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ORIGINAL ARTICLE

Prolonged survival of rat whole-limb allografts treated with cyclophosphamide, granulocyte colony-stimulation factor and FK506

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Keywords
chimerism, cyclophosphamide, graft versus host disease, granulocyte colony-stimulation factor, limb graft, polymerase chain reaction.

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Summary

We evaluated the efficacy of a new protocol using cyclophosphamide (CYP), granulocyte colony-stimulation factor (G-CSF) and FK506 to induce high level chimerism following rat whole-limb allotransplantation. The present study investigated the dose requirement and toxicity of CYP monotherapy in inducing stable bone marrow chimerism. Fifty-six whole-limb allotransplants from LacZ transgenic rats to LEW rats were performed. CYP at a dose of 100 mg to 200 mg/kg was injected 2 days before transplantation and G-CSF of 25 μg/kg/day was given for 4 days. FK506 was used for 28 days at 1 mg/kg/day. The level of chimerism was evaluated by semi-quantitative polymerase chain reaction. The survival of limb allografts in recipients treated with CYP of 150 mg/kg was significantly prolonged to 107 days. The onset of rejection was more prolonged to 158 days in recipients with CYP of 200 mg/kg, with two of eight grafts surviving >1 year and three recipients (38%) showed chronic, nonlethal GVHD with a high level of bone marrow chimerism. Limb allografting could contribute to chimerism in the recipient. Pretreatment with CYP had the dose-dependent effects of prolonging the survival of limb allografts. A CYP dose of 200 mg/kg appears to significantly prolong limb graft survival but frequently causes chronic nonlethal GVHD in the longer surviving recipients.

Introduction

The establishment of a high-level of chimerism, or so-called *macrochimerism*, may be the most stable strategy for donor-specific tolerance [1,2]. Pretransplant bone marrow transfusion (BMT) in a rodent model is an effective inducer of macrochimerism and donor-specific tolerance to a variety of allografts such as skin, heart, lung and pancreatic islets. Colson *et al.* [3] first achieved reliable, mixed allogeneic chimerism in a rat cardiac allograft model by transplanting a mixture of T cell-depleted bone marrow cells into pretreated recipients.

Composite tissue allografting (CTA) is very difficult transplantation because CTA consists of various components with strong antigenicity [4]. Hewitt *et al.* [5] first applied pretransplant BMT for rat limb allotransplants and showed that low-level, mixed lymphocyte chimerism was associated with the induction of tolerance after limb transplantation. Foster *et al.* [6,7] first developed reliable tolerance across a strong histocompatibility barrier by inducing a state of mixed chimerism with BMT in a rat limb transplant model. The conclusion from these studies was that development of high-level, mixed chimerism allowed the long-term survival of limb allotransplants similar to that of other visceral organ allotransplants. Conventional protocols for inducing macrochimerism involve a sequential course of pretransplant recipient conditioning, donor bone marrow transfusion, assessment of

the level of chimerism, followed by donor allograft transplantation. The delay period required between the induction of chimerism and transplantation is not likely to be an issue in select cases of living, solid-organ transplantation. However, this delay is not possible for CTA, where for example a hand allograft is always procured from a cadaveric donor and preconditioning of the recipient is therefore impossible.

The present study concerns two issues. The first is whether the whole-limb allograft works as a vascularized bone marrow transplant because the bone marrow is surgically grafted with its microenvironment intact. Our previous study showed donor-origin cells migration into the recipient's lymphoid tissues but the ratio of donor to recipient cells remained unexpectedly low, resulting in *microchimerism* [8, 9]. The second issue concerns preconditioning of the recipient in order to raise the level of chimerism. Foster *et al.* [6]. and other studies [10, 11] used total body irradiation (TBI) to suppress recipient bone marrow cells. In the present study, we used cyclophosphamide (CYP, Shionogi Pharmaceuticals, Japan) to achieve this. To our knowledge, the rodent limb allograft model has not been used to evaluate the toxicity and dose requirements of CYP monotherapy to induce stable bone marrow chimerism across the MHC barrier.

The present study was designed to test a new protocol. Cyclophosphamide was used to precondition the recipient bone marrow, granulocyte colony-stimulation factor (G-CSF; Chugai Pharmaceuticals, Tokyo, Japan) was administered for 4 days after limb allografting to activate donor cell migration, while temporal FK506 (Astellas Pharmaceuticals, Osaka, Japan) was used to immunosuppress the recipient.

Materials and methods

Animal genetics

Hemizygous LacZ transgenic rats of Dark Agouti rat background and weighing 200–220 g were used as donors in this study. These animals were provided by Jichi Medical University and their generation has been described previously by Takahashi *et al.* [12,13]. Adult male Lewis (LEW) rats (genetic expression, $RT1^{l}$) weighing 220–260 g were used as recipients and were obtained from Charles River Breeding Laboratories, Shizuoka, Japan.

Transplantation procedure

The rat hind limb replantation model previously described by our group was used in the present study [14]. Briefly, the donor right hind limb, including bone, muscles, femoral vessels, sciatic nerve and skin was amputated at the mid-femoral level. The skin distal to the knee joint was preserved to monitor circulation and skin rejection. The recipient's ipsilateral hind limb was amputated in a similar manner. Femoral osteosynthesis was performed using an 18-gauge needle as an intermedullary rod. The femoral vessels were anastomosed microsurgically with 10-0 nylon and the sciatic nerve was sutured epineurally with 8-0 nylon. The duration of ischemia averaged 30 min. All rats were housed in cages and allowed normal cage activity without restriction. Special splints were used for all recipients in order to prevent automutilation.

The protocol for this experiment was reviewed by the Ethics Committee for Animal Experimentation at Yamaguchi University School of Medicine and was carried out in accordance with the Guidelines for Animal Experimentation at the Yamaguchi University School of Medicine and Law (No. 105) and also Notification (No. 6) of the Government.

Experimental design (summarized in Table 1)

Fifty-six animals were divided into six groups. Recipients in group I ($n = 6$) were allograft controls (Table 1). Recipients in group II ($n = 5$) were given CYP at a dose of 150 mg/kg 2 days prior to transplantation and FK506 therapy at a dose of 1 mg/kg/day by intramuscular injection for 28 days after transplantation. Group III ($n = 12$) rats were immunosuppressed with FK506. Group IV recipients ($n = 5$) were given CYP at a dose of 100 mg/kg, G-CSF for 4 days after transplantation at a dose of 25 μg/kg/day and FK506 therapy. Rats in group V ($n = 20$) were administered CYP at 150 mg/kg, G-CSF and FK506 therapy. Group VI ($n = 8$) were given CYP at 200 mg/kg, G-CSF and FK506. No recipients were sacrificed during the course of the experiment and the observation period was for up to 1 year after transplant.

Evaluation methods

Clinical evaluation

The general condition, survival of the recipients and operated limbs were checked daily. Peripheral blood leukocyte counts and body weight were measured in some groups III and V recipients at various time points. Radiographs of the operated limbs were obtained at bi-weekly intervals after surgery to evaluate the presence of bony union between graft and recipient femurs. Skin rejection of the grafted limb was diagnosed by the finding of reddish discoloration as reported previously. Statistical analysis was performed by using Statview 5.0 (SAS Institute, Cary, NC, USA). Survival times for recipients and limb grafts between groups were calculated and compared according to the Kaplan–Meier method. Differences were considered to be significant with a P-value of <0.05.

Table 1. Experimental designs and results ($n = 56$).

Group	*n*	CYP* (mg/kg)	G-CSF†	FK506‡	Death within 2 weeks (%)	Limb survival >1 year	Onset of skin rejection (days)	GVHD	Onset of GVHD (days)
I	6	–	–	–	0	0	3,3,4,5,5,5 (4.2)	0	–
II	5	150	–	+	5 (100)	0	–	0	–
III	12	–	+	+	0	0	47,52,53,56,57,67,67, 68,70,71,72,88 (64)	0	–
IV	5	100	+	+	0	0	85,87,91,92,95 (90)	0	–
V	20	150	+	+	5 (25)	1	90,90,99,100,100,101, 135,138 (107)	7	92,95,98,124, 142,164,201
VI	8	200	+	+	3 (38)	2	137,165,171 (158)	3	117,171,191

All transplantations were from LacZ Tg rats to LEW.

*CYP, cyclophosphamide was administered on two days before transplantation.

†G-CSF, granulocyte-colony simulating factor was administered for 4 days from transplantation at a dose of 25 μg/kg/day.

‡FK506 was given at a dose of 1 mg/kg/day by intramuscular injection for 28 days after transplantation.

Histology

Rejection of transplanted limbs was evaluated at the time of necropsy by examining each component tissue, including bone, marrow, muscle and skin. Bone samples were fixed in 4% formaldehyde solution and decalcified in 10% EDTA solution, embedded in paraffin, sectioned at 5 μm thickness and stained with hematoxylin and eosin for routine light microscopy.

Assessment of GVHD

Animals with GVHD were evaluated clinically and histopathologically. Clinical signs of GVHD onset included nonreversible weight loss, diarrhoea and hair loss. At the time of necropsy, sections of skin, liver and small intestine were stained with hematoxylin and eosin and examined for the presence of lymphoid infiltration, subepidermal cleft formation and epidermal necrosis.

Detection of the LacZ-Transgenic Gene by semiquantitative PCR

The LacZ gene in grafted tissues was analyzed semiquantitatively using PCR. The relative amount of LacZ gene was compared with a known autosomal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The ratio of PCR product obtained from the LacZ-specific primer to that from the GAPDH-specific primer (rat GAPDH gene oligonucleotide primers: 5′-GTG GTG CAG GAT GCA TTG CTG A-3′ and 5′-GAT GCT GGT GCT GAG TAT GTC G-3′) was used to determine the relative amount of LacZ-containing cells in the samples. The PCR mixture contained 0.5 μg of genomic DNA, 25 pmol of LacZ-specific primers (LacZ-specific oligonucleotide primers: 5′-TAA TCA ATT ACG GGG TCA TTA GTT CAT AGC-3′ and 5′-TCC CAT AAG GTC ATG TAC TGG GCA TAA TGC -3′), PCR Beads (Ready-To-Go™; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and sterile distilled water in a final volume of 22 μL. PCR was carried out in a programmable thermal cycler (Intermountain scientific corporation, Kaysville, UT, USA) for 35 cycles of denaturation (94 °C for 15 s), annealing (55 °C for 15 s) and extension (72 °C for 15 s). Each PCR product was analyzed by electrophoresis on a 2% agarose gel in parallel with a 50-bp ladder of standard markers (Boehringer Mannheim, Indianapolis, IN, USA). Gels were stained with ethidium bromide and exposed to UV light to visualize the PCR product. The specificity and sensitivity of PCR was evaluated using serial dilutions of LacZ-positive and LEW (LacZ-negative) DNA as templates. DNA from LacZ and LEW animals was mixed in ratios varying from 1:0 to 1:10 000. Each DNA mixture underwent PCR using the same conditions. After 35 PCR cycles, the LacZ band was stronger than GADPH at a 1:1 DNA ratio, identical at 1:10, lower at 1:100 and present only faintly at 1:1,000 dilution. No LacZ band was detected at a 1:10 000 ratio (Fig. 1).

At the time of final evaluation, the bone marrow was taken from of the left femur and tibia and genomic DNAs were prepared by the use of DNAZOL® Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA). The qualification of DNA was assessed spectrophotometrically. Thirty-five PCR cycles were used for DNA samples from the recipient's bone marrow.

Results

Clinical observations

Recipient survival

Group I (allograft control) and group III (treated with FK506) animals showed no serious symptoms and

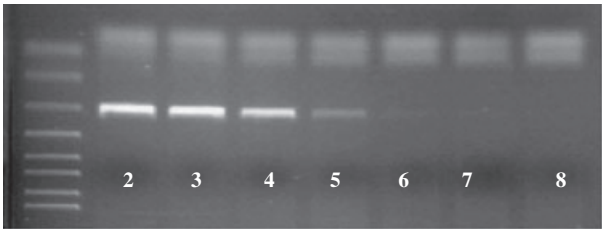

Figure 1 Determination of donor-to-recipient cell ratio by semiquantitative PCR (control experiment). LacZ-positive and LEW (LacZ-negative) DNA were mixed female rat cells in ratio varying from 1:0 to 1:10 000. PCR products following 35 cycles were electrophoresed on 2% agarose gel. These conditions allow detection of male cells in a 1:1000 dilution. Lane 1: PCR marker. Lane 2: LacZ DNA:LEW DNA = 1:0. Lane 3 = 1:10. Lane 4 = $1:10^2$. Lane 5 = $1:10^3$. Lane 6 = $1:10^4$. Lane 7 = $1:10^5$. Lane 8 = 0:1.

all survived for more than a year. Body weight decreased acutely up to 8 days but recovered by 28 days to the weight before operation. Group II (CYP150 mg/kg + FK506) animals suffered severe weight loss and all died within 2 weeks after surgery. Group IV (CYP100/G-CSF/FK506) showed weight loss but all survived after transplantation. Group V (CYP150/G-CSF/FK506) showed severe weight loss and 5 recipients (25%) died within 2 weeks post-transplant. Three of the Group VI (CYP200/G-CSF/FK506) animals (38%) died within 2 weeks of surgery, however, of the remaining five animals, three survived >100 days and two survived >300 days.

WBC count

Four of the Groups III and V animals were analyzed for WBC count. In Group V, the leukocyte count dropped to $<1000/mm^3$ at 1 week after transplant but recovered by 2 weeks. In Group III, leucopenia $<5000/mm^3$ was continuously observed up to 3 months after transplant.

Radiograph

Group III recipients showed sclerotic change (osteonecrosis) in the grafted bone after onset of graft rejection. In the Group VI long-term recipient survivors, solid bony unions between the femur junctions were confirmed by 4 weeks after transplant (Fig. 2).

Onset of skin rejection (summarized Table 1)

Limb allografts in Group I showed skin rejection on average at 4.2 days (3–5) after transplant and were acutely mummified thereafter. In Group III, the onset of skin rejection varied from 47 to 88 days and the mean time was 64 days. In Group IV, the mean onset was 90 days (85–95). In Group V recipients, skin rejection was observed in eight recipients and the mean onset was 107 days (90–138). One recipient (5%) showed no skin rejection after more than one year. In Group VI, three limb allografts showed skin rejection and the mean onset was 158 days (137–171). Two limb allografts (25%) showed no skin rejection after >1 year (Fig. 3). The onset time for skin rejection was significantly prolonged with CYP/G-CSF therapy prior to FK506 administration.

Histological study

In Group I, all components in the limb allograft were rejected simultaneously after the onset of skin rejection. In Groups III–VI, even if the skin component of the limb allograft was rejected, other components such as muscle, bone and small vessels showed no evidence of rejection over a long period. Bone marrow cells in the grafted limb were quite few, showing aplastic marrow whereas the recipient showed hypoplastic marrow (Fig. 4).

Occurrence of GVHD

No recipients in Groups I–IV showed clinical evidence of GVHD. In Group V, the occurrence of chronic GVHD was confirmed in seven recipients with a mean onset time of 131 days (92–201; Table 1) These rats showed severe hair loss but no dermatitis. Five of the animals showed rejection-free limb allografts but two showed signs of skin rejection. Weight loss was not as severe and all rats survived for 1–3 months after the onset of GVHD. Histopathology

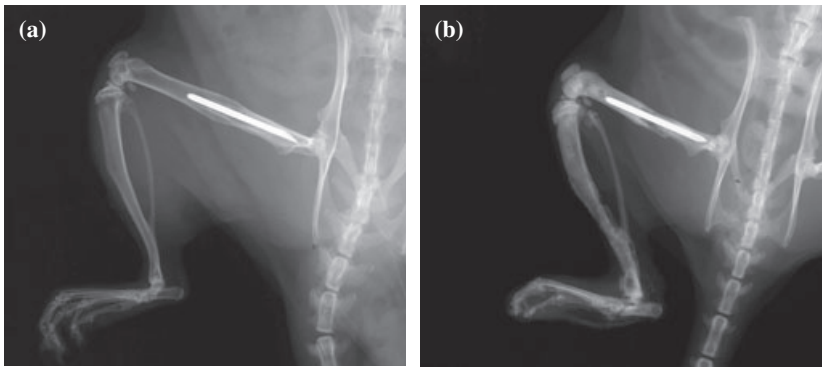

Figure 2 Radiographical evaluation. (a) In long-term recipient survivor in group VI, solid bony unions between the femur junctions were maintained without any abnormal findings. (b) Group III recipients showed sclerotic change (osteonecrosis) in the grafted bone after onset of graft rejection.

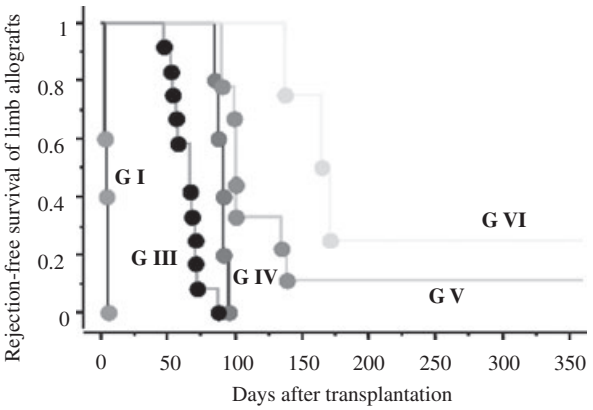

Figure 3 Onset of skin rejection. Limb allografts in group I showed skin rejection on average at 4.2 days. In group III, the mean onset time was 64 days (47–88). In group IV, the mean onset was 90 days (85–95). In group V recipients, skin rejection was observed in eight recipients and the mean onset was 107 days (90–138). One recipient (5%) showed no skin rejection after >1 year. In group VI, three limb allografts showed skin rejection and the mean onset was 158 days (137–171). Two limb allografts (25%) showed no skin rejection after >1 year. The onset time for skin rejection was significantly prolonged with CYP/G-CSF therapy prior to FK506 administration.

showed mild infiltration of inflammatory cells in the skin, small intestine and lung tissues. Three recipients in Group VI showed clinical signs of chronic GVHD and the mean onset for this was 160 days (117–191; Table 1). Two recipients survived >1 year without any rejection of the grafted limbs. At the final examination of 14 months after transplant, histopathology showed no inflammatory cell infiltration in the skin, liver and small intestine (Fig. 5).

Detection of donor-derived LacZ genes in recipient bone marrow using PCR

Bone marrow chimerism was confirmed in 5, 5, 10 and 3 recipients of Groups III, IV, V and VI, respectively, using the LacZ-specific PCR technique. Bone marrow chimerism was not found in Groups III and IV recipients with rejected limbs. In Group V, three recipients showed a high level of chimerism (10% level), whereas the seven recipients with rejected limbs showed no chimerism. A high level (10%) of bone marrow chimerism was found in the three Group VI recipients with GVHD. The level of bone marrow chimerism correlated with the occurrence of GVHD.

Discussion

Pretreatment protocol with CYP, G-CSF and FK506 in CTA

The present study is the first to demonstrate the induction of fully allogeneic bone marrow chimerism in whole-limb

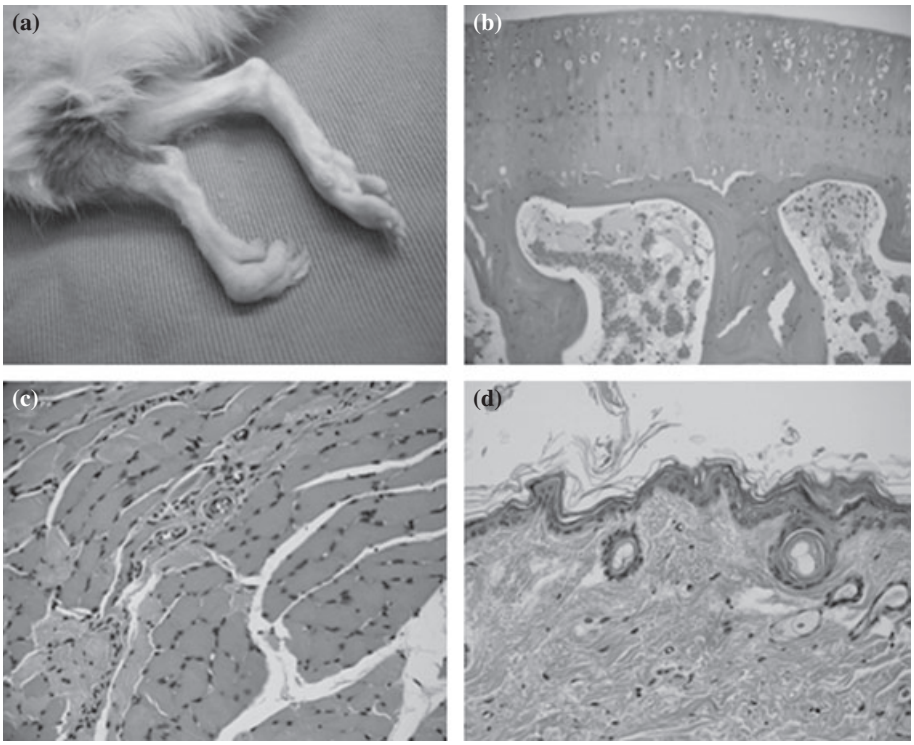

Figure 4 Long-term survived recipient in groups VI at 14 months after transplant (a). The limb allograft showed no evidence of rejection in components of bone (b), muscle (c), and skin (d). Bone marrow in the grafted limb were quite few, showing aplastic marrow.

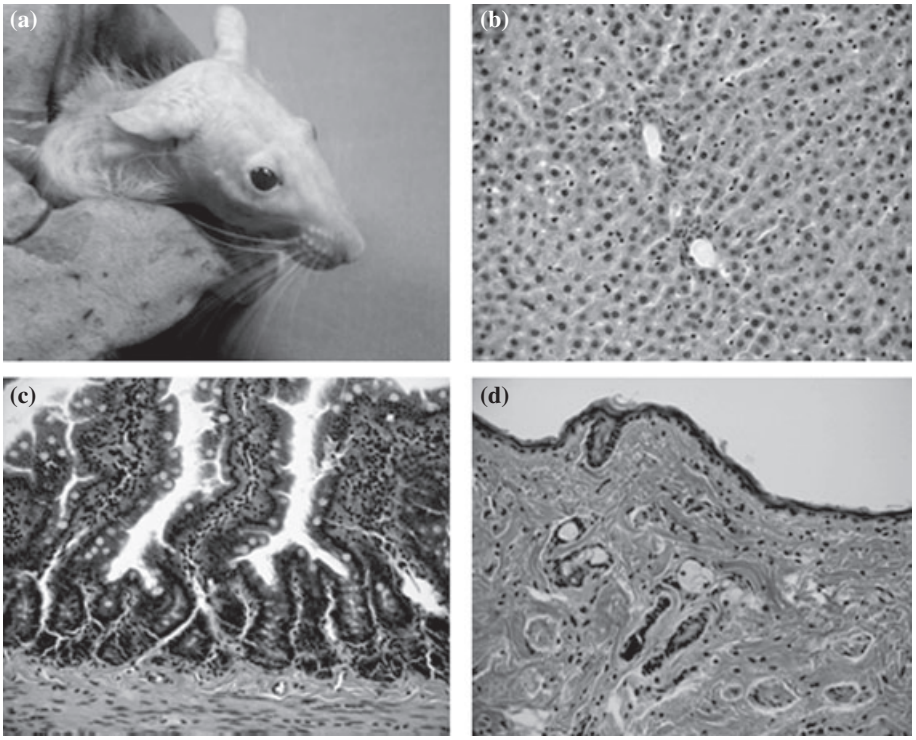

Figure 5 Group VI recipient in showed clinical signs of chronic GVHD with severe hair loss (a). Histopathology showed no inflammatory cell infiltration in the liver (b), small intestine (c) and skin (d).

allografting using a simple pretreatment protocol involving CYP, G-CSF and FK506 combination therapy.

Tomita *et al.* [15] reported the induction of skin allograft tolerance by using CYP and BMT in a murine model. The same group, Zhang *et al.* [16], also reported the induction of heart allograft tolerance in the same experimental model. Several mechanisms for the efficacy of CYP in inducing allograft acceptance have been considered, with perhaps the most important being the specific effect of CYP on proliferating T cells. Mature T or B cells reactive against allo-antigen cause clonal expansion after the transplant of allogeneic cells and CYP may selectively destroy these allo-stimulated mature reactive T cells. Proliferating cells are especially sensitive to CYP and thus the clones are selectively destroyed with this agent [17].

Dosage and timing of CYP administration and G-CSF

The dose and timing of CYP injection appear to be critical for limb graft acceptance. Okayama *et al.* [18] evaluated the required dosage of CYP required to induce macrochimerism prior to BMT. They used a single-dose of CYP ranging from 50 to 200 mg/kg and found that pretreatment with 200 mg/kg induced macrochimerism but caused lethal GVHD. A 150 mg/kg dose of CYP appears to be optimal for the induction of tolerance without GVHD, whereas lower dosages cannot induce high enough levels of chimerism for graft acceptance. Iwai *et al.* [19] performed a similar study with mouse skin allografts and found that pretreatment with a single dose of 200 mg/kg CYP induced a significantly higher degree of chimerism compared to a 100 mg/kg dose. In our study, 100 mg/kg of CYP were not enough to induce stable bone marrow chimerism. While recipients treated with 150 mg/kg of CYP showed prolonged survival of limb allografts and high levels of chimerism, only one of 20 recipients (5%) showed long-term acceptance of the graft. Two of eight recipients (25%) treated with 200 mg/kg of CYP showed long-term acceptance of limb allografts, however, toxicity was more severe than with 150 mg/kg treatment. Hence, the current findings demonstrate that CYP has dose-dependent effects on the survival of limb allografts and on the induction of chimerism.

Mayumi *et al.* [17] reported that CYP injection on day 2 after the infusion of donor-derived bone marrow and splenocytes induced the acceptance of allogeneic skin grafts in a mouse model, whereas CYP injection before BMT failed to induce skin graft acceptance. In contrast, Okayama *et al.* [18] studied the timing of CYP administration in a rat heart transplant model and found that

injection one day before BMT was the most effective for induction of allogeneic macrochimerism. We cannot assess which protocol is superior, however, CYP injection followed by the BMT protocol worked in the present limb allograft study.

Recent studies have demonstrated the development of chimerism following limb transplant. Ajiki *et al.* [20] showed the proportion of donor cells in recipient bone marrow was about 1% at 48 weeks post-transplantation [20]. A previous our study demonstrated similar results, with the level of bone marrow chimerism found to be about 1% at 24 weeks and 10% at 48 weeks. However, Mathes *et al.* [21] using a miniature swine model and flow cytometry found no evidence of donor cell engraftment in the recipient animal. Similarly, Granger *et al.* [22] reported that donor cell microchimerism in clinical hand transplant patients was barely detectable in some of the early post-transplantation specimens and undetectable thereafter. These findings indicate the level of chimerism following limb allotransplantation is unexpectedly low, resulting in *microchimerism*.

Okabe *et al.* [23] investigated the effect of G-CSF on mice pretreated with CYP. An acute, CYP-induced drop in neutrophil count was successfully reversed by G-CSF administration at a dose of 25 μg/kg/day for 4 days. In the present study, we used G-CSF at 25 μg/kg/day for 4 days to stimulate donor cell migration into the recipient and thus demonstrate that the level of bone marrow chimerism could be raised by G-CSF therapy. From the histopathological results of bone marrow, the majority of donor marrow cells migrated into recipient marrow space and lymphoid tissues, resulting aplastic marrow.

Occurrence of GVHD

Although increased chimerism and significantly prolonged limb allograft survival could be induced with CYP and G-CSF treatments, recipients frequently showed chronic nonlethal GVHD. Ramsamooj *et al.* [24] transplanted limb allografts from Lewis to Lewis x Brown Norway F1 rats and reported that seven of 19 recipients (38%) with rejection-free limb allografts showed lethal acute or chronic GVHD. To prevent GVHD in chimeric hosts, Gorantla *et al.* [11] transplanted irradiated limb allografts into BMT pretreated recipients. All limb allografts survived up to 5 months without clinical signs of GVHD. The same group, Prabhume *et al.* [10], then transplanted irradiated limb allografts and BMT simultaneously in order to raise the level of chimerism and prevent GVHD. Following 28 days immunosuppression with FK506 and mycophenolate mofetil, limb allografts survived without rejection and without GVHD in the recipient.

To prevent GVHD after limb allografting, it may be necessary to regulate the chimeric cell. Lethal GVHD after BMT has not been experienced until now because T cells were depleted *in vitro* and the bone marrow cell count was adjusted to 1×10^7–1×10^8 [6,7,10,11]. Following limb allograft or vascularized bone marrow allograft, all donor cell types including hematopoietic stem cells and stromal cells can migrate into the recipient. We have no data on the total number of migrating bone marrow cells into the limb graft. CYP/G-CSF/FK506 combination therapy resulted in prolonged survival of limb allografts but more studies aimed at controlling chronic GVHD are necessary.

Conclusion

The present study showed that pretreatment with appropriate doses of chemotherapeutic drugs can be used to induce macrochimerism for the acceptance of donor-specific limb transplants. The dose-dependent effects of CYP on the induction of chimerism and on the development of chronic GVHD provide useful information for determining the optimal dose of this drug.

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