Longer survival of rat limb allograft

Combined immunosuppression of FK-506 and 15-deoxyspergualin

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We studied the individual and synergistic effect of 3 immunosuppressive drugs, FK-506 (1 mg/kg/day), 15-deoxyspergualin (2.5mg/kg/day) and cyclosporine (15 mg/kg/day) in a DA/Lewis rat limb allotransplantation model. 74 right hindlimb transplantations were performed. The median time for onset of rejection was 4 days in animals without immunosuppression, 37 days in animals receiving cyclosporine immunosuppression for 30 days, 61 days in animals receiving FK-506 for 30 days, 36 days in animals receiving a 30-day course of cyclosporine and, in the first 15 days, a course of 15-deoxyspergualin, and 76 days in animals receiving a 30-day course of FK-506 and 15-deoxyspergualin in the first 15 days. The combination of cyclosporine with 15-deoxyspergualin did not prolong graft survival and no synergistic effect was evident. In contrast, survival time in rat limb allografts receiving FK-506 and 15-deoxyspergualin was longer than in those receiving single FK-506 therapy. Our findings suggest a positive synergistic immunosuppressive effect with FK-506 and 15-deoxyspergualin in limb allotransplantation.

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Research in visceral organ allotransplantation has made great progress in the past 20 years and is currently focused on developing more potent immunosuppressive drugs. The strong immunosuppressive action of cyclosporine (CS) was described by Borel et. al in 1976 and this drug has greatly improved the survival of clinical allotransplants. CS does not totally suppress acute rejection and prolonged use of it includes the risk of nephrotoxity and neuropathy (Muramatsu et al. 1995). Goto et al. described in 1987 the immunosuppressant drug FK-506 (FK). Many experiments have shown that FK has a stronger immunosuppressive effect than CS (Arai et al. 1989, Kuroki et al. 1991, Fealy et al. 1994). 15-deoxyspergualin (DSG), described by Umezawa in 1985, has an immunosuppressive action very different from that of FK and CS. Walter et al. (1987) induced donor-specific immunotolerance in rat kidney allotransplantats, using a short-course of DSG therapy.

To evaluate synergistic effects, we administered CS, FK and DSG in varying combinations after limb allotransplantation.

Animals and methods

The donors were adult Dark Agouti rats (DA; $RT1^a$) weighing 250–300 g, and the recipients were Lewis rats (LEW; $RT1^a$) weighing 300–350 g. Thus, transplantation from DA to LEW was across a major histocompatibility barrier (Muramatsu et al. 1995). The experiment was reviewed by the Committee on Ethics in Animal Experiments in Yamaguchi University School of Medicine and was carried out using the Guidelines for Animal Experiments in Yamaguchi University School of Medicine and the Law of the Government.

Operative procedure

We used the limb replantation model reported by Doi (1979) and Hotokebuchi et al. (1989). The donor right hindlimb was amputated at the mid-femur level. Care was taken to preserve the skin of the grafted hindlimb because it was used to monitor the circulation and rejection. A similar defect was created at the recipient site of the host animal's right hindlimb. After the limb had been orthotopically transplanted into the recipient, osteosynthesis was performed with an Kirschner wire (1.2 mm) as an intramedullary rod. The femoral vessels were anastomosed and the sciatic nerve was

Rejection period and survival of limb allograft (n 74)

Group		n	Death n (%)	Rejection period, day	Mean (SD)	Necrosis [a]	Viable toe	CMAP	Microangiography [b]
I	Autograft	8	0 (0)	> 130	> 130	0	8	8	3.0 (0)
II	No therapy	6	1 (17) [c]	4, 3, 5, 5, 5, 3	4 (1.0)	3	0	0	
III	CS30	8	0 (0)	38, 33, 35, 36, 36, 37, 44, 34	37 (3.4)	3	0	0	1.4 (0.9)
IV	DSG30	9	1 (11) [c]	35, 43, 37, 51, 48, 41,52, 46	44 (6.2)	3	0	0	1.4 (0.5)
V	FK30	12	3 (25) [c]	88, 70, 68, 57, 56, 71, 52, 53, 47	61 (13.3)	0	0	9	2.4 (0.5
VI	CS30-DSG15	9	2 (22) [d]	34, 35, 35, 38, 36, 38, 36	36 (1.5)	1	0	0	1.0 (0)
VII	CS30-DSG30	3	3 (100) [d]						
VIII	FK30-DSG15	9	1 (11) [c]	74, 80, 76, 79, 83, 76, 77, 62	76 (6.3)	0	3	8	2.8 (0.4)
IX	FK30-DSG30	10	2 (20) [d]	80, 78, 76, 78, 75, 74, 73, 73	76 (2.6)	0	7	8	3.0 (0)

CS30 cyclosporine was administered for 30 days postoperatively in a dose of 15 mg/kg/day, DSG30 15-deoxyspergualin was administered for 30 days postoperatively in a dose of 2.5 mg/kg/day, FK30 FK 506 was administered for 30 days postoperatively in a dose of 1 mg/kg/day.

[a] Evaluations were performed 120 days postoperatively.
Necrosis—the foot component mummified or fell off.
Viable toe—all of 5 toes were viable and preserved.
CMAP was obtained from the gastrocnemius muscle.

[b] Microangiographic findings were divided into 4 grades (0–3) and the mean (SD) grade was calculated.

[c] Early death after surgery or technical error of operation

[d] Death from adverse effects of immunosuppressants (weight loss and diarrhea)

epineurally sutured with 11-0 nylon suture, using microsurgical technique.

Experimental groups

The animals were divided into 9 groups according to the administration of immunosuppressant (Table). CS was given in a dose of 15 mg/kg/day, DSG in a dose of 2.5 mg/kg/day and FK, 1 mg/kg/day. Based on previous reports on limb allotransplantation, the dose of immunosuppressant given was 70–80% of the lethal dose (Black et al. 1982, Min et al. 1995). The immunosuppressive drugs were injected intramuscularly into the grafted limbs.

Evaluation

The general condition of the animals was observed daily. The onset of rejection of the grafted limbs was taken as the time when the grafted skin had erythema for 24 hours. It was assumed that necrosis had occurred when the foot mummified or fell off. The animals were killed at 120–130 (except for animals in the no immunosuppressive group, which were killed after 4 weeks postoperatively) days postoperatively and bony union between the donor and recipient was assessed on soft X-ray films. The vascularity of the grafted limbs was evaluated by microangiography and divided into 4 grades and the average was calculated: 0-no vascular shadow, 1-only the femoral artery was patent (distal to the anastomosis), 2-the vessels of the foot were patent, 3-both the femoral artery and vein were patent. The sciatic nerve was stimulated electrically (2.4 mA) at the proximal anastomosed site and an electromyogram (compound muscle action potential; CMAP) was obtained from the gastrocnemius muscle. Statistical analyses were done using the Student t-test and p-values less than 0.05 were considered significant.

Results

Group I (autografts, n 8)

All the grafted limbs survived and developed a drop-foot contracture. Bony union was achieved. CMAP from the gastrocnemius muscle was present in all cases.

Group II (allograft with no immunosuppression, n 6)

All but 1 of the limbs survived. The rejected limbs showed progressive skin erythema, swelling, skin ulcer, crest formation at the foot, mummification and finally fell off. The mean onset of rejection was 4 days postoperatively. The mean time to mummification was 11 days.

Group III (CS30, n 8)

All the grafted limbs survived. Rejection was completely suppressed during CS therapy. The mean onset of rejection was 37 days and no statistically significant difference in the delay was found between groups III and II. Bony union of the femur was achieved in all cases. Mummification of the foot occurred in 3 cases, but in 5 cases the feet and toes were covered with scar tissue. Microangiography showed vascularity of the foot. CMAP was not recordable in all animals.

Figure 1. Group V. Allografts with FK immunosuppression at 30 days postoperatively.

At 68 days, the grafted limb was rejected with desquamation and the foot was covered with scar tissue at 120 days postoperatively.

Sclerotic changes in the grafted bone and patency of the anastomosed femoral artery (grade 1).

Figure 2. Group VII. The allografts with FK therapy for 30 days and DSG therapy for 15 days postoperatively.

The grafted limb had a normal appearance at 120 days postoperatively.

Bony union and good patency of both the femoral artery and vein.

Group IV (DSG30, n 9)

All animals showed deterioration in their general condition (weight loss and diarrhea) during DSG therapy. The mean onset of rejection was 44 days, which was longer than that in the CS group ($p < 0.01$). At killing, 3 feet had mummified, but in 5 allotransplants the feet and toes were covered with scar tissue. Microangiography showed vascularity in these 5 allotransplants. As in the CS30 group, no CMAP was recordable.

Group V (FK30, n 12)

All animals had good general condition during FK therapy. Adenocarcinoma was observed in the epigastric region in one. The mean onset of rejection was 61 days, which was longer than that in the CS-30 group ($p < 0.01$) and DSG-30 group ($p < 0.05$). All femurs showed bony union at killing. Microangiography showed that the femoral vessels were patent (Figure1). CMAP was obtainable in all animals.

Group VI (CS30-DSG15, n 9)

2 animals died on days 10 and 28. The mean onset of rejection was 36 days, which is not significantly different from the CS30 group. Bony union had occurred in all, but there was a supracondylar fracture in 1 and necrosis of the foot in another. Microangiography showed no vascular shadow in the grafted limb. No CMAP was obtainable, as in the CS30 group.

Group VII (CS30-DSG30, n 3)

Severe weight loss occurred and all animals died on days 12, 15 and 28.

Group VIII (FK30-DSG15, n 9)

Weight loss and diarrhea were observed until 15 days postoperatively. The mean onset of rejection was 76 days which was longer than that in the FK30 group ($p < 0.05$). Bony union was achieved in all allotransplants and the allografted feet appeared normal in 3. Microangiography showed good patency of the femoral arteries and veins (Figure 2). CMAP was record-

able in all animals, as in the FK30 group.

Group IX (FK30-DSG30, n 10)

2 rats died on days 14 and 18. The mean onset of rejection was 76 days, longer than that in the FK30 group ($p < 0.01$). At killing, 7 of the allografted feet had a normal appearance. Microangiography showed good patency of the anastomosed vessels. CMAP was recordable in all animals.

Discussion

15-deoxyspergualin, reported by Umezawa in 1985, has unique immunosuppressive effects and is mainly used as rescue therapy for rejection. As to the mechanism of action of DSG, Suzuki et al. (1989) described the specific clonal deletion of activated lymphocytes at the site of the rejection reaction. Combination with other immunosuppressants had a synergistic effect. Tufveson and Gannedahl (1994) reported that DSG and CS potentiated each other in a monkey heart allograft study. Similar data have been presented by Schubert et al. (1989), Yuh and Morris (1993) and Nemoto et al. (1992). Collier et al. (1988), however, reported no synergistic effect with DSG and CS in a dog kidney allograft study. In contrast, Nakajima et al. (1988) and Carobbi et al. (1993) reported a synergistic effect with FK, resulting in prolonged survival of the grafts in hamster and rat heart xenotransplantations. However, the effectiveness of the combination of FK and DSG is not proven in allotransplantation.

Walter et al. (1989) have published the only experimental study of limb allotransplantation, using DSG. They reported that DSG prolonged survival of the allografted limbs. DSG therapy showed better immunosuppressive effect than CS therapy in the dose we used, but this does not mean that DSG is a better immunosuppressant than CS because the effective doses were different for each drug. Experimental studies of limb allografts, using FK, have been reported by Arai et al. (1989), Kuroki et al. (1991) and Min and Jones (1995). Min made a comparative study between CS and FK and reported that allografted limbs treated with FK survived significantly longer than with CS therapy. Therefore, FK was expected to be a more potent and safer immunosuppressant than CS, which was confirmed by our results.

In our study, combination therapies involving a few immunosuppressive drugs were used in clinical visceral organ allotransplantation. However, this was not done in limb allografts. Combination therapy with FK and DSG, used in this study, showed a synergistic effect in prolonging survival of the limb allografts and we suggest that this drug combination may also be an important clinical application.

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