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BRIEF REPORT

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Restoration of tolerance to rat hepatic allografts by spleen-derived passenger leukocytes

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Abstract The tolerance induced by orthotopic liver transplantation (OLT) in certain combinations of rat strains can be prevented by total body irradiation (TBI) of the donor. We demonstrate here that the intravenous inoculation of splenic leukocytes into irradiated donors before OLT could re-establish tolerance in association with a state of microchimerism detected in the recipients. When donor DA ($RT1^a$) strain rats were irradiated with 1000 rad 24 h before liver harvesting and subsequent liver implantation into PVG recipients, five out of six rats died from rejection in this normally tolerogenic OLT (DA-PVG) combination. Injection of 1.5×10^8 splenic leukocytes from naive DA rats into the irradiated DA donor rats 24 h before OLT restored the tolerogenic potential of the liver allografts. Immunofluorescence assay revealed an increased number of donor (DA) type cells in the PVG recipient bearing a repopulated DA liver, compared to the PVG recipient of an irradiated liver. These results suggest that passenger leukocytes reconstituted by splenic leukocytes have the capacity to protect liver allografts.

Key words Passenger leukocytes, tolerance, rat · Tolerance, rat, liver transplantation · Rat, liver transplantation, tolerance · Liver transplantation, rat, tolerance

Introduction

The role of passenger leukocytes in liver transplantation has been extensively reported [3, 5–7, 9, 10]. Total body irradiation (TBI) of the donor has previously been shown to be an effective method for significantly reducing passenger leukocyte load [4]. It has recently been reported by Sun J. et al. [9] that the tolerance induced by orthotopic liver transplantation (OLT) can be prevented by TBI of the donor and that the tolerogenic effect can be re-established by preceding transplantation of the liver in a normal rat for 36 h. This implies the important role of passenger leukocytes in the induction of tolerance. Our studies have shown here that it is possible to re-establish tolerance by injecting spleen cells into irradiated donors 24 h before OLT.

Materials and methods

Surgery

Male DA ($RT1^a$) and PVG ($RT1^c$) rats weighing 250–300 g were used as donors and recipients. OLT was carried out as we have previously reported [2]. In group 1, untreated DA livers taken from naive DA rats were implanted into PVG recipients. In group 2, DA rats were lethally irradiated with 1000 rad using 20 MEB Electron (Varian, Clinac 850, USA). Twenty-four hours later, the irradiated DA livers were harvested and transplanted into naive PVG recipients. In groups 3 and 4, DA rats received the same dose of irradiation as in group 1. Immediately after irradiation, DA rats were intravenously injected with 1.5×10^8 (group 3) or 5×10^7 (group 4) DA leukocytes purified from the spleen of naive DA rats. Twenty-four hours later, the irradiated and reconstituted livers were implanted into naive PVG recipients. To assess the effect of irradiation on liver graft viability, the irradiated DA livers were implanted into naive DA recipients (group 5). All rats surviving

Table 1 The effect of donor irradiation and repopulation on liver allograft survival

Groups	Treatment of DA donor rats	Recipient	Survival (days)	Two-month survival (%)
1	None	PVG	> 60 (× 6)	6/6 (100)
2	Irradiation[a]	PVG	5, 8, 11, 17, 18, > 60	1/6 (16.7*)
3	Irradiation+repopulation (1.5×10^8 DA splenocytes)	PVG	> 60 (× 6)	6/6 (100)
4	Irradiation+repopulation (5×10^7 DA splenocytes)	PVG	15, 17, 18, 19	0/4 (0)
5	Irradiation	DA	> 60 (× 6)	6/6 (100)

* $P < 0.05$ vs group 1 (Fisher's exact test)
[a] DA rats were irradiated with 1000 rad 24 h prior to liver harvesting

Table 2 mAb FITC-labeled donor (DA) cells per mm^2 of PVG recipient spleen taken 15 days after OLT. Normal DA and PVG livers were used as positive and negative controls, respectively

Groups	Treatment of DA donor rats	Recipient	Number	Labeled cells per mm^2 (mean ± SD)
1	None	PVG	3	18.8 ± 5.2
2	Irradiation[a]	PVG	3[b]	4.4 ± 1.3
3	Irradiation+repopulation (1.5×10^8 DA splenocytes)	PVG	3	13.2 ± 3.7*

* $P < 0.05$ vs group 2 (Student's t-test)
[a] DA rats were irradiated with 1000 rad 24 h prior to liver harvesting
[b] Six OLTs were performed to obtain three survivors on the 15th day post-OLT

beyond 60 days received both DA and LEW ($RT1^l$) skin grafts to confirm whether tolerance was established. For histology and immunofluorescence studies, several rats were sacrificed at various days after OLT in order to obtain serum, liver, and spleen samples.

Immunofluorescence assay for detection of DA class I in PVG spleen

Spleen samples taken at day 15 from groups 1,2, and 3 ($n = 3$/group) were immediately frozen in liquid nitrogen and stored at –70 °C until used. The frozen sections were cut to 6 μm in thickness in a cryostat, fixed with –20 °C acetone, and blocked with 5% low-fat milk powder in TRIS (hydroxymethyl) aminomethione buffered saline (TBS, pH 7.4). The sections were treated with a directly labeled fluorescein isothiocyanate (FITC)-conjugated mAb that reacts only with DA ($RT1^a$) MHC class I (mAb C3, Pharmingen, San Diego, Calif., USA). The FITC-conjugated mAb was diluted 1:100 in TBS, incubated with the section for 1 h at 37 °C, washed with TBS, and mounted in a solution of 50 mg N-propyl-gallate (Sigma) in 200 μl 2 M TRIS and 800 μl glycerol. Immunofluorescence was observed using an Olympus Fluorescence microscope (Model BH2-RFL, Tokyo, Japan). Then, mAb C3-labelled cells were counted in five representative fields of view (magnification × 400) is the average number was expressed as the number of labelled cells per mm^2 of recipient splenic tissue.

Results

We have previously demonstrated that PVG rats grafted with DA livers naturally overcome rejection and that tolerance is induced [1]. This was confirmed with group 1 in the present study (Table 1). When the donor rats were irradiated (1000 rad) 1 day prior to liver harvesting, five out of six rats died between days 5 and 18 after OLT (group 2; Table 1). Histological findings revealed that the cause of death was rejection. When DA leukocytes (1.5×10^8) taken from naive DA rats were transferred intravenously into the irradiated DA rats (group 3), all recipients survived for more than 60 days (Table 1). However, inoculation of less than 5×10^7 DA leukocytes failed to reintroduce tolerance in PVG recipients (group 4). To assess the effect of irradiation on liver graft viability, the irradiated DA livers were implanted into naive DA recipients (group 5). In this group, all naive DA recipients ($n = 4$) survived for more than 60 days (Table 1), suggesting that the graft viability was not affected by irradiation. All surviving OLT rats in all groups received DA and LEW skin grafts 60–70 days after OLT in order to confirm whether donor specific tolerance had been established. All long-term surviving rats accepted DA skin but not third party LEW skin. As shown in Table 2, the immunofluorescence studies using PVG spleen taken at day 15 after OLT revealed that the number of cells expressing DA class I antigen in PVG spleen (group 2; irradiated DA liver into PVG) was smaller than that in group 3, in which the DA rats were reconstituted by naive DA splenic leukocytes after irradiation.

Discussion

Using a normally tolerogenic OLT combination (DA liver into PVG), we demonstrated here that TBI of the DA donor failed to induce tolerance in PVG recipients

but that liver-induced tolerance could be re-established by the injection of naive DA spleen cells into the irradiated DA donor rats. This is the first report that shows that liver passenger leukocytes may be reconstituted by splenic leukocytes.

Sun J. et al. [9] first reported that TBI prevented liver-induced tolerance in the rat model of OLT using the nonrejector PVG-DA combination. Conversely, it has been reported by others [10] that TBI prolonged allograft survival when using the rejector ACI-LEW combination. These reports have shown further that depletion of liver leukocytes including class II-positive cells, macrophages, interstitial dendritic cells, T cells, and B cells by irradiation resulted in deferring rejection [10] or preventing spontaneous graft acceptance [9]. Regardless of which combination (nonrejector or rejector) is used in the rat model of OLT, donor type passenger leukocytes in donor livers appear to be necessary for both the induction of tolerance and the rejection response in the recipient. TBI was demonstrated to be a successful technique for removing the radiosensitive mobile function of the passenger leukocytes [4]. We have confirmed this using immunofluorescence techniques by showing the markedly reduced number of migrating DA class I-positive cells in the PVG recipient bearing an irradiated DA liver. This may be one of the reasons why TBI of donor rats prevented the induction of tolerance [8]. The reconstitution of irradiated donor rats with naive DA splenic leukocytes caused the increased distribution of cells expressing DA type class I antigen in the PVG recipient. The number of injected DA leukocytes was also critical, as inoculation of less than 5×10^7 failed to reintroduce tolerance in the PVG recipient. These results suggest that the initial interaction between a sufficient number of donor passenger leukocytes and recipient leukocytes is essential to regulate the subsequent recipient immune reaction against the donor antigen in the liver grafts.

In conclusion, our results suggest that there is a specific cell population present in the inoculated splenic leukocytes that repopulates the irradiated liver. Whether these cells are then reprogrammed by the liver in order to re-establish tolerance has yet to be determined. An investigation is currently being carried out to determine which fraction of spleen cells (macrophages, T cells, or B cells) protects the recipient from the rejection reaction and helps to re-establish tolerance.

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