Long-term survival of rat cardiac allografts by intrathymic plus portal venous injections of donor bone marrow cells and short-term tacrolimus immunosuppression

Abstract Intrathymic (IT) or portal venous (PV) injection of donor antigens has been shown to prolong organ acceptance in low responder rat strain combinations. We determined whether a combination of these strategies would prolong cardiac allograft survival in high responder combinations. Wistar Furth rats received $1 \times 10^8$ ACI rat bone marrow cells (BMCs) via IT, intravenous (IV), PV, IV + PV, IT + IV or IT + PV route at the time of ACI cardiac transplantation. Without tacrolimus (FK), all grafts were acutely rejected. With FK immunosuppression (1.5 mg/kg per day, I.M., days 0–4), single BMC injection did not increase graft survival beyond 93 days, whereas 70% of grafts survived indefinitely ($>150$ days) when IT and PV BMCs were combined. Animals receiving IT and PV BMCs also had less allograft vasculopathy. Thus, IT and PV injections of donor BMCs under a brief course of FK synergistically improve cardiac allograft survival.

Keywords Rat cardiac allograft · Intrathymic injection · Portal venous injection · Bone marrow cells · Allografts vasculopathy

Abbreviations BMCs Bone marrow cells · CAV Coronary artery vasculopathy · CPM Counts per minute · FK Tacrolimus · IT Intrathymic · IV Intravenous · LEW Lewis · MLR Mixed leukocyte reaction · MST Mean graft survival time · PV Portal venous · WF Wistar Furth

Introduction Despite recent refinements in immunosuppressive agents, side effects associated with the lifetime use of non-specific immunosuppression, including organ toxicity, allograft vasculopathy, post-transplant lymphoproliferative disease, and opportunistic infection, remain a barrier to successful clinical solid organ transplantation [24]. The ultimate goal of clinical transplantation, therefore, is to induce a permanent state of donor-specific tolerance that will allow weaning the recipient from long-term use of immunosuppressive agents. Strategies that promote graft acceptance, such as intrathymic (IT) [1, 17, 20, 21, 25, 29, 30, 33], intravenous (IV)² [32], and por-
tal venous (PV)\textsuperscript{3} injections [5, 9, 11, 12, 13, 26, 35, 36] of donor alloantigens, or creation of mixed allogeneic chimerism [3, 6, 7, 8], have been extensively studied in rodents, however, none have been successful in humans. Obstacles that hinder the clinical application of these strategies include the timing of the pre-treatment which, in some models, requires 1–3 weeks prior to transplantation in order to promote a tolerant state [1, 13, 20, 21, 29, 30], the limited animal strain combinations in which tolerance could be promoted [33, 1, 13], and the risk associated with conditioning regimens. Although strategies utilizing either IT or PV injection of donor antigens have resulted in long-term graft acceptance in certain rat-strain combinations, the various strategies have proven successful in high responder strains. Furthermore, combining these two treatments has not, to date, been reported. In the present study, we report for the first time that a combination of IT and PV injections of donor bone marrow cells (BMCs) at the time of transplantation, along with a short course of tacrolimus (FK), results in long-term acceptance of rat cardiac allografts in a high responder strain combination.

Materials and methods

Animals

Four to five-week-old male ACI (RT1A\textsuperscript{a}) and Wistar Furth (WF\textsuperscript{b}; RT1A\textsuperscript{a}) rats were used as donors and recipients, respectively. This strain combination involves a complete disparity in both major and minor histocompatibility antigens. Lewis (LEW\textsuperscript{c}; RT1A\textsuperscript{a}) rats were used as third-party donors. Rats were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA) and housed in a pathogen-free facility at the Biomedical Science Center at the University of Pittsburgh. All animals received human care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985).

Cardiac transplantation

Heterotopic cardiac transplantation was performed as described by Ono and Lindsey [22]. Donor ACI or third-party LEW hearts were procured and stored in a cold saline bath. Recipient (WF) rats were anesthetized with methoxyflurane and the sorta and inferior vena cava were excised through median laparotomy. Arterial and venous anastomoses were constructed with 8–0 monofilament sutures. Allograft survival was assessed by daily palpation. Graft rejection was defined as complete cessation of ventricular contraction, and further confirmed by histological examination.

Preparation of BMCs

Fresh BMCs were harvested from femurs, tibias, and humeri of ACI or WF rats and resuspended in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA), using sterile techniques. BMC suspensions were adjusted to 1 × 10\textsuperscript{6} cells/ml in medium for IT injection and 1 × 10\textsuperscript{6} cells/ml in medium for IV and PV injections. Cell viability was over 95% as determined by trypan blue dye exclusion.

IT injection of BMCs

After cardiac transplantation, the thymus of the intubated WF recipient was widely exposed by upper median sternotomy. With the aid of an operating microscope, a total of 1 × 10\textsuperscript{6} cells in 0.1 ml of medium were meticulously injected into both lobes of the thymus with a 30-gauge needle. Animals that received the same volume of medium alone served as controls. All animals in whom intralobular hemorrhage or leakage of BMCs into- or out of the capsule occurred were excluded from the study.

PV or IV injection of BMCs

Under methoxyflurane anesthesia, a total of 1.0 × 10\textsuperscript{6} cells in 1 ml of medium or 1 ml of medium alone (for control) were injected into superior mesenteric (PV route) or penile (IV route) veins with a 30-gauge needle, respectively.

Immunosuppression

A short course of FK (generously provided by Fujisawa Pharmaceutical Co., Ltd., Osaka, Japan) was administered at a dose of 1.5 mg/kg per day, intramuscularly, for a period of 5 days, starting on the day of transplantation. Treatment groups are depicted in Figure 1.

Mixed leukocyte reaction (MLR)

Three animals from each group were tested for in vitro alloreponsiveness to donor antigens using one-way MLR assay. Gamma irradiated splenocytes (2000 rads from \textsuperscript{137}Cs source) obtained from naive ACI, WF, or LEW, were used as stimulators. Responder cells were harvested from cervical and mesenteric lymph nodes of the WF recipients. Ficoll (Amersham Pharmacia Biotech, Uppsala, Sweden) purified mononuclear cells were washed and resuspended at a concentration of 1 × 10\textsuperscript{6} cells/ml in a medium consisting of DMEM (Life Technologies) supplemented with 0.5% fresh normal ACI serum, 2 mM L-glutamine (Life Technologies), 25 mM HEPES buffer solution (Life Technologies), and 56 ng/ml gentamicin (Life Technologies). Responders (10\textsuperscript{5}) and stimulator cells (10\textsuperscript{5}) were co-cultured in a total volume of 200 μl of medium in 96-well, round-bottomed microtiter plates (Costar, Cambridge, MA, USA). Cultures were incubated at 37 °C in 10% CO\textsubscript{2}, pulsed on the fourth day with 1.0 μCi of \textsuperscript{3}H thymidine, harvested on the fifth day with an automated cell harvester (Tomtec, Hameden, CT, USA), and were counted in a beta scintillation counter (Wallac, Gaithersburg, Mass, USA). All assays were performed in triplicate. Results were expressed as counts per minute (CPM) ± standard deviation. The stimulation index is the ratio of the CPM generated in response to a given stimulator over the baseline CPM generated in response to the host [3].