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A Canine Model of Chronic Graft-versus-Host Disease

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A B S T R A C T

In long-term survivors of allogeneic hematopoietic cell transplantation (HCT), chronic graft-versus-host disease (GVHD) is the major cause of morbidity and mortality and a major determinant of quality of life. Chronic GVHD responds poorly to current immunosuppressive drugs, and while T cell depletion may be preventive, this gain is offset by increased relapse rates. A significant impediment to progress in treating chronic GVHD has been the limitations of existing animal models. The goal of this study was to develop a reproducible comprehensive model of chronic GVHD in the dog. Ten recipient dogs received 920 cGy total body irradiation, infusion of marrow, and an infusion of buffy coat cells from a dog leukocyte antigen (DLA)–mismatched unrelated donor. Postgrafting immunosuppression consisted of methotrexate (days 1, 3, 6, 11) and cyclosporine. The duration of cyclosporine administration was limited to 80 days instead of the clinically used 180 days. This was done to contain costs, as chronic GVHD was expected to develop at earlier time points. All recipients were given ursodiol for liver protection. One dog had graft failure and 9 dogs showed stable engraftment. Eight of the 9 developed de novo chronic GVHD. Dogs progressed with clinical signs of chronic GVHD over a period of 43 to 164 (median, 88) days after discontinuation of cyclosporine. Target organs showed the spectrum of chronic GVHD manifestations that are typically seen clinically. These included lichenoid changes of the skin, fasciitis, ocular involvement (xerophthalmia), conjunctivitis, bronchiolitis obliterans, salivary gland involvement, gingivitis, esophageal involvement, and hepatic involvement. Peripheral blood lymphocyte surface antigen expression of CD28 and inducible costimulator was elevated in dogs with GHVD compared with those in normal dogs, but not significantly so. Serum levels of IL-8 and monocyte chemotactic protein-1 in GVHD-affected dogs at time of euthanasia were elevated, whereas levels of IL-15 were depressed compared with those in normal dogs. Results indicate that the canine model is well suited for future studies aimed at preventing or treating chronic GVHD.

INTRODUCTION

Chronic graft-versus-host disease (GVHD), first reported in the 1970s for human patients undergoing allogeneic hematopoietic cell transplantation (HCT) [1-4], has remained a major determinant of morbidity and mortality. Its manifestations resemble those of autoimmune, systemic collagen, and vascular diseases. Among patients undergoing transplantation for hematologic malignancies, a beneficial graft-versus-tumor effect has been described that has a significant association with chronic GVHD [5]. However, this benefit is offset by recurrent and often fatal bacterial and fungal infections due to the impaired immune function both from chronic GVHD itself and from the extended immunosuppressive treatment. The reported cumulative incidences of chronic GVHD range from 25% to 50% in survivors of allogeneic transplantation [6]. Chronic GVHD responds only slowly and often incompletely to current immunosuppressive drugs, with the median duration of treatment among surviving patients ranging from 2.5 to 3 years [7-9]. The mortality rate associated with chronic GVHD is approximately 25%. One way of reducing the incidence of chronic GVHD has been through T cell depletion, which can either be accomplished in vitro by removing T cells from the grafts or in vivo by treating patients with antithymocyte globulin, an antibody to CD52, or post-transplantation cyclophosphamide [10-13]. However, the benefit from decreasing the risk of chronic GVHD by T cell depletion may be offset by an increased risk of relapse [10].

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Therefore, the challenge is to retain the beneficial graft-versus-tumor effect of chronic GVHD while significantly shortening the current lengthy immunosuppressive treatment and its associated high risk of morbidity and mortality.

The first report on treatment of patients with chronic GVHD using combinations of steroids and other immunosuppressive agents was published in 1981 [14]. Since then, treatment efforts of chronic GVHD have been characterized by a lack of progress, despite intense clinical investigations in the form of phase I/II and randomized controlled phase III clinical trials [15-18]. This lack of progress against chronic GVHD has been disappointing and has not been helped by the limitations of existing animal (mostly murine) models of chronic GVHD [19]. The existing models do not replicate the full spectrum of the clinical disease and, to date, have not produced clinical advances comparable to those achieved in acute GVHD. We described a chronic GVHD model in allografted dogs in 1982 [20] but did not pursue these observations because of competing priorities and the belief that the chronic GVHD problem would be resolved in humans before canine studies could get underway, which was clearly an incorrect assessment. In light of the lack of success of human trials described above, we redeveloped a canine model of chronic GVHD, which we describe in this report. A reproducible model of chronic GVHD in a clinically highly relevant large animal will set the stage for a systematic evaluation of specific biological reagents directed at T cell checkpoints for more effective and definitive treatment of chronic GVHD.

MATERIALS AND METHODS

Experimental Animals

Random-bred litters of beagles and mini-mongrel cross-breeds were raised at the Fred Hutchinson Cancer Research Center in Seattle Washington. The dogs weighed from 8.3 to 15.3 (median, 10.6) kg and were 6.5 to 15 (median, 9.3) months old. They were observed for disease at least 20 days before study. The institutional care and use committee of the Fred Hutchinson Cancer Research Center approved the research protocols and the American Association for the Accreditation of Laboratory Animal Care certified the facility. Ten donors and 10 recipients were unrelated for at least 5 generations and were mismatched for highly polymorphic major histocompatibility complex (dog leukocyte antigen [DLA]) class I and class II associated microsatellite markers [21,22]. DLA mismatching was confirmed by direct sequencing for DLA-DRB1 alleles [23].

HCT

On day 0, HCT recipients were conditioned with a single dose of 920 cGy total body irradiation (TBI) delivered at a rate of 7 cGy/minute from a high-energy linear accelerator (Varian Clinac 6, Palo Alto, CA) (Figure 1). Within 4 hours after TBI, the recipients were given an intravenous (i.v.) infusion of 2.0×10^8 to 5.2×10^8 (median, 4.2×10^8) nucleated donor marrow cells/kg. Twenty-four hours later, the recipients were given an i.v. infusion of $.2 \times 10^8$ to 3.5×10^8 (median, 1.4×10^8) peripheral blood mononuclear cells/kg obtained by COBE Spectra Apheresis System (Gambro BCT, Lakewood, CO) from the marrow donor. Postgrafting immunosuppression consisted of i.v. methotrexate (MTX), .4 mg/kg/day on days 1, 3, 6, and 11, and cyclosporine (CSP), given twice daily starting on day –1 through 80 at a dose of 7.5 mg/kg to 15 mg/kg, adjusted to maintain a blood CSP level between 100 ng/mL to 300 ng/mL. Marrow recipients were given ursodiol (.75 mg/kg, twice daily, days –1 to 80) for protection of the liver. All dogs were given standard postgrafting care, including constant rate infusion of lactated Ringers solution while receiving MTX. Fevers were treated as sepsis and dogs were given antibiotics. Hematopoietic engraftment was assessed by chimerism studies using microsatellite markers [24,25].

Evaluation of GVHD

A diagnosis of GVHD was based on clinical findings, which included generalized skin ulcerations or scleroderma, facial edema, dry eye syndrome, erythema of the sclera, nasal occlusion, gingivitis, elevated liver enzymes, anorexia, vomiting, and/or diarrhea. The dogs were monitored at a minimum twice daily and the progression of GVHD was recorded in a digital program DVMax (Veterinary Health Management Software, Westbrook, ME). When GVHD had progressed to the point of diminished activity level, weight loss greater than 30%, failure to eat, or signs of distress or if a requirement of critical care procedures were noted, the decision was made to euthanize the animal after establishing consensus of the principle investigator, clinical veterinarian, and animal technicians currently on hand. After euthanasia, a complete necropsy was performed and representative tissues fixed in 10% buffered formalin, embedded in paraffin, cut, and stained with hematoxylin and eosin for evaluation by a pathologist (G.S.). Chronic GVHD was graded from mild to severe based on the degree of lymphocyte infiltration, and the degree of tissue damage (apoptosis, fibrosis, lichenoid formation, and sclerodermatous foci).

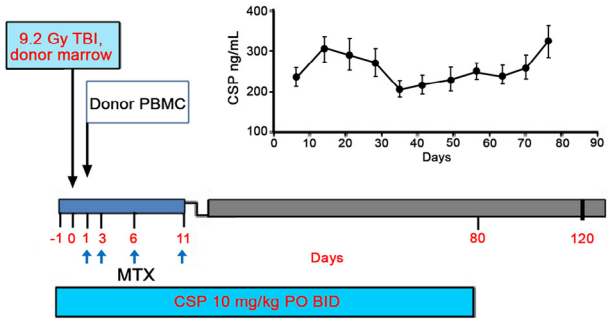

Figure 1. Treatment schema for induction of chronic GVHD in DLA-mismatched unrelated HCT recipients. Recipients were given 920 cGy TBI and donor marrow on day 0. On day 1, recipients were injected with buffy coat cells from a COBE apheresis from their respective donor. Immunosuppression consisted of methotrexate (MTX) administered on days 1, 3, 6, and 11 and cyclosporine (CSP) administered on days –1 through 80 at a dose to maintain serum levels between 100 ng/mL and 300 ng/mL. The average CSP serum level for the 10 dogs on study is shown graphically in the top right portion of the figure. Ursodiol was given on days –1 through 80 to reduce the incidence of liver GVHD.

Peripheral Blood Cell Surface Antigen Expression

Peripheral blood was collected from dogs in 10% heparin, and centrifuged over Ficoll-Paque-Plus (Sigma-Aldrich, St Louis, MO) (adjusted to 1.074 density with water) using standard methods. After washing in PBS +2% horse serum, the cells were resuspended in the same buffer and labeled with the following fluorochrome-conjugated mouse anticanine specific antibodies: anti-CD3 (17.6F9), CD28 (1C6), and anti-inducible costimulator (ICOS) (3F12) using standard labeling techniques. Commercial rat anticanine CD4 and CD8-labeled antibodies were used for single and double staining (eBiosciences Inc., San Diego, CA). The cells were stained and analyzed for positive expression using BD Canto-2 flow cytometer (BD Biosciences). The percentage of positive cells was determined using FlowJo software (Ashland, OR).

Major antigenic protein-1 Cytokine Expression

IL-2, IL-6, IL-8, IL-10, IL-15, TNF-α, and monocyte chemotactic protein (MCP)-1 levels were measured using CYTOMAG-90K Milliplex major antigenic protein-1 (MAP) kit (Millipore, Billerica, MA). Sera were collected from normal dogs and dogs with GVHD before euthanasia and were tested according to manufacturer's specifications. Levels of cytokines and chemokines were expressed in picograms/mL (pg/mL).

RESULTS

Hematopoietic Cell Engraftment

All 10 dogs had hematopoietic cell engraftment after HCT. Mononuclear cell and granulocyte counts recovered rapidly after nadirs on days 5 through 10; however, full granulocyte count recoveries for dogs H694 and H720 were delayed and platelet recoveries were also delayed to approximately day 50 (Figure 2). Donor cell chimerism (Figure 3) was apparent after 1 week and was sustained for both mononuclear cells and granulocytes. We assume the apparent loss in mononuclear chimerism for H633 after day 20 was likely due to the dilution effect of preceding multiple transfusions that were given because of thrombocytopenia. H633 died on day 30 from a hemorrhagic cerebellar stroke and was excluded from further analysis. All dogs with the exception of H694 became febrile for a brief period of time (temperature >39.2°C)