Allogeneic Cell Therapy for a Murine Mammary Carcinoma

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ABSTRACT

The effect of allogeneic cell therapy on tumor growth was studied in a murine model of mammary carcinoma (4T1) as an experimental model of solid tumors in humans. I.v. inoculation of 4T1 (H-2d) cells into syngeneic mice [BALB/c or (BALB/c×C57BL/6)F, (F,)] carrying the H-2d histocompatible antigens resulted in tumor colonies in the lungs that finally cause the death of all of the mice. Sublethally irradiated F, mice were inoculated with 4T1 cells to simulate minimal residual disease and with immunocompetent splenocytes derived from naive donors of F, (syngeneic), BALB/c (syngeneic to the tumor but semiallogeneic to the host), or C57BL/6 (allogeneic to the tumor and semiallogeneic to the host) mice. The survival of F, tumor-bearing mice that were treated with allogeneic C57BL/6 splenocytes was significantly prolonged (P < 0.02) compared with hosts given F, or BALB/c-derived splenocytes that are syngeneic to 4T1 tumor cells. Adoptive transfer of lung cells that were isolated from F, primary mice inoculated with 4T1 cells and syngeneic BALB/c or F, splenocytes led to local tumor growth and death in secondary recipients. In contrast, only 1 of 22 secondary recipients developed tumors when inoculated with lung cells derived from F, mice given allogeneic C57BL/6 splenocytes. All of the 21 secondary hosts survived disease-free for a follow-up time of >200 days. These results indicate that immunocompetent cells allogeneic to the mammary carcinoma cells were able to inhibit tumor development in the primary hosts and to prevent tumor growth in the adoptive recipients, which suggests that allogeneic cell therapy may be an efficient antitumor tool to eradicate minimal residual disease in human solid tumors.

INTRODUCTION

Disease recurrence of malignant hematopoietic cells and certain solid tumors is the most frequent complication after conventional and even after high-dose chemoradiotherapy combined with autologous stem cell transplantation (1, 2). In patients with acute and chronic leukemia as well as in patients with other malignancies of the hematopoietic system, eradication of residual disease or reversing relapse can be accomplished by infusion of allogeneic immunocompetent cells that induce the GVL effect, as has been shown at our center and can be accomplished by infusion of allogeneic immunocompetent cells allogeneic to the mammary carcinoma cells were able to inhibit tumor development in the primary hosts and to prevent tumor growth in the adoptive recipients, which suggests that allogeneic cell therapy may be an efficient antitumor tool to eradicate minimal residual disease in human solid tumors.

8 and 9) or for women with metastatic disease (10). As of yet, with the exception of anecdotal cases (11–13), convincing evidence of GVT effects has not been documented in metastatic breast cancer or in any other metastatic solid tumor. Therefore, the potential use of immunocompetent allogeneic cells as a therapy (alloCT) for treatment of metastatic solid tumors remains speculative.

We have been studying a murine model of mammary carcinoma (4T1) showing that these tumor cells express H-2d class I histocompatibility antigens which allow growth in hosts carrying the H-2d antigen but lead to recognition as "foreign" and rejection in histocompatible mismatched H-2b mice (14). The present work provides the first conclusive evidence that alloCT can eradicate tumor cells in a murine model of metastatic breast cancer. The data support our previous observations suggesting that alloBMT may induce measurable responses against metastatic breast cancer (14).

MATERIALS AND METHODS

Mice. BALB, C57, and F, mice aged 10–12 weeks, were purchased from Harlan Sprague Dawley and maintained in the animal facility of the Hadassah University Hospital, with food and water ad libitum, in full compliance with all of the regulations for protection of animal rights.

Tumor. 4T1 is a BALB-derived tumor cell line (H-2d) established from a cell subpopulation isolated from a single, spontaneously arising mammary tumor of a BALB/c×C3H mouse where the "r" stands for fostering (15). 4T1 cells were maintained by passage in vitro in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, and 1% nonessential amino acids. Preparation of cells for injection included harvesting by 0.25% trypsin in 0.05% EDTA, washing with RPMI 1640 and resuspension in Hank’s medium for i.d. or i.v. injection into mice in a volume of 0.1 ml or 0.25 ml, respectively. All of the tissue culture media and reagents were purchased from Biological Industries (Beit Ha’emek, Israel). Cells were kept at 37°C in a humidified 5% CO2/air incubator.

Measurement of Primary Tumor Growth in Vivo. Local i.d. tumors were measured once a week in two perpendicular dimensions with a Taxol- (r) rimeter caliper. Tumor size in cm3 was calculated by the formula (a x b²)/2, where b is the smaller dimension of the tumor.

Experimental Design of Cell Therapy. F1 mice were conditioned by nonlethal TBI (4 Gy) or lethal TBI (11 Gy), using a 6-MeV linear accelerator at a dose rate of 1.9 Gy/min. Sublethally irradiated F, recipients were inoculated i.v. 24 h later with 2 x 104 4T1 cells and 4 h afterward with 15 x 106 splenocytes derived from F1, BALB, or C57 mice. F1 mice conditioned with lethal TBI (11 Gy) were reconstituted 24 h later with 15 x 106 syngeneic F1 BM cells given together with the splenocytes. Twenty-four h after BM reconstitution and splenocytes inoculation, mice were inoculated i.v. with 0.25 ml of 2 x 104 4T1 tumor cells. In some experiments, as detailed in the "Results," mice were inoculated i.d. with 0.1 ml of 2 x 104 4T1 tumor cells. BM cells were prepared by flushing RPMI 1640 through the shafts of the femora of donors with a 25-gauge needle followed by centrifugation at 230 x g and resuspension in Hank’s medium for i.v. injection. Splenocytes were prepared by teasing spleens into single cell suspensions, centrifugation at 230 x g, and resuspension in Hank’s medium for an i.v. injection in a volume of 0.25 ml. For adoptive transfer experiments, lungs were removed from these F1 mice on day 12 after tumor inoculation and, after teasing into single cell suspensions, were injected i.d. in a volume of 0.1 ml into naive secondary BALB hosts.

Statistical Analysis. The statistical significance of tumor size was evaluated by the standard two-tailed, unpaired, Student t test. The Kaplan-Meier method was used to calculate the probability of survival as a function of time after tumor inoculation or after adoptive transfer of lung cells into naive
RESULTS

Induction of i.d. and Lung Tumors

Injection of 4T1 (H-2d) cells into syngeneic hosts carrying the H-2d histocompatible antigens resulted in local tumor growth after i.d. inoculation or in tumor colonies in the lung after i.v. injection. Tumor colonies were isolated from the lungs of mice injected i.v. with 4T1 tumor cells and adoptively transferred i.d. into naive syngeneic recipients leading to local tumor growth and the death of the secondary hosts from pulmonary metastases. Adoptive transfer of 4T1 from secondary recipients was used to determine the presence or elimination of minimal residual disease. Only if allowing for a sufficient time in the primary hosts before isolation of tumor cells for adoptive transfer into the secondary recipients (Fig. 1). Lung cells isolated 7 days after i.v. inoculation with $2 \times 10^4$ 4T1 cells did not result in tumor development in the secondary hosts for a follow-up period of $>42$ days whereas the same number of lung cells isolated after 14 and 22 days led to marked local tumor growth (0.819 and 1.763 cm$^3$, respectively) as measured in naive secondary BALB recipients on day 28 after adoptive cell transfer (Fig. 1). The consistent kinetics for adoptive transfer of lung metastases was used to design an experimental system to test the effect of alloCT on metastatic and local tumor cell growth as described below. Adoptive transfer of lung tumor colonies to secondary recipients was used to determine the presence or elimination of minimal residual disease after alloCT.

The Effect of alloCT on i.d. Tumor Growth

F1(H-2d/h) mice were exposed to nonlethal whole body irradiation (4Gy) and on the next day were inoculated i.d. with $2 \times 10^4$ 4T1 cells to simulate minimal residual disease. On the day of tumor inoculation, all of the 22 mice were assigned to 4 experimental groups, each given i.v. $15 \times 10^6$ splenocytes derived from F1(H-2d/h), BALB(H-2d), or C57(H-2b) mice. A control group of mice did not receive splenocytes. The effect of allogeneic splenocytes on tumor growth was evaluated by serial tumor measurements as shown in Table 1. Tumor size was significantly lower in mice receiving C57 cells fully allogeneic to the 4T1 tumor cells compared with tumor sizes measured in mice inoculated with BALB- or F1-derived splenocytes syngeneic to the 4T1 tumor cells ($P = 0.04$ and 0.03 for day 28 and 36, respectively). Tumor size measurements in the control group of mice which did not receive splenocytes were similar to numbers measured in experimental groups given syngeneic splenocytes. Reduced tumor size did not affect the survival of C57-inoculated F1 mice (median = 45 days) which followed a pattern similar to that of BALB-treated F1 mice (median = 47 days). In these two experimental groups, BALB → F1 and C57 → F1, splenocytes were semiallogeneic to F1 hosts and may have caused GVHD; however, survival of these mice was not statistically different ($P > 0.1$) from survival of untreated controls (median = 56 days) or F1 mice inoculated with F1 splenocytes (median = 68 days). The same experimental design used for mice that received syngeneic and allogeneic splenocytes i.d. into the tumor area, did not affect tumor size (data not shown).

The Effect of alloCT on Growth of Lung Metastases Induced by i.v. Inoculation of 4T1 Tumor Cells

The fact that alloCT with donor lymphocytes that was given i.v. effectively reduced i.d. tumor growth (Table 1) led us to test the response to i.v. alloCT on lung tumors induced by i.v. inoculation of 4T1 cells. Inoculation of allogeneic splenocytes into recipients exposed to nonlethal or lethal irradiation induces a GVHD disease that may cause the death of tumor-bearing recipients. Because our final goal was to detect evidence of GVT effects, we decided to bypass lethal GVHD by designing adoptive transfer experiments in which lung tumour cells isolated from primary hosts were injected i.d. into secondary syngeneic recipients; therefore, local tumor growth could be monitored by serial measurements and death in these secondary hosts would be related to tumors only.

A. alloCT after Sublethal Irradiation. F1 mice were exposed to nonlethal irradiation (4Gy) and 24 h later were inoculated with $2 \times 10^4$ 4T1 cells and $15 \times 10^6$ splenocytes derived from either syngeneic F1 mice (group 1); BALB donors (group 2), semiallogeneic to the host but syngeneic to the tumor; or C57 donors (group 3), semiallogeneic to the host and allogeneic to the tumor. Twelve days later, lungs were removed and $1.5 \times 10^6$ cells were adoptively transferred i.d. into naive BALB mice. In each experimental group, five mice of the original F1 hosts were kept to determine survival of the primary tumor recipients. Fig. 2 presents the probability of survival of these primary F1 mice shown by Kaplan-Meier curves. The probability of survival at day 60 was 0%, 7%, and 50% in F1 mice treated with splenocytes derived from F1, BALB, and C57 mice, respectively. Survival time of mice treated with C57-derived splenocytes fully allogeneic to the 4T1 tumor cells was significantly longer than the survival times of mice treated with F1- or BALB-derived splenocytes (Table 1). The longer survival time of C57 mice treated with splenocytes from F1 donors is consistent with the immunosuppressive effects of GVHD on F1 mice within the tumor-bearing hosts. The survival time of F1 mice treated with splenocytes from BALB donors was intermediate between the survival times of F1 mice treated with syngeneic splenocytes or with C57-derived splenocytes (Table 1). The results of this experiment confirm the immunosuppressive effects of GVHD and suggest that the survival of mice treated with splenocytes from F1 donors was significantly longer than the survival time of mice treated with splenocytes from BALB donors (log-rank test, $P = 0.01$). The significance of the survival difference between mice treated with syngeneic splenocytes and mice treated with C57-derived splenocytes was also evaluated using the log-rank test (Fig. 2). The statistical significance between pairs of Kaplan-Meier curves was evaluated by the log-rank test (17).
Fig. 2. Probability of survival of (BALB×C57) F₁ mice that were sublethally irradiated (4 Gy) and inoculated i.v. 24 h later with 2 × 10⁴ 4T1 (H-²) tumor cells and 15 × 10⁶ splenocytes, syngeneic [BALB (H-²) and F₁ (H-²/h)] or allogeneic [C57(H-²)] to the tumor cells. Experimental groups treated with syngeneic or allogeneic splenocytes consisted of 10–14 mice pooled from three experiments.

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Fig. 3. Tumor sizes measured in adoptive transfer recipients (BALB, H-²) inoculated i.d. with 1.5 × 10⁶ lung cells derived from (BALB×C57) F₁ (H-²/h) mice that were sublethally irradiated (4 Gy) 13 days earlier and inoculated i.v. 24 h later with 2 × 10⁴ 4T1 tumor cells (H-²) and 15 × 10⁶ splenocytes, syngeneic (BALB and F₁) or allogeneic (C57(H-¹)) to the tumor cells. Experimental groups treated with syngeneic or allogeneic splenocytes consisted of 16–22 mice pooled from three experiments.

B. alloCT after Lethal TBI and Syngeneic Reconstitution. F₁ mice exposed to 11 Gy and reconstituted with syngeneic F₁-derived BM cells were inoculated with 15 × 10⁶ spleenocytes derived from 1) F₁ mice, 2) BALB mice, or 3) C57 mice; 24 h later, they were inoculated i.v. with 2 × 10⁴ 4T1 tumor cells to simulate minimal residual disease after autologous BM transplantation. Nine F₁ mice given F₁ spleenocytes died of lung metastases within a median of 29 days (range, 24–52) after tumor inoculation as did the control group of 11 naive F₁ mice inoculated with tumor cells only (median, 31 days; range, 24–52 days). F₁ mice that were reconstituted with F₁-derived BM after lethal TBI and given BALB splenocytes or C57 splenocytes developed GVHD, which was severe in 10 BALB-treated F₁ mice leading to their death without lung metastases within a median of 10 days (range, 7–11 days,) and moderate GVHD in 7 C57-treated F₁ mice, which died with very small lung metastases within a median of 29 days (range, 17–70). In parallel, control groups of F₁ mice that were exposed to lethal TBI, given syngeneic F₁ BM cells, and treated with splenocytes derived from F₁, BALB, or C57 mice but not with tumor cells were monitored for GVHD-related death. Mice inoculated with BALB or C57 splenocytes developed severe or moderate GVHD, respectively, and died within a median of 12 and 22 days, respectively, whereas mice that were given F₁ splenocytes without subsequent tumor cells survived for over 140 days. These results indicate that mice of experimental groups 2 and 3 died of GVHD, whereas mice in group 1 died because of tumor development.

Adoptive transfer experiments were carried out under the same conditions as described for mice exposed to sublethal irradiation (section A). i.d. tumor sizes in the secondary recipients are listed in Fig. 5: a marked antitumor effect of alloCT was noted when using C57 splenocytes that are allogeneic to the 4T1 tumor cells. On day 50 after adoptive transfer, no tumor development was observed in secondary recipients inoculated with lung cells derived from F₁ mice and given allogeneic C57 splenocytes, whereas lung cells derived from F₁ mice inoculated with BALB or F₁ splenocytes induced large tumors comparable to those induced by lung cells derived from naive F₁ mice than for mice treated with F₁ (P = 0.0035) or BALB (P = 0.0293) splenocytes, which were both syngeneic to the 4T1 cells.

Adoptive transfer of lung cells isolated from additional mice of these same experimental groups (F₁ and BALB, group 1 and 2, respectively), resulted in local i.d. tumor growth in secondary BALB hosts (Fig. 3). In contrast, local tumor growth was totally absent in secondary BALB hosts that were inoculated with lung cells obtained from F₁ mice treated with C57 splenocytes allogeneic to the tumor cells (group 3). Of 22 secondary hosts that received lung cells isolated from F₁ mice inoculated with C57 splenocytes, only 1 mouse developed a tumor on day 60, and this mouse died on day 85 after adoptive transfer. The remaining 21 mice were disease-free for a period of >200 days (Fig. 3). A control group of secondary BALB hosts were inoculated with lung cells derived from F₁ mice that received 2 × 10⁴ 4T1 tumor cells with no cell therapy. Mice of all of the experimental groups with growing tumors died between day 35 and 104 (median, day 69). Probability of survival on day 200, calculated from Kaplan-Meier curves, was 0%, 21%, and 95.5% in adoptive recipients inoculated with lung cells derived from original hosts of groups 1, 2, and 3, respectively (Fig. 4). These results demonstrate the efficient antitumor effect of C57 splenocytes against metastatic disease in mice bearing 4T1 tumor cells whenever fully allogeneic immunocompetent cells are used for immunotherapy. Disease-free survival for the C57→F₁ experimental group was significantly prolonged (P = 0.0001) compared with that of secondary recipients inoculated with lung cells derived from F₁→F₁ recipients (group 1) or BALB→F₁ recipients (group 2).
interactions with target tumor cells, the adoptive transfer of lung cells obtained from mice with metastatic disease in the context of alloantigens and being presented to MHC-restricted T lymphocytes. A direct identification of GVT effector cells will help in designing an efficient therapy in tumor-bearing hosts and may be safer for application in cancer patients.

GVT effects against solid tumors were documented previously in our laboratory in (NZB × NZW) F1 female mice developing spontaneous sarcoma (18); 24% of (NZB × NZW) F1 recipients developed spontaneous sarcoma at 1 year of age, whereas no tumor was observed in BALB/c→NZB × NZW F1 chimeras with no clinical manifestations of GVHD. Similarly, our recent publication (14), with the same 4T1 metastatic mammary carcinoma model, indicated statistically significant reduced primary tumor cell growth in C57→(BALB × C57) F1 and DBA2→BALB chimeras in comparison with untreated controls, again pointing to the existence of GVT effects. Preliminary clinical experience at our center suggests that GVT effects may also be induced by allogeneic peripheral blood lymphocytes derived from histocompatible siblings in patients with metastatic breast cancer after autoBMT (19) or alloBMT (11). Transient elimination of metastatic liver disease in one patient and prolongation of disease-free survival in five additional patients was documented in patients undergoing alloCT with donor lymphocyte infusion after autoBMT (19). Prolonged alloCT effects may be achieved in recipients after alloBMT when host-versus-graft tolerance, induced by infusion of donor stem cells, may enable durable GVT effects in stable chimeras (11).

The data presented here, together with our work published recently (14), suggest that GVT effects that are mediated by allogeneic lymphocytes could be used as a new modality in patients who are fully resistant to available chemotherapy. Although GVL effects (20-22) and GVT effects (11, 14)—independent of GVHD—were previously demonstrated both in experimental models and in humans, in clinical practice, one should always be aware of the relationship between beneficial GVT effects and detrimental GVHD. Induction of GVL or GVT effects by alloCT carries the risk of uncontrolled GVHD (3, 4). One way to overcome the development of uncontrolled GVHD is donor lymphocyte infusion in graded increments (4). We have shown...
previously that by increasing the time interval between alloBMT and alloCT, a greater resistance to GVHD develops (21, 23). This phenomenon, also confirmed by others (24), suggests that alloCT may be induced late after alloBMT, preferably in an outpatient setting, while controlling for GVHD by infusion of graded increments of donor lymphocytes with the option to discontinue therapy as soon as the threshold of GVHD is reached. In the future, better control of GVHD, while exploiting the GVL and GVT potential of donor lymphocytes, may be accomplished by introducing the thymidine kinase suicide gene into donor lymphocytes before stem cell infusion, thus providing the option to eradicate donor lymphocytes if uncontrolled GVHD develops (25).

An immunological approach such as alloCT may be a practical way of controlling residual disease in patients with metastatic breast cancer or other metastatic solid tumors who are at high risk to relapse or with evidence of disease fully resistant to all of the other available modalities. Our experimental system could be useful for testing GVT effects mediated by allogeneic lymphocytes leading to the more effective and safer cure of metastatic diseases.

ACKNOWLEDGMENTS

We thank Judy Schwartz for generous support.

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