The Role of Host Lymphoid Populations in the Response of Mouse EMT6 Tumor to Photodynamic Therapy

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ABSTRACT

Photodynamic therapy (PDT) treatment of murine EMT6 mammary sarcoma using Photofrin (10 mg/kg) and light (110 J/cm²) cured all these lesions growing in syngeneic BALB/c mice. In contrast, the same treatment produced initial ablation but no long-term cures of EMT6 tumors growing in either scid or nude mice, the immunodeficient strains sharing the same genetic background with BALB/c mice. No difference was detected in either the level of Photofrin accumulated per g of tumor tissue or the extent of tumor cell killing during the first 24 h after PDT of EMT6 tumors growing in BALB/c or scid mice. The assumption that the difference in tumor cures could be ascribed to the absence of functional lymphoid cells in scid and nude mice was supported by the results of experiments involving the adoptive T-cell transfer into scid mice or bone marrow transfer between BALB/c and scid mice. The adoptive transfer of splenic virgin T lymphocytes from BALB/c mice into scid mice performed 9 days before PDT of EMT6 tumors growing in the recipients was successful in delaying the recurrence of treated tumors. Adoptive transfer done immediately after PDT or 7 days after PDT had no obvious benefit. Even better improvement and a high cure rate of PDT-treated tumors was obtained with scid mice reconstituted with BALB/c bone marrow. In contrast, a marked drop in tumor cure rate was observed with BALB/c mice reconstituted with scid bone marrow. These results suggest that the activity of host lymphoid populations was essential for preventing the recurrence of EMT6 tumors following the PDT treatment used in this study. The contribution of PDT-induced immune reaction may, therefore, be of critical importance for the cure with at least some tumors.

INTRODUCTION

One of the established strategies for the control of solid cancers involves the induction of inflammation within these lesions by either nonspecific agents such as bacterial vaccines (1, 2) or specific immune agents such as cytokines interleukin 4, interleukin 7, and granulocyte colony-stimulating factor (3). Such inflammation can lead to the induction of specific immunity against cancer cells and result in the regression of local as well as metastasized tumors (1, 2, 4, 5). In contrast, inflammation of normal tissues generally has no significant effect on tumor growth in the same host. The presence of nonviable cancerous cells damaged by the inflammatory insult and an associated influx of inflammatory cells generates conditions for the evolution of an inflammation-primed immune development cascade (3, 6). The ingestion of dead and damaged tumor cells by macrophages and other "professional" antigen-presenting cells may stimulate the recognition of tumor epitopes by CD4⁺ T cells, leading, in turn, to the generation and expansion of tumor-specific CTLs. Recently, it has become evident that PDT, a newly established modality for cancer treatment, exerts a very strong local inflammatory reaction at the treated tumor site (7, 8). This is characterized by the release of various cytokines and other inflammatory mediators (7, 9) as well as by a massive accumulation of nonspecific effector cells (i.e., neutrophils, mast cells, and monocytes/macrophages) in PDT-treated tumors (8). Functional activation of these inflammatory cells is indicated by their elevated tumoricidal activity (8). The objective of the present study was to test the hypothesis that, in agreement with the effects of other inflammatory insults on cancerous tissue, this event in PDT-treated tumors also leads to the induction of tumor-specific immunity mediated by host lymphoid populations. The role of lymphoid cells in the curative effect of PDT was studied using a murine tumor model, EMT6, growing in syngeneic immunocompetent or immunodeficient mice.

MATERIALS AND METHODS

Tumor Model and Mice. The EMT6 mammary sarcoma (10) was maintained in syngeneic BALB/c mice by biweekly i.m. passage (11). For experiments, this tumor was implanted s.c. by injecting 6–10 × 10⁶ EMT6 cells into a dorsal site. In addition to BALB/c mice, the tumor was implanted into scid mice (BALB/cBy-j/scid/TO) and nu/nu mice (BALB/cBy-nu). Both of these immunodeficient mouse strains, hereafter referred to as scid and nude mice, share a common genetic background with BALB/c mice. The rate of EMT6 tumor growth was similar in all these strains. Only female mice were used in the experiments. Their age was 7–9 weeks at the time of PDT treatment, except for those used in the experiments involving bone marrow transfer (as specified below).

PDT. The mice were administered Photofrin (10 mg/kg i.v. in most cases) 6 days after tumor inoculation, which was followed by light treatment (630 ± 10 nm, 110 J/cm², 130 mW/cm²) 24 h later. The largest tumor diameter at the time of treatment was 5–7 mm, and the thickness never exceeded 3.5 mm. The monodirectional light beam was delivered from a tunable light source (model A5000 with a 1-kW xenon bulb, manufactured by Photon Technology International, Inc.) using a 5-mm core diameter liquid light guide 2000A (Luminex, Munich, Germany).

The end point in most experiments was tumor cure/recurrence. Individual treatment groups consisted of 8–12 mice. The mice were observed for up to 90 days after PDT for signs of tumor regrowth; no sign of tumor at the end of this time interval qualified as the cure. In some experiments, the mice were sacrificed at 24 h after Photofrin administration or at different times following photodynamic light treatment. The excised tumors were either digested and a fluorometric assay (12) or enzymatically dissociated into a single-cell suspension. The latter case, the tumor cell yield was determined, and aliquots of cell suspension were plated into Petri dishes with DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). Tumor cell colonies were stained and counted 7–8 days later. The clonogenicity was calculated based on the plating efficiency and cell yield normalized to the values obtained with tumors from untreated animals (12). These values were not changed in the control groups receiving Photofrin only.

For the fluorescent assay, the tumors were minced and completely digested in ScintiGest (Fisher Scientific Co., Fair Lawn, NJ). Photofrin concentration in tumor tissue lysates was determined from fluorescence intensities (405 nm excitation, 625 nm emission) using standard calibration curves (12).

Adaptive Transfer of T Lymphocytes and Bone Marrow Transplantation. For the adoptive transfer studies, spleens taken from donor animals were carefully teased apart to yield splenocyte suspensions without enzymatic digestion. After removal of erythrocytes by lysis in ice-cold ammonium chloride buffer (13), T lymphocytes were separated from other splenocytes by...
a standard purification procedure using nylon wool fiber (Polysciences Inc., Warington, PA). Immediately thereafter, these cells were transferred into recipient mice by injecting $1 \times 10^7$ cells/mouse into the tail vein. Examination by flow cytometry (described below) verified that the purity of T-cell populations eluted through nylon wool columns was $>85\%$.

For reconstitution of the hemopoietic system by bone marrow transplantation, bone marrow was collected from femurs of donor mice and injected immediately into lethally irradiated mice ($1 \times 10^7$ cells/recipient, i.v.). Lethal whole-body $\gamma$-radiation (Shepherd Irradiator, cesium 137 unit) was used to destroy the bone marrow of the recipient and facilitate the reconstitution of the hemopoietic system from the transplanted bone marrow. scid mice received 4.5 Gy (0.128 Gy/min), and BALB/c mice were irradiated to 7 Gy (0.94 Gy/min).

Flow Cytometry. The purity of the spleen-derived T-cell populations used for adoptive transfer as well as the lymphocyte levels in the blood of mice that received T cells or bone marrow transplant were analyzed by standard flow cytometry techniques. Blood samples (0.1 ml) were taken from tail veins of mice at different times after adoptive T-cell transfer or bone marrow reconstitutions. After lysis of erythrocytes, aliquots of WBC suspensions were stained with monoclonal antibodies specific for mouse leukocyte antigens that were conjugated with FITC or phycoerythrin. In addition to identifying T lymphocytes by their common marker, CD3, helper T cells were distinguished by positive staining with anti-CD4, cytotoxic/suppressor T cells by staining with anti-CD8, and B lymphocytes by staining with anti-B220. All monoclonal antibodies were from PharMingen (San Diego, CA). For staining (30 min at 0°C), the antibodies were diluted in HBSS containing 1% fetal bovine serum and 0.05% sodium azide. Flow cytometry analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA) or Coulter Epics Elite ESP (Coulter Electronics, Hialeah, FL).

RESULTS

Tumor Cell Killing and Photofrin Accumulation. The direct killing of EMT6 cells by PDT treatment of tumors growing in BALB/c or scid mice was examined by excising tumors immediately after the termination of photodynamic light treatment and plating cells dissociated from these tumors for the colony formation assay (Fig. 1a). Two PDT treatments based on different Photofrin doses were used in these experiments. As will be shown later, even the lower PDT dose was sufficient to attain a 100% cure of EMT6 tumor growing in BALB/c mice. No significant difference in cell killing was noted for the EMT6 tumor growing in BALB/c compared to the same tumor growing in scid mice. Very little, if any, increase in tumor cell death was reached with the higher compared to the lower Photofrin dose.

In additional experiments, this type of analysis was expanded to the determination of the indirect lethal effect of PDT by delaying tumor excision for up to 24 h posttreatment (Fig. 1b). The PDT dose used resulted in the killing of 60–70% of tumor cells when assayed immediately after light treatment. In accordance with previous studies using the same tumor model (14), the survival of tumor cells decreased markedly with the time elapsed between the light treatment and tumor excision. The extent of tumor cell killing at 0, 12, and 24 h post-PDT was not dependent on the host (BALB/c or scid mice); i.e., there was no significant difference in the survival of cells in PDT-treated tumors growing in these two different mouse strains.

In addition, no significant difference was detected in the accumulation of Photofrin in EMT6 tumors growing in either BALB/c or scid mice. Based on the standard fluorometric assay, the Photofrin levels determined 24 h after photosensitizer administration (25 mg/kg, i.v.) were 37.2 ± 6.1 and 35.3 ± 4.2 µg per g of tumor tissue (±SD) for the tumors growing in BALB/c and scid mice, respectively.

PDT Response of Tumors Growing in BALB/c, scid, and Nude Mice. The photodynamic treatment combining a Photofrin dose of 10 mg/kg and a light dose of 110 J/cm² was curative for all EMT6 tumors growing in BALB/c and scid mice (Fig. 2). The same PDT treatment of EMT6 tumors growing in scid mice produced a similar initial response, with all the tumors becoming impalpable within 1–2 days after light delivery. However, in scid mice, all these tumors regrew between 14 and 21 days after the treatment. Even doubling the PDT dose by increasing the light exposure to 220 J/cm² was not sufficient to produce any cures of the EMT6 tumor growing in scid mice, although the average tumor recurrence time was delayed by approximately 5 days.

The same type of comparison for the EMT6 tumor growing in either BALB/c or nude mice produced very similar results (Fig. 3).
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Fig. 2. The response of EMT6 tumors growing in BALB/c or scid mice to PDT. Tumor-bearing mice received Photofrin (10 mg/kg, i.v.), followed 24 h later by the light treatment of either 110 or 220 J/cm². The mice were observed afterward for signs of tumor growth. Tumor-free mice at 90 days post-PDT were considered cured. Each treatment group contained eight mice.

Again, the PDT treatment used resulted in a complete initial ablation of tumors in both groups and cured all the EMT6 tumors growing in BALB/c mice, but none of the tumors growing in nude mice. Most of the tumors in the latter group recurred by day 10 post-PDT, i.e., somewhat earlier than those in scid mice (Fig. 2).

Modulation of Tumor Response by Adoptive Transfer of T Cells. In the next series of experiments, attempts were made to determine whether the response of EMT6 tumors growing in scid mice to PDT can be improved by restoring the T-lymphocyte populations, which are drastically reduced or nonexistent in these hosts. For this purpose, spleens were taken from nontreated BALB/c mice, and T cells were separated from other splenocytes by elution through nylon wool columns and transferred into scid mice. Flow cytometry analysis of blood samples taken from the recipient mice 1–3 weeks after T-cell transfer confirmed the presence of CD4⁺ and CD8⁺ cells at levels that were in most cases normal or near-normal for BALB/c mice. Accumulation of T cells in the spleens of these mice was also obvious. As expected, the number of CD4⁺ cells in the blood of nontreated scid mice was found to be extremely low (0–2%), and CD8⁺ cells were practically nonexistent.

Adoptive T-cell transfer was performed at different times relative to the PDT treatment of EMT6 tumors growing in the recipient mice. The PDT dose was 110 J/cm² (10 mg/kg Photofrin). There was no indication of an improvement in the tumor response to PDT (compared to that with scid mice that have not received T lymphocytes) when adoptive T-cell transfer was done either immediately after PDT or at 7 days post-PDT (Fig. 4a). However, the recurrence of EMT6 tumors was delayed for 1 week in the scid mice that received the T cells 9 days before PDT (i.e., 2 days before the tumor implant; Fig. 4b). The average time of tumor recurrence in these mice compared to the mice not receiving the adoptive T-cell transfer (26 and 18 days, respectively) was statistically different (P < 0.01, Student’s t test). The transfer to scid mice of nonselected BALB/c splenocytes 9 days before PDT resulted in a similar benefit to the tumor response. In this case, the data even suggest a low level of tumor cures, but statistically (log-rank test) there was no significant difference in cures between the results obtained with the transfer of complete splenocytes and selected T cells. The transfer of splenocytes or T cells under the experimental protocol used in this study (2 days before the tumor implant) had no obvious effect on the growth rate of EMT6 tumors in the absence of PDT treatment. The volumes at 7 days postimplantation for tumors growing in control scid mice, T cell recipients, and recipients of nonselected splenocytes were 63 ± 11, 60 ± 12, and 56 ± 7 mm³ (mean ± SD), respectively.

The Effect of PDT following Bone Marrow Transfer. The role of immune cell populations in the response of EMT6 tumors to PDT was also studied by performing different combinations of bone marrow transfer. This included the transfer of bone marrow from BALB/c to scid mice and from scid to BALB/c mice, as well as the scid-to-scid transfer in a control group of mice. Two protocols were tested for the BALB/c bone marrow transfer to scid mice. The difference between protocols 1 and 2 was the age of the scid mice at the time they received the bone marrow, which was 7 and 3 weeks, respectively. Flow cytometry analysis confirmed the presence of mature T and B
lymphocytes in the blood of scid mice 8 weeks after they received the BALB/c bone marrow. The blood levels of CD4⁺, CD8⁺, and B220⁺ cells in these mice were similar or even higher than the average levels in nontreated BALB/c mice. A similar analysis performed at 6 weeks after bone marrow transfer suggested that the reconstitution of the hemopoietic system was incomplete at that time.

The attempt to replace the hemopoiesis in BALB/c mice with the scid bone marrow-based hemopoiesis was not completely successful. Flow cytometry analysis indicated the presence of CD4⁺ cells (range, 21—34%) and CD8⁺ cells (5—8%) but only marginal levels of B lymphocytes (0—2% of B220⁺ cells) in the blood of these mice. Our experience suggests that, at least with BALB/c mice, it may not be possible to achieve by radiation a 100% destruction of hemopoietic cells of the recipient in a successful bone marrow transplantation experiment. These mice received whole-body γ-irradiation (7 Gy) that would be lethal to them if they were not rescued by the bone marrow transfer (as confirmed with control mice treated with the radiation only). Despite this, some irradiated bone marrow cells have obviously survived and eventually reconstituted the T-lymphoid populations in these mice.

The EMT6 tumors inoculated in mice that received a bone marrow transplant 8 weeks earlier showed no apparent difference in the growth rate in these hosts compared to the growth rate in control scid or BALB/c mice. When they reached the appropriate size, these tumors were treated by PDT, again using the dose of 110 J/cm² (Photofrin 10 mg/kg). The results (Fig. 5) demonstrate the restoration of the curative effect of PDT in the scid mice that received BALB/c bone marrow with the cure rates of 63% with protocol 1 and 80% with protocol 2. On the other hand, the PDT-based cure rate of EMT6 tumors growing in BALB/c mice that had their hemopoietic system reconstituted with the scid bone marrow was reduced drastically compared to the effects seen in normal BALB/c hosts (Figs. 2 and 3). Also shown in Fig. 5 is the PDT response of EMT6 tumors growing in scid mice reconstituted with the scid bone marrow. This transplant has not changed the immunodeficient status of the recipients, and, as expected, the PDT treatment was not curative in these mice.

DISCUSSION

A striking difference was demonstrated between the response of EMT6 tumor growing in immunocompetent BALB/c mice and immunodeficient scid or nude mice (Figs. 2 and 3). The PDT treatment used cured all the tumors growing in BALB/c mice, whereas it produced no cure of tumors growing in either scid or nude mice. It is also shown that the photosensitizer Photofrin accumulated at similar levels in EMT6 tumors growing in either BALB/c or scid mice and that the extent of tumor cell killing between 0 and 24 h after PDT was not different with these two host strains. This suggests that the difference in tumor cures originated in the lack of activity of lymphoid cells in severely immunocompromised scid and nude mouse strains. Nude mice, which are characterized by the congenital absence of a thymus, have severely reduced numbers of mature T cells, whereas the levels of B lymphocytes and natural killer cells are normal (15). Scid mice have a defect in rejoining of double-strand DNA breaks (required in the V(D)J recombination during normal maturation of T and B cells) due to a mutation in a gene encoding for a protein...
This consideration prompted us to include in the study the transfer of tumors growing in these mice 9 weeks later was superior to that BALB/c bone marrow into scid mice that were only 3 weeks old (protocol 1) and close to the fully curative effect seen with BALB/c (protocol 2 in Fig. 5). The average cure rate for PDT-treated EMT6 also very small (17). In addition, histological abnormalities are discompleteness of scid mice into immune equivalents of cell types present within scid mice. This is an aspect that requires may have hindered the orderly reconstitution of lymphoid populations impacted on the functional activity of macrophages and other immune regrowth after PDT in a small percentage of animals. The effects of may have proved useful for this purpose.

The contribution of lymphoid populations to the cure of EMT6 tumor by PDT was demonstrated further by the adoptive transfer of T cells to the scid mice and different combinations of bone marrow transplantation. The adoptive transfer of virgin T lymphocytes or nonselected splenocytes from BALB/c mice to scid mice that served as the hosts of PDT-treated EMT6 tumors delayed the recurrence of these lesions when it was performed at 9 days before PDT. Even more complete restoration of the sensitivity of EMT6 tumors to PDT was observed with scid mice that had their hemopoietic system reconstituted with the BALB/c bone marrow. Another finding that supports the importance of host lymphoid populations in the response of the EMT6 tumor to PDT is the loss of the curative effect in BALB/c mice reconstituted with scid bone marrow.

Interestingly, reconstituted bone marrow from scid mice introduced into other scid mice appears to have produced a delay in tumor regrowth after PDT in a small percentage of animals. The effects of the conditioning regimen as well as the transplant procedure used (involving, e.g., protracted cycles of cytokine release) may have impacted on the functional activity of macrophages and other immune cell types present within scid mice. This is an aspect that requires additional investigation.

The abnormalities in lymphoid tissues and sites in adult scid mice may have hindered the orderly reconstitution of lymphoid populations after the T-cell or bone marrow transfer, and this would prevent a complete “transformation” of scid mice into immune equivalents of BALB/c mice. The thymus in scid mice is severely hypoplastic and overgrown with fibrocollagenous stroma, whereas lymph nodes are also very small (17). In addition, histological abnormalities are discernible in the spleen, where lymphoid follicles and germinal centers are replaced by nodular structures consisting mainly of stromal cells. This consideration prompted us to include in the study the transfer of BALB/c bone marrow into scid mice that were only 3 weeks old (protocol 2 in Fig. 5). The average cure rate for PDT-treated EMT6 tumors growing in these mice 9 weeks later was superior to that obtained when adult scid mice were used for the bone marrow transfer (protocol 1) and close to the fully curative effect seen with BALB/c mice. This supports the assumption that the restoration of the sensivity to PDT of EMT6 tumors is affected by how successfully the lymphoid sites in these animals were reconstituted.

Timing was shown to be important for the optimal benefit of the adoptive transfer of T cells into scid mice to be realized (Fig. 4). The activation and expansion of transferred T lymphocytes requires time and a suitable environment for optimal homing of these cells. An additional factor with the timing of T-cell transfer may be the need to avoid the correlation with the immunosuppressive effect induced by PDT. Nearly complete depletion of peritoneal lymphocytes and a loss of responsiveness of splenic lymphocytes to concanavalin A and lipopolysaccharide mitogens was observed in mice after PDT treatment to the exposed musculoperitoneal layer (abdominal skin removed; Ref. 18). The systemic immunosuppression was reported to persist for several weeks after such treatment protocol with Photofrin-based PDT (19). This appears to be mediated by adoptively transferable suppressor cells that are not antigen-specific and was shown not to be reversed by adoptively transferred virgin splenic lymphoid cells (20). However, the immunosuppressive effect seems less severe after PDT treatment protocols of s.c.-growing EMT6 tumors such as those used in this study,4 and even milder after cutaneous PDT exposure (19).

Additional research is required to elucidate specific roles of distinct populations of lymphoid cells in PDT-induced immune reaction. Perhaps other models of immunodeficient mice (e.g., CD4 and CD8 knockout mutants), in which the problem with the reconstitution of lymphoid populations encountered with scid mice could be avoided, may prove useful for this purpose.

Most likely, PDT-induced immune activation does not substantially contribute to the initial ablation of treated lesions but may be of critical importance in the eradication of remaining foci of cancerous cells under the reduced tumor burden. This, as suggested by the results of the present study, appears to be an essential requirement for preventing the recurrence of PDT-treated EMT6 tumors. A relevant factor in the development of a PDT-induced immune reaction may be the immunogenicity of treated tumors. The EMT6 tumor is strongly immunogenic, and this may facilitate the recognition of tumor antigens following PDT by lymphoid cells. However, current investigations in our laboratory indicate that lymphoid populations may also play a role in the response to PDT of tumors that are less immunogenic (21). The induction by PDT of tumor-specific immunity against a weakly immunogenic murine fibrosarcoma was described by Canti et al. (22).

4 M. Korbelik, unpublished results.
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In summary, this study demonstrates that host lymphoid populations can participate in the antitumor effect of PDT, and that the contribution of immune mechanisms may be of critical importance for the cure with at least some tumors. Additional investigation is warranted to define the exact nature of the underlying mechanisms responsible for this effect and to help determine how it might be exploited to enhance the antitumor effect of PDT in the clinic. An important implication for preclinical research is that the response to PDT of human and other tumors (even those of poor immunogenicity) xenografted in immunodeficient mice may not adequately reflect the response of these lesions in original hosts.

In ongoing studies, we are continuing to exploit the difference in sensitivity to PDT of tumors growing in syngeneic immunocompetent and immunodeficient mice for further characterization of PDT-induced tumor immunity (21).

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REFERENCES

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