowth distance was bone in the allografts but only one tenth was bone in the xenografts. In empty chambers, two thirds of the total ingrowth distance was bone (unpublished data). Thus, ossification was much more impaired than soft-tissue ingrowth into xenografts, as compared to allografts and empty chambers. These differences were unaffected by cyclosporin A treatment. In the present investigation, T-cell-dependent immune reactions consequently had no great effect on the osteoconductive properties of the graft: neither the ingrowth of soft tissue into the graft nor the ossification of this tissue. The confidence interval for an effect of CsA on bone ingrowth excludes the possibility that T-cell-dependent mechanisms are largely responsible for the smaller ingrowth distance in xenografts. These findings are somewhat surprising, since animal studies have shown that the incorporation of bone allografts is influenced by T-lymphocyte-dependent immune reactions (Bos et al. 1983, Kirkeby et al. 1991, 1992, Stevenson et al. 1997). Furthermore, improved incorporation of bone allografts and enhanced osteogenesis in xenogeneic bone transplants have been reported after immunosuppressive treatment (Burchart et al. 1977, Goldberg et al. 1984, 1985, Fukunaga et al. 1995).

Cyclosporin A acts primarily by inhibiting the production of certain cytokines (interleukins and interferon gamma (IFN-χ)) in T-cells (Bunjes et al. 1981, Russel et al. 1992, Borel et al. 1996). In vitro, interleukin production and release are almost totally blocked at a cyclosporin concentration of 100 ng/mL, and toxic side-effects are commonly seen when the concentration reaches 800 ng/mL (Irschik et al. 1984, Holt et al. 1986, Szturm et al. 1989). Thus, cyclosporin A concentrations were in the therapeutic interval in all tested animals at the end of the experiment, and immunosuppression can be assumed to have been effective during the study. This is also in accordance with earlier studies on bone induction, where similar doses of cyclosporin A treatment normalized bone induction in heterotopically placed demineralized xenogeneic (rabbit) bone matrix in rats (Ekelund and Nilsson 1992, Ekelund et al. 1997).

The impaired bone formation in xenografts we found may be caused by immune reactions that are unaffected by cyclosporin A, or by non-immunogenic mechanisms such as inhibitory cell/matrix interactions and gross structural differences between allo- and xenografts. Studies on bone ingrowth into porous surfaces of metals used for joint replacements have shown that the size of the pores is important with respect to optimal ingrowth (Bobyn et al. 1980, Martens et al. 1980). Furthermore, interactions may occur between the cytokines produced by immune cells and the cells involved in bone formation (Bertolini et al. 1986, Evans et al. 1990, Gowen et al. 1990, Frost et al. 1997). Moreover, nerves containing peptides that can affect bone cells appear simultaneously with the mineralization process in fracture healing and in bone graft incorporation (Hukkanen et al. 1993, Madsen et al. 1996). An involvement of these nerves and their neuropeptides in the revascularization of the graft and in the recruitment and activity of the osteogenic cells was suggested. The temporal occurrence and quantity of these nerves may be different in allo- and xenografts, and such differences may contribute to the inferior incorporation of the xenograft.

In conclusion, xenografts showed less bone and soft tissue ingrowth than allografts. Bone formation in the xenografts was more affected than the total tissue ingrowth. These differences were not significantly affected by immunosuppressive treatment with cyclosporin A. Thus, in our study, osteoconduction was not greatly affected by T-cell-dependent immune reactions.

Aspenberg P, Wang J S. A new bone chamber used for measuring osteoconduction in rats. Eur J Exp Musculoskel Res 1993; 2: 69-74.

Aspenberg P, Wang J S. The bone conduction chamber, materials and growth factors. Cells & Material 1995; 5: 337-44.

Berrey B H, Lord C F, Gebhart M C, Mankin H J. Fractures in allografts. J Bone Joint Surg (Am) 1990; 72: 825-33.

Bertolini D R, Nedwin G E, Bringman T S, Smith D D, Mundy G R. Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors. Nature 1986; 319: 516-8.

Bobyn J D, Pilliar R M, Cameron H V, Weatherly G C. The optimum pore size for the fixation of porous surfaces. Clin Orthop 1980; 150: 263-70.

Borel J F, Bauman G, Chapman I, et al. In vivo pharmacological effects of cyclosporin and some analogues. Adv Pharmacol 1996, 35: 115-246.

Bos G D, Goldberg V M, Zika J M, Heiple K G, Powell A E. Immune responses of rats to frozen bone grafts. J Bone Joint Surg (Am) 1983; 65: 239-46.

Bunjes D, Hardt C, Röllinghoff M, Wagner H. Cyclosporin A mediates immunosuppression of primary cytotoxic T-cell responses by impairing the release of interleukin-1 and interleukin-2. Eur J Immunol 1981; 11: 657-61.

Burchart H, Glowczewskie F P, Enneking W F. Allogeneic segmental fibular transplants in azathioprine-immunosuppressed dogs. J Bone Joint Surg (Am) 1977; 59: 881-94.

Ekelund A, Nilsson O. Effects of cyclosporin A on experimental new bone formation in rats. Clin Orthop 1992; 284: 288-98.

Ekelund A, Ahmed M, Bjurholm A, Nilsson O. Neuropeptides in heterotopic bone induced by bone matrix in immunosuppressed rats. Clin Orthop 1997; 345: 229-38.

Enneking W F, Mindell E R. Observations on massive retrieved allografts. J Bone Joint Surg (Am) 1991; 73: 1123-42.

Evans D B, Bunning R A D, Russel R G G. The effects of recombinant human interleukin-1ß on cellular proliferation and production of prostaglandin E2, plasminogen activator, osteocalcin and alkaline phosphatase by osteoblast-like cells derived from human bone. Biochem Biophys Res Comm 1990; 166: 208-16.

Frost A, Jonsson K B, Nilsson O, Ljunggren Ö. Inflammatory cytokines regulate proliferation of cultured human osteoblasts. Acta Orthop Scand 1997; 68: 91-6.

Fukunaga T, Masumi S, Yano H, Ikebe S, Shimizu K. Osteogenesis in xenogeneic bone transplantation, using an immunosuppressant. Acta Orthop Scand 1995; 66: 557-60.

Goldberg V M, Bos G D, Heiple K G, Zika J M, Powell A. Improved acceptance of frozen bone allografts in genetically mismatched dogs by immunosuppression. J Bone Joint Surg (Am) 1984; 66: 937-50.

Goldberg V M, Powell A, Shaffer J W, Zika J, Bos G D, Heiple K G. Bone grafting: role of histocompatibility in transplantation. J Orthop Res 1985, 3: 389-404.

Gowen M, Chapman K, Littlewood A, Hughes D, Evans D, Russel G. Production of tumour necrosis factor by human osteoblasts is modulated by other cytokines, but not by osteotropic hormones. Endocrinology 1990; 126: 1250-5.

Hallén I G. Heterologous transplantation with Kiel bone. Acta Orthop Scand 1966; 37: 1-19.

Holt D W, Marsden J T, Johnston A, Bewick M, Taube D H. Blood cyclosporin concentrations and renal allograft dysfunction. BMJ 1986; 293: 1057-9.

Hooten J P, Engh C A, Heekin R D, Vinh T N. Structural bulk allografts in acetabular reconstruction. J Bone Joint Surg (Br) 1996; 78: 270-5.

Horowitz M C, Friedlander G E. Induction of specific T-cell responsiveness to allogeneic bone. J Bone Joint Surg (Am) 1991; 73: 1157-68.

Hukkanen M, Konttinen Y T, Santavirta S, Paavolainen P, Gu X-H, Terenghi G, Polak J M. Rapid proliferation of calcitonin gene-related peptide-immunoactive nerves during healing of rat tibial fracture suggests neural involvement in bone growth and remodeling. Neuroscience 1993, 54: 969-79.

Irschik E, Tilg H, Niederwieser D, Gastl G A, Huber C H, Margreiter R. Cyclosporin blood levels do correlate with clinical complications. Lancet 1984; II: 692-3.

Kirkeby O J, Larsen T B, Lereim P. Bone grafts in T-cell-deficient rats. Acta Orthop Scand 1991; 62: 459-62.

Kirkeby O J, Nordsletten L, Skjeldal S. Healing of cortical bone grafts in athymic rats. Acta Orthop Scand 1992; 63: 318-22.

Madsen J E, Wang J S, Hukkanen M, Nordsletten L, Konttinen Y T, Santavirta S, Polak J M, Aspenberg P. Sensory nerve ingrowth during graft incorporation in the rat. Acta Orthop Scand 1996; 67: 217-20.

Mankin H J. Complications in allograft surgery In: Osteochondral allografts (Eds. Friedlander G E, Mankin H J, Sell K W). Little Brown, Boston 1983; 259-74.

Mankin H J, Doppelt S, Tomford W. Clinical experience with allograft implantation. Clin Orthop 1983; 174: 69-86.

Martens M, Ducheyne P, De Meester P, Mulier J C. Skeletal fixation of implants by bone ingrowth into surface pores. Arch Orthop Traum Surg 1980; 97: 111-6.

Russel G, Graveley R, Seid J, Al-Humidan A-K, Skjodt H. Mechanisms of action of cyclosporine and effects on connective tissues. Semin Arthritis Rheum 1992; 21: 16-22.

Salama R. Xenogeneic bone grafting in humans. Clin Orthop 1983; 174: 113-21.

Stevenson S, Li X Q, Davy D T, Klein L, Goldberg V M. Critical biological determinants of incorporation of non-vascularized cortical bone grafts. J Bone Joint Surg (Am) 1997; 79: 1-16.

Szturm K, Jeffrey J R, Rush D N, McKenna R M. Cyclosporin A and G inhibition of cytokine production. Transpl Proc 1989; 21: 857.

Thoren K, Aspenberg P, Thorngren K G. Lipid extraction decreases the specific immunologic response to bone allografts in rabbits. Acta Orthop Scand 1993; 64: 44-6.

Urist MR. Bone: Formation by autoinduction. Science 1965, 150: 893-9.