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Establishment of chimerism in donor liver with recipient-type bone marrow cells prior to liver transplantation produces marked suppression of allograft rejection in rats

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Abstract In this study, we investigated whether establishment of chimerism in donor liver with recipient-type bone marrow cells (BMCs) prior to liver transplantation could prolong the liver allograft survival. Donor female ACI rats were inoculated with recipient-type BMCs of male LEW rats via the portal vein, with or without irradiation as cytoablation, followed by intramuscular administration of FK506 for 5 days. At 1–2 months later, livers were harvested and transplanted into naive female LEW rats. No immunosuppressants were used. Chimerism in donor rats was confirmed by primers specific for the sex determinant Y chromosome of rats. With livers from rats pretreated with recipient-type BMCs, survival of liver allografts was significantly extended, irrespective of irradiation. These results showed that modification of the donor liver by intraportal injection of recipient-type BMCs and concomitant administration of FK506 prior to liver transplantation prolonged liver allograft survival in rats.

Key words Liver transplantation · Chimerism · Bone marrow transplantation · Rat · FK506

Introduction

The availability of non-specific immunosuppressive agents has allowed liver transplantation to become an established treatment of end-stage liver disease. However, allograft rejection is still a limiting factor to sucessful liver transplantation and continuous requirement for non-specific immunosuppression is responsible for an increased incidence of infections, malignancies, and drug toxicity. The induction of donor-specific unresponsiveness without systemic or chronic immunosuppression is an elusive goal in organ transplantation. Suppression of allograft rejection by immunological modification of the donor graft is one possibility, and may allow a reduction of the total dose of immunosuppressants in the recipient, thus minimizing the attendant risks.

It is generally agreed that liver is a more tolerant organ with regard to graft rejection than other organs such as the heart, kidneys, and pancreas [1, 2]. Among the numerous mechanism advanced to explain this privileged status of liver allografts, replacement of donor Kupffer cells by host cells after liver transplantation has been cited both in animal models [3–5] and clinical practice [6]. The immunogenicity of liver allografts depends on the dendritic cells (DCs) recruited from bone marrow-derived immature cells. Therefore, it is anticipated that replacement of donor liver DCs with recipient-type cells before liver transplantation may lead to decreased immunogenicity and reduced rejection following liver transplantation.

In this study, we investigated whether replacement of donor liver DCs by recipient-type cells, by inducing hematopoietic chimerism in the donor with recipient-type bone marrow cell (BMC) Cs transplantation (BMTx) before orthotopic liver transplantation (OLT), could prolong liver graft survival in rats.

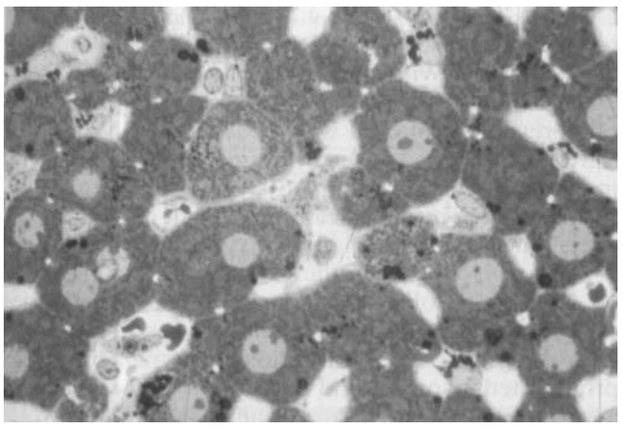

Fig. 1 Histological findings of donor liver 2 weeks after bone marrow cell transplantation (BMTx). Livers from rats killed 2 weeks after BMTx revealed a histologically normal cytoarchitecture in the lobular and portal regions (toluidine blue, × 1000)

Materials and methods

Animals

Inbred female and male LEW ($RT1^l$), female ACI ($RT1^a$), and male BN ($RT1^n$) rats weighing 180–250 g were purchased from Charles River Japan (Atsugi, Kanagawa, Japan).

Female LEW rats were used as recipients and female ACI rats were used as donors for OLT. Male LEW rats served as donors for BMTx to ACI rats before OLT. Male BN rats served as the source of BMCs as a third party.

Using this different sex combination, subsequent PCR analysis with primers specific for the sex determinant Y chromosome to confirm chimerism in donor rats after BMTx is possible.

The rats were fed with solid rat food. Tap water was given to the rats between the following procedures.

Bone marrow transplantation

Bone marrow was flushed from the tibia and femur of male LEW rats, washed, resuspended in phosphate-buffered solution (PBS), filtered through a nylon mesh. The pooled BMCs were washed and adjusted to a concentration of 2.0×10^8 cells in 1 ml of PBS. A total of 300 or 600×10^6 unfractionated BMCs of male LEW rats was infused via the portal vein (PV) of female ACI rats 2 h after they had been totally irradiated with 600 cGy from a ^{137}Cs source at 0.6 Gy/min. After the BMTx, 1 mg/kg per day of FK506 was given by intramuscular injection for 5 days from day 0 to day 4 after BMTx. FK506 was kindly provided by Fujisawa Pharmaceutical (Osaka, Japan).

Surgical procedures

OLT without rearterialization was carried out according to Kamada's cuff technique, with some modifications, 1–2 months after BMTx. Briefly, the rats were anesthetized with diethyl ether. The donor liver was completely skeletonized, and its hepatic artery was ligated. After injection of 150 IU heparin intravenously, 5 ml of cold Ringer's lactate solution was perfused via the PV and the liver was removed. The isolated liver was stored in cold Ringer's lactate solution during the recipient preparation. The liver was transplanted orthotopically into the recipient rat after the recipient liver was separated from surrounding structures and the entire liver was resected. The suprahepatic vena cava of the graft was anastomosed to the recipient's vena cava with a running suture. The graft PV and infrahepatic vena cava connected to those of the recipient using the cuff technique and the bile ducts of the graft and recipient were connected. The average non-hepatic time was about 15 min.

Experimental design

Donor ACI rats were subdivided into the following seven groups:

Group 1, untreated donor rats (control group).
Group 2, irradiated donor rats (600 cGy) subjected to BMTx via the PV; 300×10^6 LEW cells.
Group 3, irradiated donor rats (600 cGy) subjected to BMTx via the PV; 600×10^6 LEW cells.
Group 4, non-irradiated donor rats subjected to BMTx via the PV; 300×10^6 LEW cells.
Group 5, non-irradiated donor rats subjected to BMTx via the PV; 600×10^6 LEW cells.
Group 6, non-irradiated donor rats subjected to BMTx via the penile vein; 300×10^6 LEW cells.
Group 7, non-irradiated donor rats subjected to BMTx via the PV; 300×10^6 BN cells.

Donor rats in all Groups except those in Group 1 received FK506 (1 mg/kg per day) intramuscularly for 5 days beginning on the day of BMTx. No immunosuppressants were administered to any of the recipient rats after OLT.

Detection of chimerism

Chimerism in female ACI rats after BMTx from male LEW rats was confirmed by PCR analysis with primers specific for the sex determinant Y chromosome [7]. Genomic DNA was prepared from peripheral blood leukocytes, bone marrow, and liver taken at the time of liver harvesting by a standard procedure and the DNA was quantified spectrophotometrically. The PCR reaction mixture contained 0.5 μg genomic DNA, 1 U *Taq* DNA polymerase (Perkin Elmer Cetus, obtained through Takara, Kyoto, Japan), 25 pmol rat Sry-specific oligonucleotide primers (KH-1) 5′-GAGAGAGGCACAAGTTGGC-3′, KH-2 5′-GCCTCCTGGAAAAAGGGCC-3′), μl 2 mM dNTP, and 5 μl 10 × PCR buffer (500 mM KCl, 20 mM $MgCl_2$, 100 mM TRIS-HCl, 0.1% gelatin, pH 8.4) in a final volume of 50 μl. PCR was carried out in a thermal cycler (Perkin Elmer Cetus Instrument, Norwalk, Conn., USA) by 30 cycles of denaturation (96 °C, 1 min), annealing (54 °C, 45 s), and extension (72 °C, 1 min).

The reaction products were analyzed by electrophoresis in 2% agarose gels, followed by ethidium bromide staining. The specificity of the amplication was confirmed by Southern blot hybridization with a rat Sry-specific probe (KH-3 5′-ATCAGCAAGCAG CTGGGA-3′) end-labeled with γ-[^{32}P]ATP at the 5′ terminus.

Histological examination

Sections of the liver of donor rats killed 2 weeks after BMTx were fixed in formalin and stained with toluidine blue for routine light microscopy.

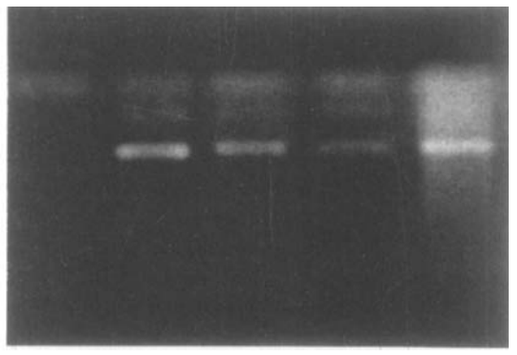

Fig. 2 Detection of chimerism in the donor rat by PCR. DNA from male LEW cells in female ACI rats was detected in peripheral blood, bone marrow, and liver in Groups 2–5 at the time of liver harvesting. Male and female (negative) controls are included. (*BM* Bone marrow, *PB* peripheral blood)

Statistical analysis

Statistical analysis was performed with the Wilcoxon rank test and differences were considered to be statistically significant when the *P* value was less than 0.01 in a two-tailed test.

Results

Histological examination of donor rats

Livers from rats killed in all groups except those in Group 1, 2 weeks after BMTx, revealed histologically normal cytoarchitecture in the lobular and portal regions (Fig. 1).

Detection of chimerism in the donor rat

DNA from male LEW cells in female ACI rats was detected in peripheral blood, bone marrow, and liver in Groups 2–5 at the time of liver harvesting (Fig. 2) but not in Group 6. Also, DNA from male BN cells in female ACI rats was detected in peripheral blood, bone marrow, and liver in Group 7.

Survival

Untreated LEW rats with naive ACI livers died after transplantation due to acute rejection; the mean survival time was 9.0 ± 0.6 days in Group 1 ($n = 6$). With livers from rats subjected to BMTx via the PV and FK506 administration beforehand, the mean survival time was significantly extended to 27.2 ± 23.3 days in Group 2 ($n = 6$), 18.3 ± 2.3 days in Group 3 ($n = 3$), 21.8 ± 10.2 days in Group 4 ($n = 4$), and 18.3 ± 3.1 days in Group 5 ($n = 3$), irrespective of irradiation and the dose of BMCs. With livers from rats previously subjected to BMTx via the penile vein and with livers from rats previously injected with BMC from BN rats as a third party, the mean survival time was 10.3 ± 1.5 days in Group 6 ($n = 3$), and 9.7 ± 0.6 days in Group 7 ($n = 3$). There were no statistically significant differences between Group 1 and the latter two groups (Table 1).

Although only one of the six rats in Group 2 survived for 74 days, histological findings revealed no evidence of rejection. The portal area showed apparent fibrosis with no lymphocytic infiltration and small bile ducts proliferated in some portal tracts (Fig. 3). These findings were consistent with those obtained when the hepatic artery was not reconstructed at the time of OLT in rats.

Discussion

Suppression of allograft rejection by immunological modification of the donor graft allows a reduction in the total dose of immunosuppressants in the recipient and minimizes the risks of infection and drug toxicity. Several approaches have therefore been tried to achieve immunological modification, such as the deletion of antigen-presenting cells (APCs) by irradiation of the graft or by some pharmacological agents [8, 9], masking of allo-antigens of APCs with anti-class II antibody [10], and gene transfer to the graft with interleukin-10 to induce suppressor cells [11].

Liver grafts are spontaneously accepted and induce systemic tolerance without the use of immunosuppressive drugs in animals. For example, in some species such as rats or pigs in some strain combinations, liver allografts are spontaneously accepted across a full MHC class I and class II barrier without immunosuppressants [1, 2, 12]. This acceptance occurs only with liver, but not with kidney, heart or skin grafts. Spontaneous acceptance of liver allografts is accompanied by full immunological tolerance of other organs from donor strains, but not from third-party strains. Liver allografting also reverses ongoing rejection of the heart [12]. Recent clinical trials have also shown that several cases of OLT are stable without immunosuppressive drugs [13].

Among the numerous mechanisms advanced to explain this privileged status of liver allografts, replacement of donor Kupffer cells by host cells after liver transplantation has been cited both in animal models [3–5] and clinical practice [6]. It is generally agreed that the immunogenicity of allografts depends on the DCs recruited from bone marrow-derived immature cells [14]. Therefore, we investigated here whether induction of chimerism in the donor with recipient-type BMTx before OLT may lead to decreased immunogenicity and reduced rejection in rats.

Table 1 Survival time of liver allografts in each group. The Wilcoxon rank test for differences between Group 1 (control) and Groups 2–5 gave values of $P < 0.01$; differences between Group 1 and Groups 6 and 7 were not significant. (*BM* Bone marrow, *BMCs* bone marrow cells, *MST* mean survival time, *PV* portal vein, *IV* intravenous injection)

Group	Donor	Recipient	BM donor	Route	BMCs ($\times 10^6$)	Irradiation (cGy)	FK506	Survival time (days)	MST ± SD (days)
1	ACI		LEW–	–	–	–	–	8, 9, 9, 9, 9, 10	9.0 ± 0.6
2	ACI	LEW	LEW	PV	300	600	+	14, 14, 15, 22, 24, 74[a]	27.2 ± 23.3
3	ACI	LEW	LEW	PV	600	600	+	17, 17, 21	18.3 ± 2.3
4	ACI	LEW	LEW	PV	300	–	+	15, 17, 18, 37	21.8 ± 10.2
5	ACI	LEW	LEW	PV	600	–	+	15, 19, 21	18.3 ± 3.1
6	ACI	LEW	LEW	IV	300	–	+	9, 10, 12	10.3 ± 1.5
7	ACI	LEW	BN	PV	300	–	+	9, 10, 10	9.7 ± 0.6

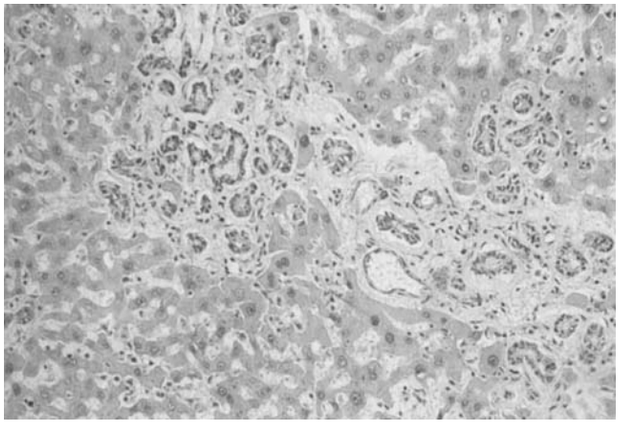

Fig. 3 Histological findings of the grafted liver that survived for 74 days after liver transplantation. Histological findings revealed no evidence of rejection. The portal area showed apparent fibrosis, with no lymphocytic infiltration and small bile ducts proliferating in some portal tracts. These findings are consistent with those observed when the hepatic artery was not reconstructed at the time of orthotopic liver transplantation in rats

Survival time was prolonged in all groups, irrespective of irradiation, when recipient strain BMCs were inoculated via the PV to the donor. PCR analysis at the time of liver harvesting confirmed the establishment of chimerism in the donor rat. Histological findings 2 weeks after BMTx showed normal cytoarchitecture in the lobular and portal regions. Although immunohistochemical studies were not done, these results may suggest that innoculated BMC Cs of LEW rats once reaching the bone marrow of ACI rats, differentiate there and then migrate to the liver as DCs or Kupffer cells. Therefore, donor passenger cells might be replaced by recipient-type cells before OLT, to a certain extent.

Recently, Starzl et al. [15–17] have demonstrated allogenic microchimerism, in which the donor passenger leukocytes migrate widely into the recipient's lymphoid tissues and their survival is associated with long-term acceptance of the liver graft. Donor leukocyte migration outside the graft, rather than recipient Kupffer cells in the liver, play a role in tolerance induction by creating a systemic tolerance. According to the concept of the two-way paradigm proposed by Starzl et al. [16, 17], clinical organ transplantation under immunosuppression involves a double-immune reaction which has host-versus-graft as well as graft-versus-host arms.

Sriwatanawongsa et al. [18] investigated the roles, microchimerism, and cellular components of the liver in rats in which liver grafting induces specific tolerance. This work showed that replacement of donor passenger leukocytes with recipient leukocytes, by parking the liver in recipient strain rats for 30 days, prevented liver-induced tolerance to skin grafts of the liver donor strain, in spite of the liver not being rejected. To obtain full tolerance, both parenchymal and passenger leukocytes of donor origin were required. Whether the microchimerism is responsible for the donor-specific unresponsiveness or is merely a consequence of it is still unclear [7, 19, 20].

In our experiment, survival time was prolonged. There seemed to be several factors responsible for the results. Without immunosuppression after OLT in rats with these kinds of MHC disparities, passenger leukocytes may act as an "enemy" for allograft acceptance in the early phase after OLT. Complete replacement of donor passenger leukocytes by recipient-type cells cannot be established because of the low irradiation dose (600 rad) in Groups 2 and 3. With this protocol, the ratio of chimerism might be less than 50% in Groups 2 and 3. In Groups 4 and 5 with no irradiation at the time of BMTx, it might be even lower. So, the passenger cells of donor origin could migrate to the recipient lymphoid tissues. One of the rats in Group 2 survived for more than 70 days, histological findings revealing no evidence of acute rejection, and this rat seemed to be able to acquire donor-specific transplantation tolerance in spite of the strongest rejection combination of ACI into LEW rats without concomitant administration of immunosuppressants after OLT. Reduced immunogenicity and subsequent migration of passenger leukocytes of donor origin might have played an important role in this rat.

Further examination will be needed to find the optimal dose of irradiation, the optimal interval between BMTx and liver transplantation, and the optimal dose of BMCs to attain tolerance. Moreover, this protocol cannot itself be applicable to clinical allotransplantation because it needs donor preparation long before OLT. We are now preparing to apply this protocol to a hamster-rat xenograft model with some modifications. Indeed, Starzl et al. [16] proposed humanized pigs as a possible strategy for pig-human xenotranplantation.

In conclusion, these results clearly show that modification of the donor liver by intraportal injection of recipient-type BMCs and the concomitant administration of FK506 prior to OLT can prolong liver allograft survival in rats.

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