□ Review Article □

Urologic Applications of Cryo-Immunology

Abhinav Sidana, Ronald Rodriguez

From the Department of Urology, James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA

Korean Journal of Urology
Vol. 50 No. 7: 629-634, July 2009

DOI: 10.4111/kju.2009.50.7.629

Purpose: Cryoablation is gaining acceptance as a primary treatment of localized metastatic urologic malignancies as well as a salvage therapy. Anecdotal clinical reports suggest that cryoablation can induce a systemic antitumor immune response, which has been confirmed in animal models also.

Materials and Methods: A review of the relevant literature was performed to suggest urologic applications of cryo-immunology.

Results: This article reviews the existing evidence regarding cryo-immunology and discusses the mechanisms for generation of an anti-tumor immune response.

Conclusions: Our findings suggest combining cryoablation with other immunotherapeutic approaches to devise a cryo-immunotherapeutic strategy with potential to impact the progression of metastatic disease. **(Korean J Urol 2009;50:629-634)**

Key Words: Cryosurgery, Prostatic neoplasms, Kidney neoplasms, Immunotherapy

Correspondence to: Ronald Rodriguez
Associate Professor of Urology, Medical Oncology, Cellular & Molecular Medicine, Viral Oncology, Johns Hopkins University School of Medicine, 600 N. Wolfe Street, Marburg Room 205A, Baltimore, MD 21287-2101, USA
TEL: 410-614-6662
FAX: 443-287-1010
E-mail: RRodriguez@jhmi.edu

INTRODUCTION

Cryoablation, or tissue destruction by deep freezing and thawing, is a widely used treatment modality for the management of both benign conditions and localized cancer. Although cold temperature had been used therapeutically for centuries,[1] Arnott[2] was the first to use it to treat cancer in the middle of the 19th century when he used iced saline solutions to control advanced breast and uterine cancers. Towards the end of the 19th century and beginning of the 20th century, liquid air and liquid oxygen began to be used for the treatment of various skin diseases.[3,4] Cryotherapy applications grew slowly until 1961, when Cooper and Lee[5] devised a cryosurgical unit capable of delivering liquid nitrogen (−196°C).[6] They used it for the treatment of Parkinsonism.[7,8] The pioneering work on cryosurgery in urology was done by Gonder et al,[9-11] who modified Cooper's unit for prostate ablation, first in dogs and then in humans. Their technique involved transurethral freezing with a single closed-probe system using liquid nitrogen. Cryosurgery was used initially for benign prostatic hypertrophy in patients unsuitable for surgery, but local complications were frequent. With the advent of better treatment modalities for benign prostatic hypertrophy, the use of cryosurgery in the management of urologic conditions was abandoned. In the meantime, cryosurgery continued to develop in other specialties with improvements made in liquid nitrogen probes and heating devices.[12-14] In the 1980s, Korpan[15,16] performed experiments to understand the mechanism of cell damage caused by freezing. His experiments, coupled with his clinical studies involving cryoablation, provided the framework for formulation of the clinical and technical requirements for modern cryosurgery.

With a growing need for minimally invasive techniques and the development of more efficient cryosurgical units, cryosurgery re-emerged about a decade ago as a clinical tool in the field of urological oncology. With the development of the percutaneous transperineal approach by Onik et al,[17,18] the application of cryoablation in the treatment of localized prostate cancer received a major boost. Since then, cryoablation has been widely used in the management of both benign and malignant prostatic conditions.[19] Analysis of data from the Cryo-OnLine Database (COLD) registry showed a 77.1% 5-year biochemical disease-free survival (bDFS) rate when cryoablation was used as the primary treatment and a 59%

5-year bDFS rate when used for salvage of prostate cancer.[20,21]

Although renal cryotherapy has been studied in animal models,[22,23] it wasn't used as a management tool for kidney neoplasms until Uchida et al[24] first reported percutaneous cryoablation for managing renal tumors. Cryoablation of small renal masses now serves as a promising nephron-sparing alternative to partial nephrectomy for small renal masses.[25] With its advantages of reduced blood loss and morbidity, cryoablation is now increasingly being offered as an alternative to partial nephrectomy for select small renal masses. A literature analysis of 6 published series of renal cryoablation showed a 97-100% cancer-specific 5-year survival rate for lesions smaller than 3 cm.[26] Currently, cryosurgery is extensively used as a minimally invasive modality both for primary treatment of localized renal cancer and for salvage treatment of metastatic renal cancer.

CRYOBIOLOGY

Cryoablation induces tumor cell death directly by damage to cell membranes and organelles and indirectly by causing vascular compromise through thrombosis of small vessels.[27-30] With falling temperatures, the cells dehydrate and proteins are damaged as the result of high solute concentration, resulting in damage to the membranes and disruption of the enzymatic machinery of the cell. With faster cooling, ice crystals form inside cells, damaging cell membranes and organelles by mechanical effects. With repeated freezing, the thermal conductivity of the tissue increases and spreads the damage.

The indirect damage to cells occurs through vascular ischemia resulting in cell death. Vessel wall damage can occur as a result of perivascular cellular hydration, resulting in vessel distension and mechanical injury or from direct cell damage to endothelial cells lining the vessels. Both pathways ultimately lead to increased permeability, edema, and a coagulation cascade giving rise to microthrombi in vessels and tissue ischemia. Reperfusion injury is also proposed to play a role in the cell damage.

Histologically, the above mechanisms culminate in coagulative necrosis of the tissue. Sindelar et al[22] studied the effects of cryosurgery in rat kidney and reported that cellular proteins were denatured and membranes disrupted within an hour of cryosurgery. Twenty-four hours after cryosurgery, the tissue had undergone complete coagulative necrosis along with mild to moderate infiltration of lymphocytes, plasma cells, and macrophages. Thus, freezing of tissue resulted in the formation of inflammatory debris.

CRYO-IMMUNOLOGY

Although freezing temperatures have been used for the treatment of diverse conditions for centuries, the systemic effects of local cryosurgery were not recognized until 50 years ago. The immunologic effects of cryosurgery were first documented by Yantorno et al[31] who demonstrated the production of antibodies against rabbit male reproductive tissues after freezing.[32] These antibodies, which reached a peak 7-10 days after cryoablation, were found to be tissue- and species-specific. Blackwood et al[33] did palliative cryotherapy in 13 patients with advanced or metastatic cancers and found elevated antibodies after freezing but did not observe any clinical response. Ablin[34] observed regression of metastatic lesions in prostate cancer patients whose primary tumors were treated with cryoablation, and coined the term "cryo-immunotherapy."[35,36] They found enhancement of this immune response after repeated *in situ* freezing, which they explained was due to multiple inoculations with the same antigen. These anecdotal reports of regression of metastases in patients whose primary tumors were treated by cryosurgery further stimulated interest in the immunostimulatory potential of cryoablation. Immunologic assays at that time were limited, and the mechanism of cryo-immune response remained controversial. This led to a host of studies in animal models to define the cryo-immune response and its mechanism. In these experiments, animals bearing a subcutaneously implanted tumor were treated with cryoablation and then re-challenged with the same tumor. Blackwood and Cooper[37] using myosarcoma MT449A and carcinosarcoma Walker 256 tumors, showed that there is a threshold to antigenic stimulation that can invoke antitumor immunity. They found that regression of challenge tumors was faster when only a small amount of tumor tissue was left in the animal but slower or nonexistent if a bulk of tissue was left. Bagley et al[38] demonstrated that lymphocytes from cryosurgically treated animals were cytotoxic to specific tumor cells for which the animal was treated, thus showing cell-mediated immunity to be at least partially responsible for the antitumor effects of cryosurgery. Matsumura et al,[39] Misao et al[40] further characterized the kinetics of cryoablation-induced

antitumor immunity by using metastasizing rat mammary tumor No. 1 (MRMT-1) in Sprague-Dawley (SD) rats. They observed a fall in antitumor immunity initially after cryosurgery, which increased gradually later, reaching a peak by 10 weeks after cryosurgery. The initial dip was considered to be due to activation of suppressor cells because of cryosurgical stress or slow and steady absorption of antigens.

Lubaroff et al[41] studied the immunologic aspects of prostate cancer by using Dunning R3327 tumors in Copenhagen rats. They were able to achieve complete cryosurgical destruction of small tumors. Partial cryosurgical ablation of large tumors in their models caused regression of the remaining lesion after cryosurgery, but the tumors eventually grew back in 4 weeks and killed the animals. Although cryosurgery alone did not protect the animals against tumor rechallenge, its combination with BCG enhanced antitumor immunity adequately to protect 50% of the animals against rechallenge. Sabel et al[42] also examined the response triggered by cryosurgery in mammary adenocarcinoma (MT-901) in BALB/c mice. They found higher levels of the type 1 helper cytokines interleukin-2 and interferon gamma and higher tumor rejection rates in animals treated by cryoablation. Cryoablation also increased natural killer cell activity as compared to surgery.

We studied the anti-tumor immune response-generating effects of cryoablation by using a metastatic murine model. Experiments in our laboratory have shown that cryoablation alone does not cause any significant prolongation of survival in mice but cyclophosphamide effectively unmasks a systemic anti-tumor effect of cryoablation, resulting in prolongation of survival and cure of half of the mice with metastatic colon cancer.[43] When these cured animals were rechallenged with double the lethal dose of tumor cells, none of the animals grew tumors. When this anti-tumor response was further studied, it was found to be transferable through splenic and lymph nodal cells of animals treated with cryoablation and cyclophosphamide combination. We showed CD8+ T cells to be the main effectors of this immune response, which agrees with prevalent knowledge. Cyclophosphamide (Cy), an alkylating agent used to treat cancer, has been shown to mitigate the suppression of anti-tumor immunity through effects on regulatory T cells (T_{regs}), which 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.[44,45] T_{regs} are actively recruited and induced by tumors to block the priming or effector function of anti-tumor T cells. Treatment with cyclophosphamide enhances the apoptosis and decreases the homeostatic proliferation of these cells.[46] Flow cytometric analysis of the splenic and lymph node suspensions showed significant reduction in the T_{regs} population in animals treated with cyclophosphamide, thus providing evidence for the effectiveness of the combination therapy. A Phase I clinical trial using cryoablation and cyclophosphamide combination in patients with metastatic prostate and renal cancers resistant to conventional therapy is currently underway. The interim analysis showed a reduction in the rate of progression of metastatic disease in the first 5 patients, although an objective clinical response was not seen.

Whereas development of an antitumor response was shown by most of the studies, some studies did not demonstrate any immune response after cryosurgery. Allen et al[47] studied the effects of cryosurgery by using hepatoma tumors in Buffalo rats. There was no acceleration or regression of tumor growth in animals treated by cryosurgery, nor was there evidence for tumor immunity after cryosurgical ablation. Similar results were reported by Hoffmann et al[48] in a Dunning AT-1 prostate cancer model.

Numerous studies have also reported that cryosurgery of the primary tumor enhanced, rather than reduced, tumor metastasis. Yamashita and Hayakawa showed that cryosurgery of 3-methylcholanthrene-induced tumors in WKA/Hok rats resulted in increased deaths due to metastasis when compared with surgical excision. When cryo-necrotized tissue was surgically removed, a reduction in tumor metastasis was seen. The cryosurgically treated mice also had reduced resistance to tumor rechallenge.[49,50] Friedman et al[51] examined the effects of cryosurgery of normal ventral prostate and intraprostatic injection of complete Freund's adjuvant (CFA) in Copenhagen rats. In contrast with the findings of Lubaroff et al,[41] cryosurgery in combination with CFA injection increased the susceptibility of the rats to tumor challenge by Dunning R3327 MatLyLu prostate adenocarcinoma.

MECHANISMS OF THE CRYO-IMMUNE RESPONSE

Cryoablation of tumor tissue leads to coagulative necrosis of cells. This causes a cellular breakdown and release of intracellular contents and proinflammatory cytokines. In addition to

cytokines, the release of heat shock proteins, DNA, RNA, or the chromosomal protein HMGB1 (high mobility group box chromosomal protein 1) serves as a stimulus for the innate immune response and attracts granulocytes, macrophages, and natural killer (NK) cells. These cells further release cytokines and chemokines. Dendritic cells are the professional antigen presenting cells (APC), which are crucially important in the capture, processing, and presentation of tumor antigens to tumor-specific T cells.[52] The necrotic tumor debris contains endogenous "danger signals," such as HMGB1, which are required for the activation and maturation of APCs. Dendritic cells reach the damaged tissue, take up tumor antigens in a background of inflammation and abundant cytokines, and undergo a change in phenotype with upregulation of cell surface markers.[53] The activated APCs migrate to tumor-draining lymph nodes where they present tumor antigens in the complex of major histocompatibility complex (MHC) molecules as well as costimulatory signals to tumor-specific T cells, inducing their activation. Activated, effector $CD4^+$ T cells can destroy MHC Class II^+ tumors or provide help to a variety of anti-tumor effector cells, including $CD8^+$ cytotoxic T cells, B cells, and macrophages.

AUGMENTATION OF CRYO-IMMUNE RESPONSE FOR IMMUNOTHERAPY OF UROLOGIC MALIGNANCY

Although cryoablation of a solitary tumor focus can provide an animal with protection against re-challenge with the same tumor, the efficacy of this immune response against established metastatic disease has been quite limited. Reports of the regression of metastatic disease in cancer patients after cryoablation of the primary tumor have been anecdotal, and strategies to induce anti-metastatic effects consistently have not been reported. Advanced or metastatic cancer is associated with a number of mechanisms inhibiting anti-tumor immunity including regulatory T cells, suppressive or tolerogenic APCs, and an unfavorable tumor microenvironment. Against these headwinds, provision of tumor antigens and danger signals may be insufficient to reverse a robust state of immunologic tolerance to the tumor. To capitalize on the stimulatory effect of cryotherapy, this response must be intensified by using other immunomodulatory agents to counteract the inhibitory influence of metastatic cancer on anti-tumor immunity. It is necessary to augment this response to achieve a reliable clinical response. Because cryoablation is already being extensively used as a salvage therapy in urologic oncology, modulation of the cryo-immune response may provide a beneficial effect against metastatic prostate and renal cancer.

This intensification can be done in multiple ways. Facilitating the antigen uptake by use of monoclonal antibodies, injecting activated dendritic cells loaded with specific tumor antigens, dendritic cell activation by cytokine injections, using inhibitors against immune checkpoints and intratumoral injection of Toll-like receptor agonists are a few approaches that can be used in conjunction with tumor cryoablation to augment anti-tumor immune responses. We have already shown the effectiveness of T_{reg} depletion in augmenting the anti-tumor immune response. Any of these approaches in combination with tumor cryoablation will give us a cryo-immunotherapeutic strategy for better control of metastatic urologic malignancies.

CONCLUSIONS

With increasing evidence of the ability of the adaptive immune system to fight cancer and the limitations of current treatment options for metastatic cancer, cryo-immunotherapy may provide a promising alternative in managing metastatic renal and prostate cancer and will possibly decrease the morbidity and mortality associated with advanced and metastatic disease.

REFERENCES

1. Korpan NN. A history of cryosurgery: its development and future. J Am Coll Surg 2007;204:314-24
2. Arnott J. Practical illustrations of the remedial efficacy of a verylow or anaesthetic temperature. Lancet 1850;2:257-9
3. Campbell-White A. Liquid air: its application in medicine and surgery. Med Rec 1899;56:109-12
4. Campbell-White A. Possibilities of liquid air to the physician. J Am Med Assoc 1901;36:1354-6
5. Cooper IS, Lee AS. Cryostatic congelation: a system for producing a limited, controlled region of cooling or freezing of biologic tissues. J Nerv Ment Dis 1961;133:259-63
6. Cooper IS. Cryogenic surgery: a new method of destruction or extirpation of benign or malignant tissues. N Engl J Med 1963;268:743-9
7. Cooper IS. Cryogenic surgery of the basal ganglia. JAMA 1962;181:600-4
8. Das K, Benzil DL, Rovit RL, Murali R, Couldwell WT. Irving

S. Cooper (1922-1985): a pioneer in functional neurosurgery. J Neurosurg 1998;89:865-73
9. Gonder MJ, Soanes WA, Smith V. Chemical and morphologic changes in the prostate following extreme cooling. Ann N Y Acad Sci 1965;125:716-29
10. Gonder MJ, Soanes WA, Smith V. Experimental prostate cryosurgery. Invest Urol 1964;1:610-9
11. Gonder MJ, Soanes WA, Shulman S. Cryosurgical treatment of the prostate. Invest Urol 1966;3:372-8
12. Zacarian SA, Adham MI. Cryotherapy of cutaneous malignancy. Cryobiology 1966;2:212-8
13. Crisp WE, Asadourian L, Romberger W. Application of cryosurgery to gynecologic malignancy. Obstet Gynecol 1967; 30:668-73
14. Pandey SK, Milverton EJ, Maloof AJ. A tribute to Charles David Kelman MD: ophthalmologist, inventor and pioneer of phacoemulsification surgery. Clin Experiment Ophthalmol 2004;32:529-33
15. Korpan NN. Hepatic cryosurgery for liver metastases. Long-term follow-up. Ann Surg 1997;225:193-201
16. Korpan NN. Basics of cryosurgery. New York: Springer; 2001
17. Onik G, Porterfield B, Rubinsky B, Cohen J. Percutaneous transperineal prostate cryosurgery using transrectal ultrasound guidance: animal model. Urology 1991;37:277-81
18. Onik GM, Cohen JK, Reyes GD, Rubinsky B, Chang Z, Baust J. Transrectal ultrasound-guided percutaneous radical cryosurgical ablation of the prostate. Cancer 1993;72:1291-9
19. Ritch CR, Katz AE. Prostate cryotherapy: current status. Curr Opin Urol 2009;19:177-81
20. Jones JS, Rewcastle JC, Donnelly BJ, Lugnani FM, Pisters LL, Katz AE. Whole gland primary prostate cryoablation: initial results from the cryo on-line data registry. J Urol 2008; 180:554-8
21. Pisters LL, Rewcastle JC, Donnelly BJ, Lugnani FM, Katz AE, Jones JS. Salvage prostate cryoablation: initial results from the cryo on-line data registry. J Urol 2008;180:559-64
22. Sindelar WF, Javadpour N, Bagley DH. Histological and ultrastructural changes in rat kidney after cryosurgery. J Surg Oncol 1981;18:363-79
23. Barone GW, Rodgers BM. Morphologic and functional effects of renal cryoinjury. Cryobiology 1988;25:363-71
24. Uchida M, Imaide Y, Sugimoto K, Uehara H, Watanabe H. Percutaneous cryosurgery for renal tumours. Br J Urol 1995; 75:132-7
25. Dominguez-Escrig JL, Sahadevan K, Johnson P. Cryoablation for small renal masses. Adv Urol 2008:479495
26. Hafron J, Kaouk JH. Cryosurgical ablation of renal cell carcinoma. Cancer Control 2007;14:211-7
27. Karlsson JO, Cravalho EG, Borel Rinkes IH, Tompkins RG, Yarmush ML, Toner M. Nucleation and growth of ice crystals inside cultured hepatocytes during freezing in the presence of dimethyl sulfoxide. Biophys J 1993;65:2524-36
28. Fraser J, Gill W. Observations on ultra-frozen tissue. Br J Surg 1967;54:770-6
29. Whittaker DK. Mechanisms of tissue destruction following cryosurgery. Ann R Coll Surg Engl 1984;66:313-8
30. Hoffmann NE, Bischof JC. The cryobiology of cryosurgical injury. Urology 2002;60(2 Suppl 1):40-9
31. Yantorno C, Soanes WA, Gonder MJ, Shulman S. Studies in cryo-immunology. I. The production of antibodies to urogenital tissue in consequence of freezing treatment. Immunology 1967;12:395-410
32. Shulman S, Brandt EJ, Yantorno C. Studies in cryo-immunology. II. Tissue and species specificity of the autoantibody response and comparison with iso-immunization. Immunology 1968;14: 149-58
33. Blackwood J, Moore FT, Pace WG. Cryotherapy for malignant tumors. Cryobiology 1967;4:33-8
34. Ablin RJ. Cryoimmunotherapy. Br Med J 1972;3:476
35. Soanes WA, Ablin RJ, Gonder MJ. Remission of metastatic lesions following cryosurgery in prostatic cancer: immunologic considerations. J Urol 1970;104:154-9
36. Soanes WA, Gonder MJ, Ablin RJ. A possible immuno-cryothermic response in prostatic cancer. Clin Radiol 1970;21: 253-5
37. Blackwood CE, Cooper IS. Response of experimental tumor systems to cryosurgery. Cryobiology 1972;9:508-15
38. Bagley DH, Faraci RP, Marrone JC, Beazley RM. Lymphocyte mediated cytotoxicity after cryosurgery of a murine sarcoma. J Surg Res 1974;17:404-6
39. Matsumura K, Sakata K, Saji S, Misao A, Kunieda T. Antitumor immunologic reactivity in the relatively early period after cryosurgery: experimental studies in the rat. Cryobiology 1982;19:263-72
40. Misao A, Sakata K, Saji S, Kunieda T. Late appearance of resistance to tumor rechallenge following cryosurgery. A study in an experimental mammary tumor of the rat. Cryobiology 1981;18:386-9
41. Lubaroff DM, Reynolds CW, Canfield L, McElligott D, Feldbush T. Immunologic aspects of the prostate. Prostate 1981;2:233-48
42. Sabel MS, Nehs MA, Su G, Lowler KP, Ferrara JL, Chang AE. Immunologic response to cryoablation of breast cancer. Breast Cancer Res Treat 2005;90:97-104
43. Levy MY, Sidana A, Chowdhury WH, Solomon SB, Drake CG, Rodriguez R, et al. Cyclophosphamide unmasks an anti-metastatic effect of local tumor cryoablation. J Pharmacol Exp Ther 2009; Epub ahead of print
44. Wang HY, Wang RF. Regulatory T cells and cancer. Curr Opin Immunol 2007;19:217-23
45. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene 2008;27:5904-12
46. Brode S, Cooke A. Immune-potentiating effects of the chemotherapeutic drug cyclophosphamide. Crit Rev Immunol

2008;28:109-26
47. Allen PJ, D'Angelica M, Hodyl C, Lee J, You YJ, Fong Y. The effects of hepatic cryosurgery on tumor growth in the liver. J Surg Res 1998;77:132-6
48. Hoffmann NE, Coad JE, Huot CS, Swanlund DJ, Bischof JC. Investigation of the mechanism and the effect of cryoimmunology in the Copenhagen rat. Cryobiology 2001;42:59-68
49. Hayakawa K, Yamashita T, Suzuki K, Tomita K, Hosokawa M, Kodama T, et al. Comparative immunological studies in rats following cryosurgery and surgical excision of 3-methylcholanthrene-induced primary autochthonous tumors. Gann 1982;73:462-9
50. Yamashita T, Hayakawa K, Hosokawa M, Kodama T, Inoue N, Tomita K, et al. Enhanced tumor metastases in rats following cryosurgery of primary tumor. Gann 1982;73:222-8
51. Friedman EJ, Orth CR, Brewton KA, Ponniah S, Alexander RB. Cryosurgical ablation of the normal ventral prostate plus adjuvant does not protect Copenhagen rats from Dunning prostatic adenocarcinoma challenge. J Urol 1997;158:1585-8
52. Steinman RM. Some interfaces of dendritic cell biology. Apmis 2003;111:675-97
53. den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Frielink C, Toonen LW, et al. Efficient loading of dendritic cells following cryo and radiofrequency ablation in combination with immune modulation induces anti-tumour immunity. Br J Cancer 2006;95:896-905