Section 2-c

Induction of High-Level Chimerism in Composite Tissue Transplants

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Introduction

Although the prolonged survival of experimental composite tissue allografts (CTAs) is achievable using immunosuppressive drugs, long-term immunosuppression of CTAs is not acceptable in the clinical setting because of serious side-effects. The development of a model for reliable CTA tolerance induction across a major histocompatibility complex (MHC) mismatch and without the need for long-term immunosuppression is highly desirable.

Chimerism is the term used to describe cell traffic from the graft to the recipient and is frequently detected following successful organ transplantation in animal models. Starzl et al. [1] were the first to demonstrate that following successful liver transplantation, small numbers (less than 1%) of donor dendritic cells migrate into the recipient's lymphoid organs, including thymus, bone marrow, spleen and lymph nodes. They referred to this phenomenon as *microchimerism*. It is still controversial as to whether microchimerism can induce immunotolerance or whether it is merely a result of successful organ transplantation [2].

At present, the establishment of a high level (>10%) of chimerism may be the most stable strategy for donor-specific tolerance. This is called *macrochimerism* [2]. Pretransplant bone marrow transfusion (BMT) is a useful tool for induction of a state with mixed chimerism and immunotolerance. BMT-induced macrochimerism is an effective inducer of donor-specific tolerance to a variety of allografts, such as skin, heart, lung and pancreatic islets in a rodent model. Conventional experimental protocols for inducing macrochimerism involve a sequential course of pretransplant recipient conditioning, donor bone marrow transfusion, control of chimerism level by flow cytometry or molecular techniques followed by donor allograft transplantation. The delay period required between induction of chimerism and transplantation might not be as important in select cases of living, solid-organ transplantation. However, this delay is not possible in the case in CTA where, for example, a hand allograft is always procured from a cadaveric donor, and preconditioning of the recipient is therefore impossible.

Since whole-limb allograft contains bone marrow, it represents a vascularised bone marrow transplant where the bone marrow is surgically grafted with its microenvironment intact and where the donor bone marrow cells and stroma are able to function immediately upon transfer. This composite/vascularised bone marrow transplant model is likely to be a better source for bone marrow reconstitution than transplantation of cellular bone marrow cells. Our previous study in a rat limb allograft model showed that donor-origin cells migrated into the recipient's lymphoid tissues, but the ratio of donor and recipient cells remained unexpectedly low, resulting in microchimerism [3–5]. To raise the level of chimerism following limb transplants, novel methods of stimulating donor-cell migration into the recipient body are

required. Our issue concerns preconditioning of the recipient in order to raise the level of chimerism. Foster et al. and other studies used total body irradiation (TBI) to suppress recipient bone marrow cells [6, 7]. In the present study, we used the common chemotherapeutic drug 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.

In this chapter, we introduce a new protocol for induction of a high level of chimerism following rat whole-limb allotransplantation. CYP was used to precondition recipient bone marrow, granulocyte colony-stimulation factor (G-CSF) (Chugai Pharma, Shionogi Pharmaceutical, Osaka, 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.

Limb Transplantation to Preconditioned Recipient Rats

Animal Genetics

Hemizygous LacZ transgenic rats of Dark Agouti rat background were used as donors. These rats were provided by Jichi Medical University (Tochigi, Japan), and their generation has been described previously by Takahashi et al. [8, 9]. Adult male Lewis (LEW) rats (genetic expression, $RT1^{l}$) were used as recipients.

Transplantation Procedure

The rat hind-limb replantation model previously described by our group was used [10]. 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. The duration of ischaemia averaged 30 min. All rats were housed in cages and allowed normal cage activity without restriction. Special splints were used for all the recipients in order to prevent automutilation.

Experimental Design

Seventy-one animals were divided into 7 groups. Recipients in group I (n=6) were allograft controls. 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 per day by intramuscular injection for 28 days after transplantation. Group III (n=12) were given CYP 2 days before transplantation and G-CSF for 4 days after transplantation at a dose of 25 μg/kg per day. Group IV recipients (n=12) were given G-CSF for 4 days after transplantation followed FK506 therapy for 28 days after transplant. Rats in group V (n=5) were administered CYP at a dose of 100 mg/kg, G-CSF in the same manner as group III and FK506 therapy for 28 days with the same dosage as group II. Rats in group VI (n=20) were administered CYP at 150 mg/kg, G-CSF and FK506 therapy for 28 days. Group VII (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 300 days after transplant (Table 1).

Evaluation Methods

Clinical Evaluation

General condition, survival and weight of the recipients and operated limbs were checked daily. Peripheral blood leukocyte counts and body weight were measured in some groups IV and VI recipients at various time points. Radiographs of the operated limbs were obtained at biweekly intervals

Table 1. Experimental designs and results (n=71)

Group	n	CYP* (mg/kg)	G-CSF**	FK506 ***	Death within 2 wks (%)	Limb survival >1 yr	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	15	150	+	-	10(67)	0	3,3,3,3,4,5,7,7,9,10 (5.4)	5	4,4,9,14,14
IV	12	-	+	+	0	0	47,52,53,56,57,67,67,68,70,71,72,88 (64)	0	-
V	5	100	+	+	0	0	85,87,91,92,95 (90)	0	-
VI	20	150	+	+	5(25)	1	90,90,99,100,100,101,135,138 (107)	7	92,95,98,124,142,164,201
VII	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 four days from transplantation at a dose of 25μg/kg/day. *** FK506 was given at a dose of 1 mg per kilogram per day by intramuscular injection for 28 days after transplantation

after surgery to evaluate presence of bony union between the graft and recipient femurs. Skin rejection of the grafted limb was diagnosed by the finding of reddish discoloration, as reported previously [10]. Survival times for recipients and limb grafts were compared according to the Kaplan-Meier method.

Histology

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

Assessment of Graft-Versus-Host Disease

Animals with graft-versus-host disease (GVHD) were evaluated clinically and histopathologically. Clinical signs of GVHD onset included nonreversible weight loss, diarrhea, unkempt appearance, hair loss and rash on the paws, snout and ears. At the time of necropsy, sections of skin, liver and small intestine were stained with H&E and examined for the presence of lymphoid infiltration, subepidermal cleft formation and epidermal necrosis.

Detection of the LacZ Transgenic Gene by Semiquantitative Polymerase Chain Reaction

The LacZ gene (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in grafted tissues was analysed semiquantitatively using polymerase chain reaction (PCR). The relative amount of GFP 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 was used to determine the relative amount of LacZ-containing cells in the samples. The PCR reaction mixture contained 0.5 mg of genomic DNA, 25 pmol of LacZ-specific primers, PCR beads

(Ready-To-Go, Amersham Pharmacia Biotech, USA) and sterile distilled water in a final volume of 22 µl. PCR was carried out in a programmable thermal cycler (Intermountain Scientific Corporation, UT, USA) for 35 cycles of denaturation (94∞C for 15 s), annealing (55∞C for 15 s) and extension (72∞C for 15 by electrophoresis on a 2% agarose gel in parallel with a 50 base pair ladder of standard markers (Boehringer Mannheim, USA). Gels were stained with ethidium bromide and exposed to ultraviolet (UV) light to visualise the PCR product.

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 GAPDH 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.

Long-Term Acceptance of Allogeneic Limb Allografts

Clinical Observations

1. Recipient survival: The animals in group I (allograft control) and group IV (treated with FK506) showed no serious symptoms, and all except one survived more than 300 days. Body weight was lost acutely (-43 g) at 8 days and recovered after 28 days to pretransplantation weight. Group II (CYP/FK506) suffered severe weight loss, and all animals died within 2 weeks posttransplantation. Group III (CYP/G-CSF) also showed severe weight loss, and 10 recipients (67%) died within 2 weeks posttransplant. Group V (CYP100/G-CSF/FK506) showed weight loss, but all survived after transplantation. Group VI (CYP150/G-CSF/FK506) showed severe weight loss, and 5 recipients (25%) died within 2 weeks posttransplantation. Three group VII (CYP200/G-CSF/FK506) animals (38%) died within 2 weeks posttransplantation; however, of the remaining 5 animals, 3 survived more than 100 days and 2 more than 300 days.
2. White blood cell (WBC) count: Four groups IV and VI animals were analysed for WBC count. In group VI, the leukocyte count dropped to <1,000/mm^3 at 1 week posttransplantation, and leucopenia less than 5,000/mm^3 was continuously observed up to 3 months posttransplantation. In group IV, leucopenia was not observed up to 10 weeks posttransplantation.
3. Radiography: In groups VI and VII, long-term recipient survivors, solid bony unions between the femur junctions were confirmed by 4 weeks posttransplantation. In group I, no recipient achieved bony union.
4. Onset of skin rejection: Limb allografts in group I showed skin rejection on average at 4.2 (range, 3–5) days posttransplantation and were acutely mummified thereafter. In group III, onset of skin rejection varied from 47 to 88 (mean 64) days. Skin rejection was confirmed in 10 group IV animals, and the mean onset time was 5.4 days. In group V, mean onset was 90 (range, 85–95) days. In group VI recipients, skin rejection was observed in 8, and the mean onset was 107 (range, 90–138) days. One recipient (5%) showed no skin rejection after more than 1 year. In group VII, three limb allografts showed skin rejection, and the mean onset was 158 (range, 137–171) days. Two limb allografts (25%) showed no skin rejection after more than 1 year (Fig. 1). 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 onset of skin rejection. In groups VI and VII, 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 few, showing aplastic, marrow whereas the recipient showed hypoplastic marrow.

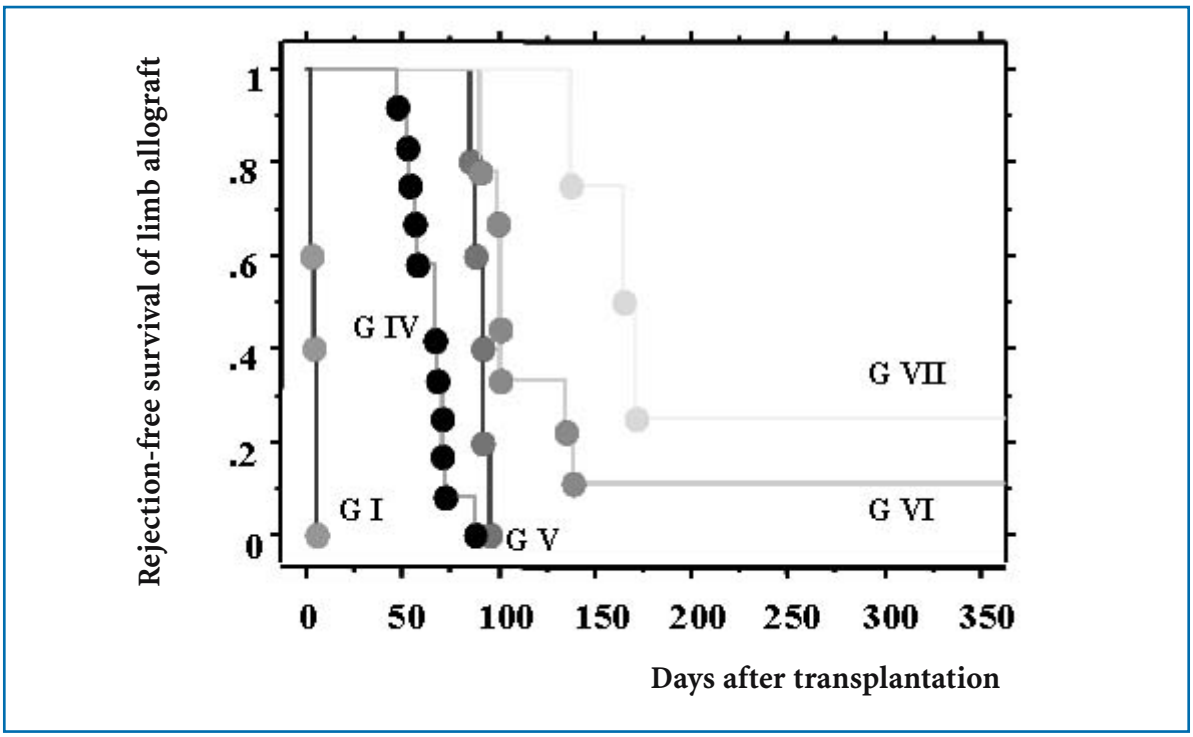

Fig. 1. Onset of skin rejection. Limb allografts in group I showed skin rejection on average at 4.2 days. In group IV, mean onset was 64 (47–88) days. In group V, mean onset was 90 (85–95) days. In group VI, skin rejection was observed in 8 recipients, and mean onset was 107 (90–138) days; one recipient (5%) showed no skin rejection after more than 1 year. In group VII, three showed skin rejection, and mean onset was 158 (137–171) days; two (25%) showed no skin rejection after more than 1 year. Onset time for skin rejection was significantly prolonged with cyclophosphamide/granulocyte colony-stimulating factor (CYP/G-CSF) therapy prior to FK506 administration

Occurrence of GVHD

No recipients in groups I, II, IV and V showed clinical evidence of GVHD. Five recipients in group III (33%) experienced acute lethal GVHD with dermatitis of the ear and paw and mean onset time of 9 days after transplant (Fig. 2a). Severe infiltration of the inflammatory cells was observed histopathologically in skin specimens of skin, small intestine and lung. All animals died within 1 week after the onset of GVHD. In group VI, occurrence of chronic GVHD was confirmed in 7 recipients, with a mean onset time of 131 (range 92–201) days. These rats showed severe hair loss but no dermatitis. Five 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 onset of GVHD. Histopathology showed mild infiltration of inflammatory cells in the skin (Fig. 2c), small intestine (Fig. 2d) and lung tissues. Three recipients in group VII showed clinical signs of chronic GVHD (Fig. 3a), and the mean onset for this was 160 (range 117–191). Two recipients survived more than 1 year without rejection of the grafted limbs. At the final examination of 14 months posttransplantation, histopathology showed no inflammatory cell infiltration in the skin (Fig. 3c), liver or small intestine (Fig. 3d).

Detection of Donor-Derived LacZ Genes in Recipient Bone Marrow Using PCR

Bone marrow chimerism was confirmed using the LacZ-specific PCR technique. In group III, 2 recipients showed a high level of chimerism (10%) (Fig. 2b), 2 showed moderate levels (1%) and 3 showed none. In the 3 recipients showing GVHD, 2 revealed 10% chimerism and the other 1% chimerism. The level of bone marrow chimerism correlated with the occurrence of

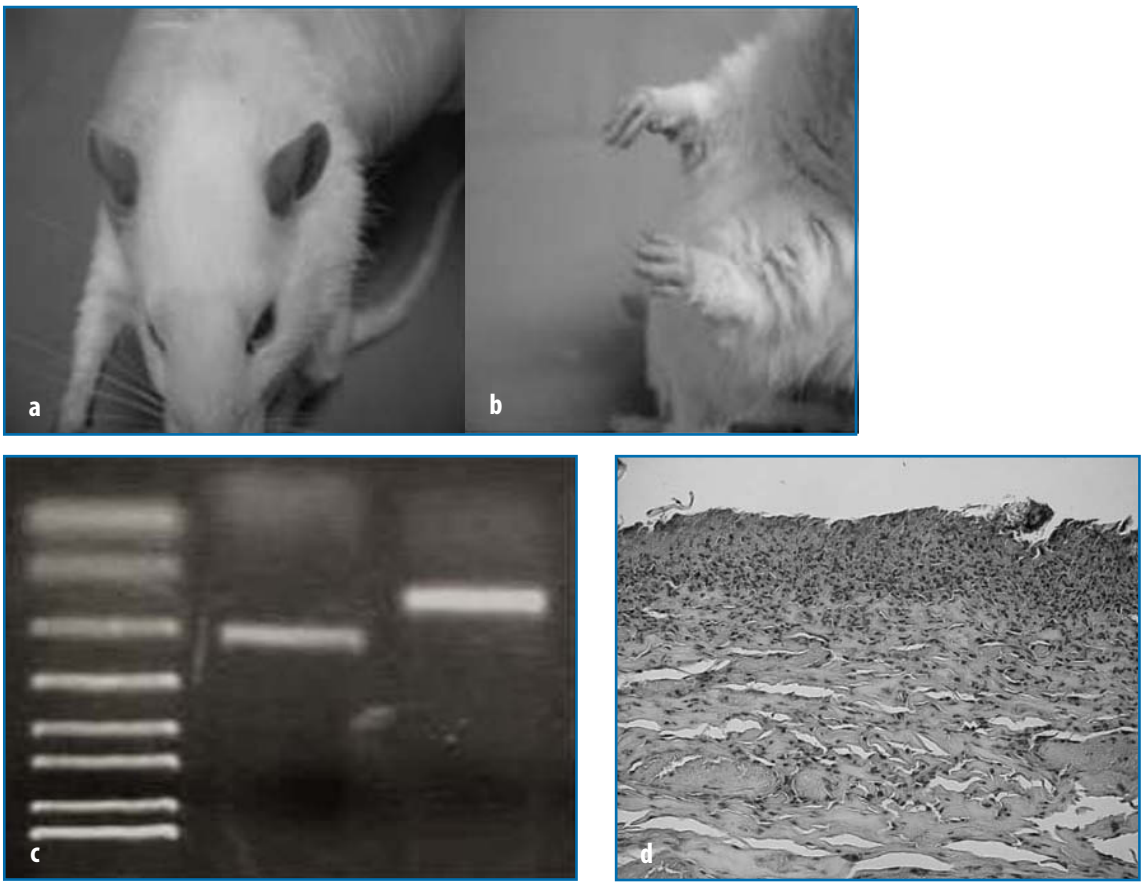

Fig. 2a-d. a Five recipients (25%) in group III [cyclophosphamide/granulocyte colony-stimulating factor (CYP/G-CSF)] experienced acute lethal graft-versus-host disease (GVHD) with dermatitis of the ear and paw and a mean onset of 9 days posttransplantation. **b** Bone marrow chimerism detected by polymerase chain reaction (PCR). *Lane 2*: LacZ band showing high level of chimerism (10%). *Lane 3*: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the same sample. **c, d** Histopathology of acute GVHD. Severe infiltration of inflammatory cells was observed in skin specimens (**c**) and small intestine (**d**)

acute GVHD. Bone marrow chimerism was not found in groups IV and V recipients with rejected limbs. In group VI, 3 recipients showed a high level of chimerism (10% level) whereas the 7 recipients with rejected limbs showed none. A high level (10%) of bone marrow chimerism was found in the 3 group VII recipients with chronic GVHD (Table 2; Fig. 3b).

Pretransplant Bone Marrow Transfusion for Composite Tissue Allografts

The ultimate goal of organ transplantation is to establish a nontoxic, easily applied regimen for induction of donor-specific tolerance so that grafts can be accepted across MHC barriers without the requirement for chronic immuno-

Table 2. Occurrence of GVHD and chimerism

Group	n	CYP (mg/kg)	FK506 (days)	Limb rejection	GVHD	Chimerism (level)
III		150	-		3 (acute)	2(10%) + 2(1%)
IV	5	-	28	5	none	none
V	5	100	28	5	none	none
VI	10	150	28	7	3 (chronic)	3 (10%)
VII	3	200	28	none	3 (chronic)	3 (10%)

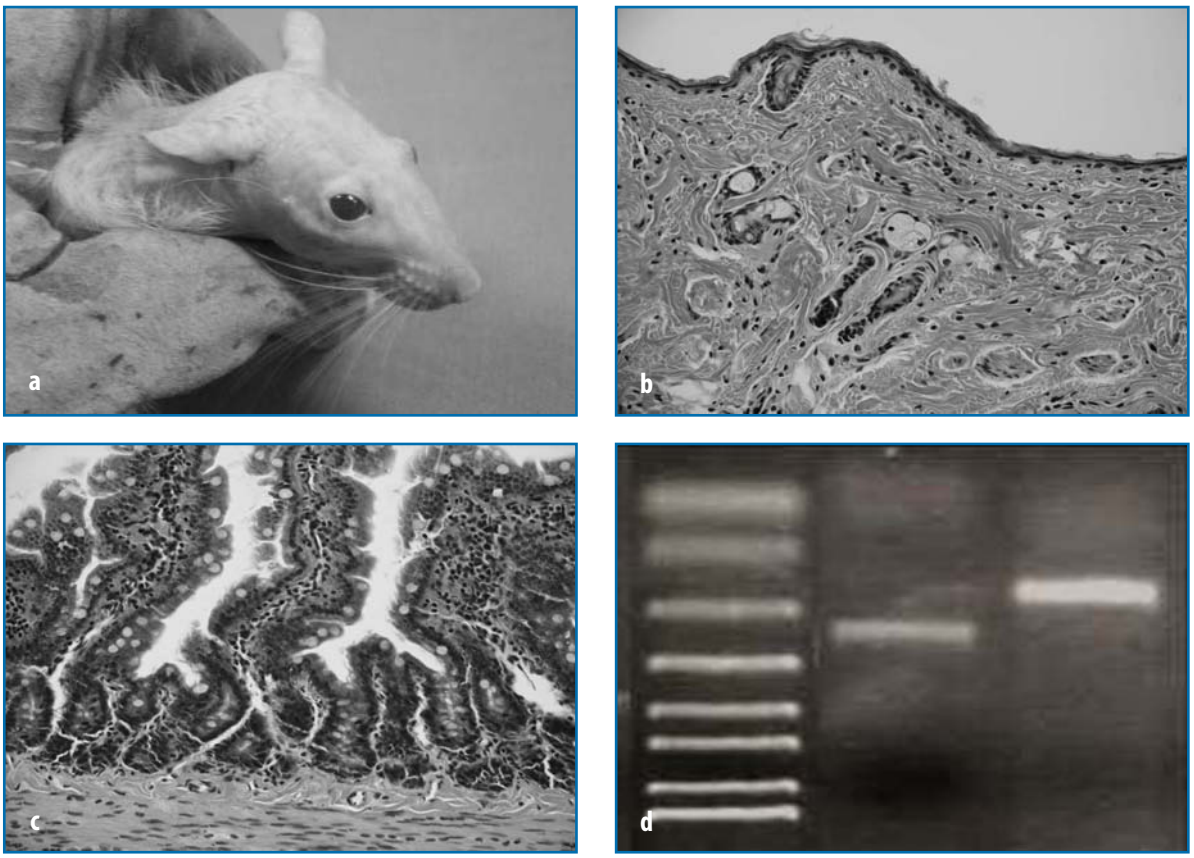

Fig. 3a-d. a Group VII recipient [cyclophosphamide/granulocyte colony-stimulating factor (CYP/G-CSF)/FK506] showing clinical signs of chronic graft-versus-host disease (GVHD) with severe hair loss. **b** Bone marrow chimerism detected by polymerase chain reaction (PCR). *Lane 2*: LacZ band showing high level of chimerism (10%). *Lane 3*: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the same sample. **c, d** Histopathology showed no inflammatory cell infiltration in the skin (**c**) and small intestine (**d**)

suppressive therapy. Macrochimerism induced by BMT is one of the strategies used to induce a state of immunotolerance. BMT patients who subsequently require a kidney transplant become tolerant to the transplanted kidney and do not require long-term immunosuppression if the kidney is transplanted from the same donor. Therefore, surgeons have attempted to establish macrochimerism by BMT after pretreatment of the recipient lymphoid system. Colson et al. first achieved reliable mixed allogeneic chimerism by transplanting a mixture of T-cell-depleted bone marrow cells into pretreated recipients in a rat cardiac allograft model [11]. Chimerism ranging between 12% and 93% was achieved in 91% of cases across several strongly antigenic, MHC-disparate strain combinations.

Hewitt et al. first applied BMT for limb allotransplant [12]. Their study showed that low-level (18 ± 3%) mixed lymphocyte chimerism was associated with induction of tolerance after limb transplantation but that a high-level (60 ± 14%) of chimerism was associated with development of GVHD. Foster et al. developed reliable tolerance across a strong histocompatibility barrier by inducing a state of mixed chimerism with BMT in a rat limb transplant model [6, 7]. There were no signs of rejection of the limb allografts for more than 100 days in the 6 out of 10 animals with a donor chimerism level greater than 60%. All 3 animals with chimerism less than 20% rejected the limb transplant. Interestingly, Esumi et al. demonstrated successful allogeneic rat limb allotransplantation using combined pretreatments of fludarabine injection, low-dose irradiation, and BMT directly into the bone marrow cavity [13]. These limb allografts survived more than 1 year without signs of rejection. The conclusion from these studies was that development of high-level mixed chimerism allowed long-

term survival of a limb allotransplant similar to that of other visceral organ allotransplants.

Raising the Level of Chimerism Following CTAs Using Pretreatment with CYP, G-CSF and FK506

Recent studies have demonstrated donor-cell migration following limb transplant. Ajiki et al. developed a green fluorescent protein transgenic rat for marking the donor cells and showed that the ratio of donor cells in recipient bone marrow was about 1% at 48 weeks posttransplantation [14]. Our previous study demonstrated similar results, and the level of bone marrow chimerism was 1% at 24 weeks and 10% at 48 weeks [3]. Mathes et al., using a miniature swine model and flow cytometry, found no evidence of donor-cell engraftment in a recipient animal [15]. Similarly, Granger et al. reported that donor-cell microchimerism in clinical hand transplant patients was barely detectable in some early posttransplantation specimens but was undetectable thereafter [16]. These results indicated the level of chimerism following limb allotransplantation was unexpectedly low, resulting in microchimerism.

To our knowledge, there have so far been no studies attempting to raise the level of chimerism following limb allograft/vascularised bone marrow transplant. The present study is the first to demonstrate the induction of fully allogeneic bone marrow chimerism in whole-limb allografting using a simple pretreatment protocol involving CYP, G-CSF and FK506 combination therapy. Destruction of recipient bone marrow cells by CYP is advantageous in that it creates a new space for the survival of donor-marrow cells. While TBI-induced chimerism and tolerance is stable, toxicity for recipients of the preconditioning regimen appears too severe to allow clinical application of this strategy. Considerable efforts have therefore been undertaken to reduce the requirement for irradiation. Chemotherapeutic drugs have been evaluated as possible substitutes for TBI, and in the present study, we focused on CYP.

Tomita et al. reported induction of skin allograft tolerance by using CYP and BMT in a murine model [17]. The same group, Zhang et al., also reported induction of heart allograft tolerance in the same experimental model [18]. 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 alloantigen 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 [19, 20].

Dosage and Timing of CYP administration and G-CSF

Dose and timing of CYP injection appear to be critical for limb graft acceptance. Establishment of stable macrochimerism and allogeneic limb graft survival are likely to depend on the CYP dose. Okayama et al. evaluated the dosage of CYP required to induce macrochimerism prior to BMT [21]. 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 induction of tolerance without GVHD whereas lower dosages cannot induce high enough levels of chimerism for graft acceptance. Iwai et al. 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 with a 100 mg/kg dose [20]. 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 1 of 20 recipients (5%) showed long-term graft acceptance. Two of 8 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 survival

of limb allografts and on induction of chimerism.

Mayumi et al. 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 [19]. In contrast, Okayama et al. studied the timing of CYP administration in a rat heart transplant model and found that injection 1 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.

Okabe et al. investigated the effect of G-CSF on mice pretreated with CYP [22]. An acute CYP-induced drop in neutrophil count was successfully reversed by G-CSF administration at a dose of 25 μg/kg per day for 4 days. To our knowledge, there have so far been no studies that have attempted to raise the level of chimerism following limb allograft or vascularised bone marrow transplant. In the present study, we used G-CSF at 25 μg/kg per 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 in 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. Other recent studies have also demonstrated GVHD following limb allografting. Ramsamooj et al. transplanted limb allografts from Lewis to Lewis x Brown Norway F1 rats and reported that 7 of 19 recipients (38%) with rejection-free limb allografts showed lethal acute or chronic GVHD [23]. To prevent GVHD in chimeric hosts, Gorantla et al. transplanted irradiated limb allografts into BMT pretreated recipients [24]. All limb allografts survived up to 5 months without clinical signs of GVHD. The same group, Prabhume et al., then transplanted irradiated limb allografts and BMT simultaneously in order to raise the level of chimerism and prevent GVHD [25]. Following 28 days immunosuppression with FK506 and mycophenolate mofetil (MMF), 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 [24]. 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. Following limb allograft or vascularised bone marrow allograft, all donor-cell types, including haematopoietic 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

Limb allografting could function as a vascularised carrier for bone marrow transplantation and contribute to a high level of chimerism in the recipient. Pretransplant CYP followed by G-CSF and FK506 treatment significantly prolonged survival of limb allografts but frequently caused chronic nonlethal GVHD in recipients. Pretreatment with CYP had dose-dependent effects, and a dose of 200 mg/kg appeared to significantly prolong limb-graft survival.

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