Cyclophosphamide Unmasks an Antimetastatic Effect of Local Tumor Cryoablation

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ABSTRACT

Cryoablation of a solitary tumor mass releases intact tumor antigens and can induce protective antitumor immunity but has limited efficacy in the treatment of established metastatic cancer. Cyclophosphamide (Cy), an anticancer drug, selectively depletes regulatory T cells (Tregs) and attenuates suppression of antitumor immunity. We used a BALB/c mouse model of metastatic colon cancer to investigate the systemic antitumor effects of in situ cryotherapy alone or in combination with 200 mg/kg i.p. Cy. When combined with Cy, cryoablation was significantly more effective than either surgical excision or cautery at inducing systemic antitumor immunity, resulting in the cure of a fraction of animals with established metastatic disease and resistance to tumor rechallenge. Lymphocytes from cured animals contained an expanded population of tumor-specific, interferon-γ-producing T cells and transferred antitumor immunity. Furthermore, treatment with Cy and cryoablation was associated with a significant decrease in the ratio of regulatory to effector CD4+ T cells. The combination of tumor cryoablation and Cy induces potent, systemic antitumor immunity in animals with established metastatic disease.

Cryoablation, or tissue destruction by freezing, is a widely used treatment of localized cancer. The procedure induces tumor cell death directly by damage to cell membranes and organelles through formation of ice crystals and indirectly by causing vascular compromise through thrombosis of small vessels (Fraser and Gill, 1967; Whittaker, 1984; Hoffmann and Bischof, 2002). Cryoablation is used as an alternative to surgical resection in the treatment of cancers of the breast, prostate, kidney, liver, and skin (Korpan, 2007). With the advent of third generation units and a decrease in the complications associated with cryoablation, this modality has become even more popular.

In addition to its effects on localized cancers, anecdotal reports from the clinic demonstrate that cryoablation can induce a systemic antitumor immune response resulting in regression of metastatic disease. The immunological effects of cryosurgery were first documented by Shulman and co-workers (Yantorno et al., 1967; Shulman et al., 1968) when they demonstrated the production of antibodies against rabbit male accessory tissues after freezing. Ablin and co-workers observed regression of metastatic lesions in prostate cancer patients whose primary tumors were treated with cryoablation (Soanes et al., 1970) and coined the term “cryo-immunotherapy” (Ablin, 1972). Further studies in animals confirmed the development of an antitumor response when cryotherapy was used for destruction of neoplasms (Tanaka, 1982; Sabel et al., 2005, 2006). It is postulated that the freezing of cells disrupts cell membranes and releases intact tumor antigens, which are captured by antigen-presenting cells for presentation to antitumor lymphocytes in tumor-draining lymph nodes (den Brok et al., 2006). Multiple studies have also demonstrated that cryoablation induces a vigorous inflammatory response to the necrotic tissue that is produced during the procedure (Sabel, 2009). This cell necrosis can be a potent stimulus for an adaptive (T cell-mediated) immune response through liberation of endogenous tumor antigens and “danger signals.” Taken together, these studies suggest that cryoablation of cancer tissue can potentially generate a clinically meaningful antitumor immune response.

The ability of the tumor-bearing host to mount an immune response to tumor antigens is down-regulated by regulatory

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ABBREVIATIONS: Treg, regulatory T cell; NK, natural killer; Cy, cyclophosphamide; MST, median survival time; HR, hazard ratio; CI, confidence interval; CTL, cytotoxic T lymphocyte.
T cells (T_{reg}s), a subset of CD4+ T cells. These cells express the interleukin 2 receptor α chain (CD25) and the forkhead box P3, or Foxp3, transcription factor. These cells have a potent ability to suppress immunity by inhibiting both cytotoxic T lymphocytes and natural killer (NK) cells and are thought to play a role in tolerance to tumor and self-antigens (Smyth et al., 2006; Wang and Wang, 2007). When T_{reg} number or function was reduced in experimental models, a surge in antitumor response was shown (Onizuka et al., 1999; Golgher et al., 2002; den Brok et al., 2006; Imai et al., 2007). Cyclophosphamide (Cy), an alkylating agent used to treat cancer, has been shown to mitigate suppression of antitumor immunity through effects on T_{reg} (Ghirgelli et al., 2004; Lutsiak et al., 2005; Brode and Cooke, 2008).

In this study, we use a mouse model of metastatic cancer to investigate the systemic antitumor effects of in situ cryotherapy with or without cyclophosphamide, with an aim to devise a clinical strategy to induce the regression of metastatic disease. To establish the efficacy of the cryoimmunologic response, we have compared cryoablation with surgery and high-temperature ablation in our tumor model.

Materials and Methods

Animals. Female BALB/c mice were purchased from Harlan (Indianapolis, IN) and kept under pathogen-free conditions at the Johns Hopkins animal care facility. All experiments were conducted on protocols approved by the institutional animal care and use committee. Six- to 12-week-old mice were used for the ablation and passive transfer experiments.

Tumor Cell Line. The mouse colon cancer cell line CT26 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 supplemented with heat-inactivated fetal bovine serum [10% (v/v)] at 37°C and 5% CO₂. The cells were allowed to grow to 80 to 90% confluence and split at 1:10 dilution.

Tumor Model. Tumor cells were reconstituted with phosphate-buffered saline to a concentration of 1 × 10⁶ cells/ml for subcutaneous injection and 2 × 10⁵ cells/ml for intravenous injection. Mice were injected with 1 × 10⁶ cells into the right flank for subcutaneous tumor. At the same time, 1 × 10³ tumor cells were injected intravenously via the tail vein for establishment of metastatic tumor. Animals were monitored daily for the establishment of subcutaneous tumor. On day 14 after tumor implantation, the tumor was cryoablated, surgically resected, or thermally ablated. Cy. Animals were given 200 mg/kg Cy on day 150, surviving animals and naive 6- to 8-week-old BALB/c mice were injected with 2 × 10⁵ CT26 cells intravenously via tail vein at a concentration of 4 × 10⁶ cells/ml. This was twice the amount we have demonstrated in previous experiments to cause 100% mortality because of metastatic tumor.

Effect of Cryoablation. Animals injected with 1 × 10⁶ CT26 cells subcutaneously and 1 × 10⁵ CT26 cells intravenously were randomized into four groups. Two groups were injected with Cy on day 13, and the other two were given an equivalent volume of saline. Tumors were removed the next day by cryoablation or surgical resection, with the mice hav-
ing a subcutaneous tumor ranging from 3 to 8 mm in greatest dimension. There were no local recurrences of tumor after either surgery or cryoablation, but neither procedure alone prevented death from systemic disease (Fig. 1A). Cyclophosphamide administration prolonged survival of animals treated with surgery [median survival time (MST) of surgery + Cy versus surgery, 48 versus 23 days; hazard ratio (HR) 5.24, 95% CI 14.54–171.7; \( p < 0.0001 \)] or with cryoablation (MST 125 versus 24 days; HR = 6.96, 95% CI = 8.14 to 61.67; \( p < 0.0001 \)). It is interesting that, although there was no significant difference in survival of animals treated with cryoablation alone versus surgery alone (\( p = 0.46 \)), recipients of cyclophosphamide plus cryoablation survived significantly longer than recipients of cyclophosphamide plus surgery (MST 125 versus 48 days; HR 4.89, 95% CI 3.4–21.4; \( p < 0.0001 \)). These results suggest that cyclophosphamide effectively unmasks a systemic antitumor effect of cryoablation. The next experiments were conducted to determine whether anamnestic antitumor immunity mediated the systemic effects of cryoablation.

**Animals Cured with Cryoablation and Cyclophosphamide Resist Tumor Rechallenge.** Four of the nine CT26-bearing animals treated with a combination of cryoablation and Cy were apparently cured (Fig. 1A) and were rechallenged with \( 2 \times 10^5 \) CT26 cells intravenously. As controls, five naive BALB/c mice were challenged with the same tumor dose. Although all of the control animals died (MST 18 days), only one of the previously cured animals died at 23 days after tumor injection (Fig. 1B). No macroscopic tumor was found on autopsy of this animal, although microscopic metastases could not be ruled out. This suggests the development of antitumor effects in these animals, which prevented tumor cells from establishing, thus significantly prolonged their survival in comparison with naive mice (\( p = 0.0051 \), HR = 9.2, 95% CI = 2.25–100.1).

**Lymphocytes from Cured Animals Transfer Antitumor Immunity to Naive Animals.** Because T and B cells are the mediators of anamnestic antitumor immunity, we sought to determine whether lymphocytes from cured animals could transfer tumor resistance to naive animals. Naive BALB/c mice (five per group) received \( 2 \times 10^5 \) CT26 cells intravenously along with graded doses, from 0 to \( 10^7 \), of a mixture of spleen and lymph node cells from mice cured of CT26 by Cy plus cryoablation (Fig. 2A). Compared with naive BALB/c recipients of CT26 tumor cells only, recipients of tumor cells plus either \( 10^6 \) or \( 10^7 \) lymphocytes from cured animals survived significantly longer (MST 25 versus 37 versus >50 days, respectively; \( p = 0.0005 \) for trend by log-rank test).

**CD8⁺ T Cells Are Important in Adoptive Transfer of Tumor Immunity.** To determine the role of specific T cell subsets in antitumor immunity induced by Cy plus cryoablation, lymphocytes from cured animals were left untreated or were depleted of CD4⁺ cells, CD8⁺ cells, or both before intravenous cotransfer with CT26 cells into naive BALB/c mice (Fig. 2B). Recipients of CD8⁺ cell-depleted lymphocytes survived significantly shorter than recipients of undepleted lymphocytes (MST 56 versus >150 days, \( p < 0.0001 \)). In contrast, depletion of CD4⁺ cells from the lymphocyte infusion had no significant effect on the survival of animals given tumor (\( p = 0.54 \)). These results suggest that CD8⁺ T cells are the major effectors of antitumor immunity in mice cured of disseminated CT26 by Cy plus cryoablation. Recipients of lymphocytes depleted of both CD4⁺ and CD8⁺ cells had a small but significant survival advantage over recipients of CT26 cells only (41 versus 38 days; \( p = 0.03 \)). This slight survival advantage may reflect a beneficial effect of non-T cells such as natural killer cells or may reflect activity of residual contaminating T cells.
Cryoablation Is More Effective Than Cautery at Promoting Systemic Antitumor Immunity. Radiofrequency ablation and high-intensity focused ultrasound are used clinically to kill tumor cells by high temperature. In contrast to freezing of tissue, which preserves the tertiary structure of proteins, burning of tissue induces protein denaturation, which may compromise immunogenicity. Because radiofrequency ablation and high-intensity focused ultrasound are not easily performed in mice with small tumors (M. Y. Levy and W. H. Chowdhury, unpublished observations), electrocautery was used to compare the immunologic effects of burning versus freezing tumors (Fig. 3). When combined with Cy, cryoablation was significantly more effective than cautery in prolonging survival (MST >125 days versus 47 days; HR 4.57, 95% CI 1.71–25.71; p = 0.0006). In fact, animals treated with Cy plus cautery did not survive significantly longer than animals treated with Cy plus surgery (MST 47 versus 53 days; HR 1.01, 95% CI 0.27–3.73; p = 0.99), suggesting no immunologic benefit to burning tumor tissue.

Cyclophosphamide Plus Cryoablation Increases Tumor-Specific T Cell Production of IFN-γ. Mice with local and metastatic disease were treated with surgery, cryoablation, Cy plus surgery, or Cy plus cryoablation. Ten days after local therapy, mice were sacrificed, spleen and lymph node cells were harvested, CD4⁺ T cells were tested for the intracellular production of IFN-γ in response to AH1, and the immunodominant H-2Ld-restricted tumor peptide was encoded by an endogenous retrovirus present in CT26 cells. The percentage of IFN-γ-positive CD4⁺ cells was significantly higher in groups treated with cyclophosphamide irrespective of the procedure performed for local tumor removal (Fig. 4). CD4⁺ T cells from mice treated with Cy plus cryoablation contained a higher proportion of IFN-γ-positive cells compared with CD4⁺ T cells from mice treated with Cy plus surgery, but the difference failed to reach statistical significance (p = 0.08).

Cyclophosphamide Suppresses Tregs. FACS analysis of fresh spleen and draining lymph node cells, harvested from the previous experiment, was done to determine whether there was a change in the T cell populations after therapy. Animals with cyclophosphamide pretreatment had a significantly lower ratio of regulatory T cells to effector cells compared with animals with only local tumor removal (p < 0.05; Fig. 5). Treg numbers were significantly lower in mice treated with Cy plus cryoablation compared with mice treated with Cy plus surgery (p < 0.05). Thus, the enhanced antitumor efficacy of Cy plus cryoablation may reflect, in part, the effect of therapy on reducing Treg, which are known to suppress antitumor immunity in animals with advanced tumors.

Discussion

Cryotherapy is used frequently to eradicate localized cancers in patients who are poor candidates for standard surgical resection. It utilizes freezing temperatures to damage tumor cells by multiple mechanisms, including destruction of local microvasculature, breakdown of cellular microtubular networks, conversion of lipid layers of cell membrane into semisolids, and the grinding action of irregular ice crystal formation (Fraser and Gill, 1967; Whittaker, 1984; Hoffmann and Bischof, 2002). Cryoablation in situ results in the generation of tumor debris in the context of an inflammatory background. This tumor debris contains endogenous danger signals required for the activation of antigen-presenting cells that are rendered competent to sensitize adaptive antitumor immunity (den Brok et al., 2006).

Cancers escape immunosurveillance by different mechanisms including anergy induction, loss of tumor antigen expression, and suppression of tumor-specific immunity by CD4⁺CD25⁺Foxp3⁻ Treg. These cells play an important role...
in immune evasion mechanisms employed by cancer (Sakaguchi et al., 2001; Terabe and Berzofsky, 2004; Wang and Wang, 2007). T_{reg}^S are actively recruited and induced by tumors to block the priming and/or effector function of antitumor T cells, thereby inhibiting the efficacy of therapeutic cancer vaccines. Treatment with cyclophosphamide enhances the apoptosis and decreases homeostatic proliferation of these cells. Expression of Foxp3, the lineage-directing transcription factor of T_{reg}s, is down-regulated after cyclophosphamide administration (Brode and Cooke, 2008; Matsushita et al., 2008).

In this report, we have developed a treatment of metastatic cancer focusing on these principles. First, in situ cryotherapy is used to generate a shower of tumor antigens in the context of tissue destruction and distress. Therefore, cryoablation effectively converts tumor tissue into an autologous tumor vaccine. Furthermore, Cy is administered to enhance apoptosis of T_{reg}s and allow for a more vigorous antitumor response. A previous study has shown that Cy pretreatment of tumor-bearing animals augments the local antitumor effect of cryoablation (Cooper et al., 1981). However, the effect of this combination on metastatic cancer had not been explored previously.

Interest in the immunostimulatory potential of cryoablation was first stimulated by anecdotal reports of regression of metastases in patients whose primary tumors were treated by cryosurgery (Soanes et al., 1970; Gursel et al., 1972). Studies in animals of the antitumor potential of cryoablation have yielded disparate results, often depending upon the read-out system. On the one hand, cryoablation of a subcutaneous tumor retards the simultaneous growth of untreated tumor on the contralateral side and induces resistance to tumor rechallenge (Neel et al., 1970; Tanaka, 1982; Sabel et al., 2005; den Brok et al., 2006). On the other hand, cryoablation of the primary tumor alone is usually, but not always (El-Shakhs et al., 1999; Joosten et al., 2001), ineffective or deleterious in controlling the growth or spread of hematogenous metastases (Javadpour et al., 1979; Yamashita et al., 1982; Wing et al., 1988; Yan et al., 2006). Our studies confirm that cryoablation of local tumor as sole therapy is unable to retard the progression of metastatic cancer. The addition of cyclophosphamide was able to unmask a systemic antitumor response and resulted in complete cure in approximately half of the animals. Cyclophosphamide selectively reduced T_{reg}^S as demonstrated by flow cytometry analysis. Reduction of T_{reg} number and function mitigates the suppression of NK cells and CD8^+ cytotoxic T lymphocytes (CTLs), providing a boost to both innate and adaptive antitumor immunity. Activation of NK cell-mediated immunity provides added protection against tumor cells that escape tumor-specific CD8^+ T cells through down-regulation of surface expression of MHC class I molecules.

We have demonstrated an increased secretion of IFN-γ by CD4^+ T cells against AH1 peptide in spleens of animals treated with the combination of cyclophosphamide plus cryoablation. IFN-γ is a proinflammatory cytokine secreted by both activated lymphocytes and natural killer cells and is considered as an antitumor agent (Doherty et al., 1996; Karami et al., 2008). Besides the direct apoptotic effects of IFN-γ on various cancer cells, it also up-regulates the expression of MHC class I on tumor cells, thus making them more susceptible to CTLs (Wadler and Schwartz, 1990; Boehm et al., 1997). The ability of cured animals to resist tumor rechallenge is dependent on the development of immunologic memory in these mice. Furthermore, this antitumor immunity can be passively transferred to naive animals, offering them a dose-dependent protection against tumor implantation. Similar results of the adoptive transfer of cells or serum from tumor-bearing animals subjected to cryoablation have been shown previously (Sabel et al., 2006). Our adoptive transfer studies reveal CD8^+ T cells as critical effectors of antitumor immunity in animals cured by cyclophosphamide plus cryoablation. This result is in accordance with the available literature on the role of CD8^+ CTLs in antitumor responses (Barth et al., 1991; Paglia et al., 1996).

We also compared the immunogenicity of burning versus freezing. In our model, cryoablation was more effective than

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**Fig. 5.** Cyclophosphamide suppresses T_{reg}s. Spleen cell suspensions used in the experiment of Fig. 4 were stained on the cell surface with anti-mouse CD4 and CD25 antibodies and intra-cellularly with anti-mouse Foxp3. Flow cytometry was done for T_{reg}s and T effector cells. Data are shown as proportion of T_{reg}s to T effector cells.
cautery at promoting a systemic antitumor immune response. Although both procedures leave tumor debris inside the host, the differential response could be due to greater damage to antigens caused by the denaturing effect of high temperature compared with the release of intact antigens by cryoablation.

The combination of cryoablation and Cy serves as an efficacious tool in our model for the development of antitumor immunity sufficient for prolongation of survival from and even cure of systemic, metastatic disease. Further studies using different cancer models and using immunomodulatory approaches such as monoclonal antibodies and/or adoptive cell therapy in combination with cryoablation and cyclophosphamide are required to devise an immunotherapeutic strategy that can affect the progression of metastatic disease in patients.

References


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