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Treatment with a Rho Kinase Inhibitor Improves Survival from Graft-Versus-Host Disease in Mice after MHC-Haploidentical Hematopoietic Cell Transplantation

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A B S T R A C T

Acute graft-versus-host disease (GVHD) is a major complication of allogeneic hematopoietic cell transplantation (HCT) and the main cause of nonrelapse mortality during the first 100 days post-transplant. Although GVHD can be prevented by extensive removal of mature donor T cells from the donor hematopoietic stem cell population, doing so eliminates any potential allogeneic graft-versus-tumor (GVT) effect also mediated by donor T cells and results in unacceptable rates of cancer relapse. One potential solution to this problem of separating GVHD development from a GVT response is to prevent T cell–mediated GVHD in the intestinal tract (IT) while preserving systemic antihost alloreactivity of donor T cells that target residual tumor cells expressing host alloantigens. We examined the ability of the anti-inflammatory rho kinase inhibitor, fasudil, given orally and intraperitoneally, to prevent GVHD in a C3H → $B6C3F_1$ mouse model of MHC-haploidentical bone marrow transplantation. Fasudil-treated recipients of anti-thy-1 mAb + C′ treated bone marrow (ATBM) cells plus T cells had a 73% 90-day survival compared with 25% among untreated ATBM + T cell recipients ($P < .0001$). Severe initial weight loss was similar in the 2 groups, but less diarrhea was observed among treated animals, and fasudil-treated survivors recovered more weight than untreated survivors. Skin inflammation occurred and resolved between weeks 2 and 8 with similar severity and kinetics in both treated and untreated surviving animals, indicating persistent alloreactivity. Day 10 post-transplantation splenocytes from fasudil-treated mice, containing mature donor T cells, and day 98 splenocytes, containing mature donor and de novo thymus-derived T cells, exhibited alloreactivity against host parental antigens, as assessed by in vitro IFN-γ production and rounds of allostimulated proliferation, respectively. These data support the idea that targeted treatment of the IT with rho kinase inhibitors can ameliorate lethal GVHD while preserving systemic alloreactivity. The results also suggest that similar mechanisms of IT-specific tolerance or resistance to GVHD operate in fasudil-treated and untreated long-term survivors of allogeneic ATBM + T cells.

INTRODUCTION

Although multiple organs are typically involved, the main cause of death in acute graft-versus-host disease (GVHD) appears to be damage to the intestinal tract (IT), especially the small and large bowel [1,2]. Whereas skin involvement is more frequent, IT GVHD is more refractory to treatment and more predictive of nonrelapse mortality (NRM) [3–5]. Prevention of GVHD by purging donor hematopoietic cell transplants (HCTs) of mature lymphocytes before transplantation leads to untenable rates of tumor relapse because of the loss of a graft-versus- tumor (GVT) effect, also mediated by mature donor lymphocytes (primarily T cells). Indeed, a roughly inverse correlation between severity of GVHD and incidence of relapse has been documented [6–8]. This conundrum has spurred efforts to mitigate GVHD while preserving a GVT effect.

One strategy is to identify tumor-specific antigens and the T cell clones recognizing them so they can be selectively expanded while all other allogeneic clones are removed [9,10]. The limited number of cancers with well-defined tumor-specific antigens is an obstacle to this approach, but so, too, is the removal of alloreactivity, which comprises a much broader, stronger, and less readily evaded tumor response repertoire than that generated against a single tumor-specific antigen. Another approach is to augment regulatory T cells (Tregs) in the graft with additional donor Tregs; however, like immunosuppression in general or removal of mature donor T cells from the donor graft, this may carry the risk of increased relapse [11].

A very different conceptual strategy is to ameliorate GVHD in the most vulnerable organs while preserving alloreactivity in general and an alloreactive GVT effect in particular. The theoretical possibility of achieving this situation has been demonstrated in mouse models by use of donor T cells genetically deficient in receptors critical for gut homing [2,12] or in cytotoxic T lymphocyte (CTL) synapse formation [13]. More directly relevant to treatment, murine GVHD has been suppressed by immunodepletion of T cells expressing gut homing receptors from the transplant [12], injection of mAb targeting neovascularizing donor endothelial cells [14,15], or administration of inhibitors of T cell receptor–coupled protein kinase C (PKC)α and PKCθ proteins

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[13]. The latter approach had the disadvantage of diminishing (although, surprisingly, not eliminating) antitumor CTL. By contrast, the homing receptor–based approaches appear to reduce the influx of inflammatory cells into the bowel mucosa while leaving the remaining systemic alloimmunity largely intact. This raises the possibility that alloreactive T cells might be redirected to the skin and non-IT organs, exacerbating morbidity and mortality from nondigestive tract GVHD. Although this was not observed in the studies just cited, there was some evidence of redirection of GVHD away from gut and into skin in mice fed a vitamin A–deficient diet leading to decreased CCR9 and $\alpha 4\beta 7$ expression on lymphocytes [16].

Importantly, an approach that, despite its potential for systemic immunosuppression, seems to target the IT has recently been translated from animals to clinical studies. The CCR5 blocking agent, maraviroc, given orally to patients from −2 to +30 days post-HCT, resulted in dramatically reduced GVHD and NRM within the first 100 days as well as 1 year post-transplantation compared with historical control subjects at the same institution [5]. In particular, there was a NRM rate of 0% within the first 100 days and no grade III or IV IT or liver GVHD. CCR5 was first shown to be important as a marker of host-reactive T cells in mouse models of GVHD targeting skin, liver, and gut tissues [17,18]. Thus, despite the expression of CCR5 on alloreactive lymphocytes throughout the body, gut-sparing effects appeared to dominate the clinical results of this trial, accounting for most of the beneficial effects.

In light of the above studies, we took an approach with anticipated systemic effects but also with the potential to preferentially target the IT. We used a rho-associated coiled coil kinase inhibitor, fasudil, demonstrated to have suppressive effects on inflammatory cell activation, motility, and homing in the context of preclinical models of autoimmune disease [19] and tumor metastasis [20–28]. Although potentially systemic in impact, we reasoned that p.o. and i.p. administration might result in predominant protection of the IT because of concentration gradients and initial local gut mucosal and serosal uptake. Fasudil is of particular interest, because it has an excellent 20-year safety record of use in Japan for prevention of poststroke cerebral artery spasm [29,30].

METHODS

Mice

Eight-week-old male C3H/HeJ (C3H; $H2^k$), (C57BL/6 × C3H)F1 ($B6C3F_1$; $H2^{b/k}$), DBA/2J ($H2^d$), and BALB/cJ ($H2^d$) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and used at 10 to 20 weeks as cell recipients or donors. Mice were kept in a pathogen-free environment in autoclaved microisolator cages and were provided with autoclaved water and food ad libitum. All protocols used in this study were approved by the Hackensack University Medical Center's Institutional Animal Care and Use Committee.

Transplantation Experiments

All preparative manipulations of donor cells were conducted in PBS supplemented with .1% bovine BSA. T cell–depleted (anti-thy-1 mAb-treated) bone marrow (ATBM) cells were prepared by flushing bone marrow cells from the femurs of donor mice, followed by incubation with J1j (anti-thy-1.2) mAb (1:100 dilution) and guinea pig complement (C′) (1:5) for 45 minutes at 37°C. T cell–enriched donor cell populations were prepared from pooled RBC-lysed spleen cell suspensions. B cells were removed by incubation with J11D2 mAb (1:500) and C′ for 45 minutes at 37°C. $B6C3F_1$ host animals were exposed to 1100 cGy irradiation (split dose of 550 cGy × 2, 4 hours apart) using a ^{137}Ce source (Gammacell 40 Exactor; MDS Nordion, Ottawa, Ontario, Canada). Within 3 to 4 hours after irradiation was completed, the mice were transplanted via tail vein injection with 2×10^6 C3H ATBM cells. For GVHD induction, mice simultaneously received 5×10^6 C3H splenic enriched T cells. Mice were weighed twice per week and evaluated daily for clinical signs, including mobility, activity, hunching, grooming, diarrhea (during observation and handling), loss of fur, and skin lesions. Weight loss up to 30% of initial weight was permitted if mice remained active, because these animals often survived and recovered some or all of the lost weight over the observation period.

Fasudil Treatment

Fasudil-treated mice started the drug 24 hours before irradiation and transplant, receiving both i.p. (fasudil-hydrochloride, 200 μg twice daily) and p.o. (fasudil-dihydrochloride, 1 mg/mL drinking water, or ~3 mg/day). This dual mode of administration was continued for 10 days post-transplantation, after which time i.p. injections were discontinued but p.o. drug was maintained for the period of observation (up to 90 days).

In Vitro Assays

IFN-γ ELISpot

Spleens from mice 10 days post-transplantation were homogenized to single-cell suspensions, pooled, and cultured at 1×10^7 responder cells with 1×10^7 irradiated (3000 cGy) stimulator cells in 2 mL for 4 days. Cells were washed and plated at 2×10^5 overnight in triplicate on nitrocellulose EIA plates coated with rat anti-mouse IFN-γ mAb (50 μg/mL), developed with biotinylated rat anti-mouse IFN-γ (4 μg/mL), and ELISpots scored by an automated Cellular Technology Limited Immunospot Series 3A reader (Shaker Heights, OH).

Carboxyfluorescein succinimidyl ester proliferation in one-way mixed lymphocyte reactions

Twelve × 10^6 splenocytes from $B6C3F_1$ hosts were harvested at day 98 post-transplantation, labeled with CFSE (5 μM), and mixed at a 3:1 responder-to-stimulator ratio with unlabeled 3000 cGy irradiated $B6C3F_1$, C3H, or BALB/c splenocytes. Cells were recovered and assayed for CFSE intensity peaks using a flow cytometer (model FC500; Beckman Coulter, Brea, CA).

Histopathology

Long-term survivor mice (12 to 13 weeks post-transplant) were killed and immediately dissected to remove liver, spleen, lung, tongue, and small intestines. Tongue was used as a substitute for skin, because it is much easier to prepare for histology and typically reflects the events simultaneously occurring in the dermis. Tissue was sliced into 2- to 5-mm sections, washed with PBS, and cryopreserved in optimal cutting temperature medium (VWR Scientific, Radnor, PA) freezing media in molds floating in liquid nitrogen. After 10 minutes, frozen samples were transferred to −80°C and stored for subsequent thin sectioning and staining with H & E. Slides were scored using a published grading system [31] for focal or diffuse infiltration of inflammatory leuokocytes into lamina propria, crypts, with or without destruction of crypt and villous architecture: 0, rare inflammatory cells in the lamina propria; 1, increased numbers of granulocytes in the lamina propria; 2, confluence of inflammatory cells extending into the submucosa; 3, transmural extension of the inflammatory infiltrate. Crypt damage was scored as follows: 0, intact crypts; 1, loss of the basal one-third; 2, loss of the basal two-thirds; 3, entire crypt loss; 4, change of epithelial surface with erosion; 5, confluent erosion. Ulceration was scored as follows: 0, absence of ulcer; 1, 1 or 2 foci of ulcerations; 2, 3 or 4 foci of ulcerations; 3, confluent or extensive ulceration. Values were added to give a maximal histological score of 11.

Statistics

Survival curves were estimated using Kaplan-Meier's product limit method. Comparison of survival curves was performed using a 2-sided log-rank test. Comparisons of treatment group weights over time were made using the nonparametric Kolmogorov-Smirnov test, with no a priori assumption of normal distribution. Proportions were compared by a 2-sided z test for significant differences. Survival data analysis was performed using SAS 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Survival

As shown in Figure 1, fasudil-treated $B6C3F_1$ mice had significantly greater 90-day survival after injection of C3H ATBM + T cells compared with untreated recipients (73% versus 25%, $P < .0001$). In the GVHD control group, most mice (70%) succumbed to disease between days 8 and 28 post-transplantation. In the fasudil-treated group, most fatalities