Prostate Cancer and Prostatic Disease (2015), 1–9

www.nature.com/pcan

REVIEW

Re-purposing cryoablation: a combinatorial 'therapy' for the destruction of tissue

JG Baust[1,2], JC Bischof[3], S Jiang-Hughes[3], TJ Polascik[4], DB Rukstalis[5], AA Gage[6] and JM Baust[7]

It is now recognized that the tumor microenvironment creates a protective neo-tissue that isolates the tumor from the various defense strategies of the body. Evidence demonstrates that, with successive therapeutic attempts, cancer cells acquire resistance to individual treatment modalities. For example, exposure to cytotoxic drugs results in the survival of approximately 20–30% of the cancer cells as only dividing cells succumb to each toxic exposure. With follow-up treatments, each additional dose results in tumor-associated fibroblasts secreting surface-protective proteins, which enhance cancer cell resistance. Similar outcomes are reported following radiotherapy. These defensive strategies are indicative of evolved capabilities of cancer to assure successful tumor growth through well-established anti-tumor-protective adaptations. As such, successful cancer management requires the activation of multiple cellular 'kill switches' to prevent initiation of diverse protective adaptations. Thermal therapies are unique treatment modalities typically applied as monotherapies (without repetition) thereby denying cancer cells the opportunity to express defensive mutations. Further, the destructive mechanisms of action involved with cryoablation (CA) include both physical and molecular insults resulting in the disruption of multiple defensive strategies that are not cell cycle dependent and adds a damaging structural (physical) element. This review discusses the application and clinical outcomes of CA with an emphasis on the mechanisms of cell death induced by structural, metabolic, vascular and immune processes. The induction of diverse cell death cascades, resulting in the activation of apoptosis and necrosis, allows CA to be characterized as a combinatorial treatment modality. Our understanding of these mechanisms now supports adjunctive therapies that can augment cell death pathways.

Prostate Cancer and Prostatic Disease advance online publication, 27 January 2015; doi:10.1038/pcan.2014.54

INTRODUCTION

The modern era of cryoablation (CA) (cryosurgery) began inauspiciously but with lasting impact with the development of a closed tip cryoprobe to treat Parkinsonism and related motion disorders.[1–3] This clinical experience demonstrated that precise, safe and effective cryoablative procedures were possible. In the decades that followed, a wide spectrum of disease states were addressed with impressive results,[4] but in some cases serious co-morbidities[5] resulted from freezing beyond the targeted tissue boundary as intra-operative visualization methodologies had not yet been developed. In the early 1990s intra-operative, real-time ultrasound was joined with CA[6] resulting in the growth of CA through the late 1990s and early 2000s.[7] Today, there is a growing list of disease states targeted with curative intent as our understanding of cancer's responses to low temperature has expanded (that is, ice per se is only a part of the story).[8] Advancements in imaging, treatment planning and devices along with the growing recognition of the curative limitations of radiation and chemotherapy[9–12] provide CA with new clinical opportunities.[13]

To better understand this opportunity, the manner in which we understand cancer has undergone a paradigm change. First, there is a growing acceptance that cancer is an age-related, natural (evolutionary) process. Seventy-seven percent of cancers are diagnosed in individuals aged >55 years.[14]

Second, the historic view of cancer as a clonal population that has lost reproductive control has evolved dramatically with the recognition of the significance of the integrated nature of the tumor microenvironment and the concept of the 'cell of origin' moving from that of a singular, cancer-initiating cell to that of a resistant population of cancer stem cells (CSCs).[15] We now recognize that cancer presents a 'moving target' through continuous mutation and recruitment of stromal cells to support tumor growth, which aids in cancer's ability to defeat today's 'gold standard' monotherapies.

THE BIOLOGY OF CANCER AND THE TUMOR MICROENVIRONMENT

Our understanding of cancer has changed in recent years due primarily to the emergence of three sentinel concepts. Following an initial report by Bonner and Dick[16] in which CSCs were first identified, CSCs are now widely accepted as a key element of tumorigenesis and the likely cell-of-origin or tumor-initiating cell. CSCs are thought to give rise to additional cancer cells both within a primary tumor and at secondary loci as well as demonstrate a capacity to dedifferentiate to form serosal cells and to resist radiation and chemotherapeutic challenges. In addition, CSCs contribute to the recruitment and 'activation' of serosal cells that

[1]Institute of Biomedical Technology, State University of New York at Binghamton, Binghamton, NY, USA; [2]Department of Biological Sciences, Binghamton University, Binghamton, NY, USA; [3]Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, USA; [4]Division of Urology, Department of Surgery, Duke University Medical Center, Durham, NC, USA; [5]Department of Urology, Wake Forest University School of Medicine, Winston-Salem, NC, USA; [6]Department of Surgery, State University of New York at Buffalo, Medical School, Buffalo, NY, USA and [7]CPSI Biotech, Owego, NY, USA. Correspondence: Dr JG Baust, Institute of Biomedical Technology, Binghamton University, State University of New York, PO Box 6000, Binghamton, NY 13902-6000, USA.
E-mail: Baustcryo@aol.com
Received 17 September 2014; revised 18 November 2014; accepted 10 December 2014

Table 1. Hallmarks of cancer

Proliferative signaling
Evade growth suppressors
Resist apoptosis
Induce angiogenesis
Avoid immune system destruction
Reprogram cellular energetics
Enable replicative immortality (CSC)
Recruit non-cancerous stromal cell to create microenvironment
Mobilization = metastasis

Abbreviation: CSC, cancer stem cell.

assume diverse cancer-supporting functions. As the communication links establish between the serosal cells, CSCs and cancer cells, a supportive tumor microenvironment is established. Tumor-associated (activated) serosal cells include endothelial cells that assist in the formation of new blood vessels, fibroblasts with highly diverse functions, including transitions to cancer cells and CSCs, various immune cells resident to the tumor that function to interfere with immuno-surveillance and the reprogramming of mesenchymal stem cells in support of differentiation plasticity.[17] The 'hallmarks of cancer' further provide insight into the defensive strategies that the tumor microenvironment activates in the face of a therapeutic challenge.[18] These characteristics are common biological responses that function to assure cancer survival and growth (Table 1). Repeating monotherapies induce mutation in cellular targets resulting in the activation of defensive strategies. By recognizing the adaptive characteristics of cancers that confer therapeutic resistance, we are better positioned to understand both the benefits and limitations of available treatment options.

IMPACT OF CANCER BIOLOGY AND TUMOR MICROENVIRONMENT ON THERAPEUTIC OUTCOME

An understanding of the scope of the defensive responses is essential to the development of new therapies as multiple 'hallmarks' must be simultaneously targeted. Cancer cells *in vivo* are not necessarily self-sustaining but often rely on cell–cell signaling from a diverse population of stromal cells. Whether paracrine or juxtacrine in origin, multiple agents (that is, hormones, cytokines, chemokines and proteases) constitute essential signaling pathways that support tumor progression. Stromal cells, including endothelial cells, fibroblasts, lymphocytes, macrophages and pericytes, may 'activate' to create a desmoplastic stroma that functions as a complex cross-talk network to support neoangiogenesis, tumor cell proliferation, survival and therapeutic avoidance, migration and invasion.[19] The activated stroma also constitutes a defensive organization of the microenvironment to assure survival through further mutation, epigenetic response and upregulation of pro-survival genes. Hence, the combined defensive capabilities of the tumor microenvironment often defeat the curative intent of today's monotherapies.[10,11] For example, following radiation therapy for prostate cancer, $>25\%$ of patients will have positive biopsies and up to one-third will receive secondary radiation treatment for cancer recurrence. Radioresistant prostate cancer is now the fourth most common genitourinary malignancy and is representative of an emerging epidemic.[20]

Central issues with both radiation and chemotherapy relate to the biology of cancer. Both therapeutic strategies primarily attack actively dividing cancer cells in specific cell cycle stages. Non-dividing cancer and CSCs are weakly affected. To address this, treatment plans are repetitive by design and aimed at inducing death in cells as cells progress through the cell cycle. Unfortunately, repetitive treatment accelerates the appearance of protective (defensive) mutations, resulting in a selected cancer population refractory to re-treatment, alternative attempts at therapeutic intervention[21] and the induction of CSC quiescence.[22]

Accordingly, today's challenge is to develop new treatment options that are (a) non-repetitive denying cancer cells the ability to activate defensive mutations; (b) capable of activating multiple cell death cascades thereby limiting cancer's ability to induce survival mechanisms; (c) not susceptible or dependent on cell cycle and (d) destructive of all cells within the tumor microenvironment eliminating both cancer cells and CSCs while launching a local immunological response not influenced by tumor-associated immune cells.

Table 2. Mechanisms of cryoablation

Hypothermia	
Metabolic uncoupling	Molecular
Energy deprivation	Molecular
Free radical—waste accumulation	Molecular
Ionic imbalance	Molecular
pH shifts (acidosis)	Molecular
Membrane phase transitions	Molecular—structural
Cytoskeletal disassembly	Molecular—structural
Freezing	
Extracellular ice	Molecular—structural
Hyperosmolality	Molecular
Cell volume disruption	Molecular—structural
Protein denaturation	Molecular
Intracellular ice	Structural—molecular
Shearing and membrane disruption	Structural
Vascular stasis	Molecular
Activation of 'local' immune response	Molecular

MECHANISMS OF CA PRECISION

An understanding of the cell-based responses to a freeze insult and the clinical schema for applying a freeze–thaw excursion reveal that a critical consequence of CA is the disruption of most of the hallmarks of cancer. This results in complete cell death provided a cell-specific nadir temperature is reached throughout the tumor . Within the frozen region where a cancer-specific nadir temperature is not attained (0° to $-30\,^{\circ}C$, referred to as the 'margin of the iceball'), physical destruction is less prevalent. It is the activation of molecular-based cell death (apoptosis and necrosis) resulting from structural, metabolic, chemical, immune and vascular perturbations that become more dominant. As such, the ablative outcome is ultimately dependent on both physical (structural) events attendant to ice formation and the initiation of cell death cascades, which effectively short circuit cancer cell's defensive capabilities. It is now well established there are multiple modes of cell death associated with CA that are dependent on stress severity. These mechanisms, categorized into molecular (apoptosis and/or necrosis) or structural (physical), are initiated by a complex series of events caused by freezing (Table 2).

The application of CA follows a series of sequenced events that constitute a 'best practice' application of CA to deliver a 'therapeutic dose'[23] (Table 3). Following cryoprobe placement and activation, heat is extracted from the targeted tissue at variable rates depending on proximity to the probe and cooling capacity of the cryogen.[8,24,25] For the tissue adjacent to the cryoprobe, rapid cooling rates form both extracellular and intracellular ice. As ice propagates distally from the cryoprobe, cooling rates are reduced wherein the cancer cells experience a myriad of other disruptive events and stress factors (Table 2), resulting in the activation of diverse cell death cascades.[26]

As freezing progresses, ice will initially form outside the cell within the extracellular space compromising tissue morphology and abrading endothelial cells from the capillaries.[6,27,28] Further, as extracellular ice forms, solutes are excluded causing osmotic pressure to rise from ~350 mOsm to ~ 8000 mOsm resulting in

cell dehydration and shrinkage creating a lethal hyperosmotic state. Ultimately, ice forms within the cell when temperatures are low enough. Rapid cooling at a rate of -20 to $-50\,^{\circ}C\,min^{-1}$ limits cellular dehydration thereby supporting intracellular ice formation. Whereas, at slower cooling rates, cancer cells can effectively dehydrate and potentially avoid intracellular freezing.

Depending on the cryogen system employed, complete freezing is indicated by both the attainment of the targeted nadir temperature within the freeze zone and distal physical boundary of freeze zone (Figures 1 and 2). For example, argon cryoprobes form a ~3.0-cm diameter freeze zone in ~10 min while a supercritical nitrogen cryoprobe will form the same size freeze zone in 3 min.[29,30] Ice per se is not an indication of complete cell death. Cells die in progressively greater numbers as temperature is lowered. As cancer cells' death to low temperature is expressed as a curvilinear response, a robust nadir temperature is indicated. Gage and Baust[31] provided a listing of cell-specific lethal temperatures but teach that $-40\,^{\circ}C$ is a clinically relevant nadir for most cancers keeping in mind that some cells within the tumor microenvironment, such as CSCs or cells demonstrating further mutation following other therapies, may be more resistant.[32]

The duration at the nadir temperature has not been studied extensively but maintenance for a period of 2–5 min is common practice. Warming is highly destructive and slow passive warming from surrounding unfrozen tissue is preferred. During thawing (Note: At the molecular level, ice surface melting begins as temperatures are elevated between -40 and $-30\,^{\circ}C$.), ice crystals re-arrange and coalesce into larger crystals (recrystallization) that may shear tissue anatomy (a phenomena akin to frost heaves on the roadway), and the functional duration of the damaging hyperosmolality is extended as is the interval of oxidative stress. An *in vitro* study of a set of parameters related to the thermal history experienced within a typical cryosurgical freeze zone suggested that in prostate cancer reducing the end temperature (-80 vs $-20\,^{\circ}C$) and/or increasing the hold time (15 vs 0 min) can increase cell death.[33] Another *in vitro* prostate cancer study demonstrated that increased hold times (5 and 10 vs 15 min) at warmer subfreezing temperatures (that is, $> -30\,^{\circ}C$) yields greater cell death, whereas once $-40\,^{\circ}C$ is reached, a hold time of ~1–2 min achieves maximal cell destruction.[34]

Table 3. Events of the freeze–thaw cycle

Event	*Best practice*
1. Cooling rate	Rapid
2. Nadir temperature	Variable (−40 °C nominal)
3. Duration at nadir temperature	5 minutes
4. Thaw rate	Slow—passive
5. Repetition of F–T cycle	2×
6. F–T interval	Duration to match freeze time
7. Second freeze	Repeat 1–4

Abbreviation: F–T, freeze–thaw. Note: Progression of the freeze event typically monitored by intra-operative, real-time ultrasound.

In practice, the nadir temperature is not found at the edge of the freeze zone but some distance within the frozen mass. This necessitates an extension of the freeze margin beyond the tumor boundary (Figure 1). In some cases the limited damage beyond the tumor boundary may be considered problematic (that is, prostate, heart, kidney and so on) due to co-morbidities. To address this, it is common practice when treating cancer with CA to apply a dual freeze cycle (freeze–thaw–freeze) providing a repeat set of stresses to augment the destructive effects of the first freeze and extend the lethal boundary (Figure 1). The interval ($\geqslant$ 3–5 min) between the first and second freeze is consequential. Following this interval, the second freeze is initiated in now hypothermic tissue, yielding more rapid and extended freezing. The lethal temperature of the targeted cells is elevated owing to the accumulation of stress factors.[35] As detailed below, current research is focused on the identification of freeze-sensitizing agents with the goal of functionally elevating the nadir temperature to approximately $-1\,^{\circ}C$.[26,36–45]

The net outcome of CA is that each of the 'hallmarks' is challenged either on a molecular and/or physical level. In effect, CA, as an energy-depletion therapy, provides a combinatorial assault overwhelming a cancer cell's defensive responses and preventing further mutation. The ablative consequences of freezing follow a relatively defined chronology. Cells that rupture release cytokines and chemokines, which function to recruit circulating immune cells launching a local, as well as potentially a systemic, immune response.[46] This freeze rupture-dependent necrosis occurs during the first hour postthaw. Apoptotic processes begin upon thawing and reach maximum levels within 12–24 h. The timing and contribution of apoptosis and necrosis has been shown to vary based on cell's sensitivity to freezing.[26,32,35,40] Indeed, endothelial cells are among the most freeze-sensitive cells.[47–49] Thus, as endothelial cells slough or die, the underlying basement membrane of the microvasculature within the tumor is exposed leading to thrombosis and circulatory stasis within a few hours. Once the vasculature is static, the tumor becomes starved for oxygen and nutrients leading to 'ischemic necrosis' peaking within a week.[48] Throughout this period, cells that were damaged but not ruptured during freezing add to a cell death continuum. In association with inflammation, delayed necrosis may continue for weeks.

ADJUVANTS FOR CRYOSURGICAL ENHANCEMENT

In recent years, new adjuvants have been sought to increase the destructive effect of freezing in an attempt to increase tissue destruction in the iceball periphery where temperatures range from 0 °C to $-40\,^{\circ}C$, nominally. Incomplete cell destruction within

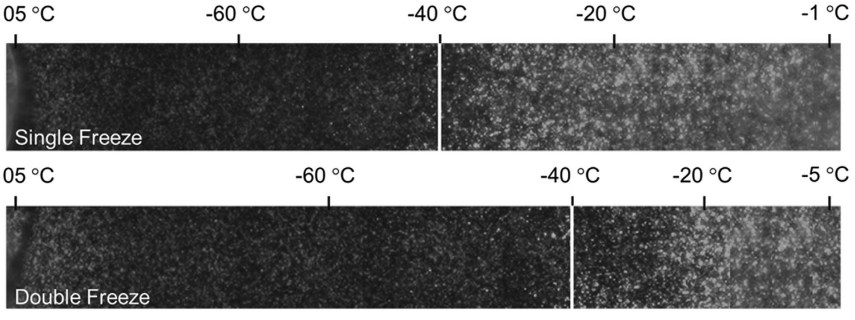

Figure 1. Comparison of the extent of cell death following a single or double freeze interval using a human prostate cancer cell TEM. TEM's were seeded with prostate cancer cells (pTEM) and the pTEMs were frozen using an argon JT cryoprobe following a single 10 min or double 10 min freeze protocol. pTEMs were assessed 24 hours following thawing using the Calcein-AM/P1 assay via fluorescence microscopy.

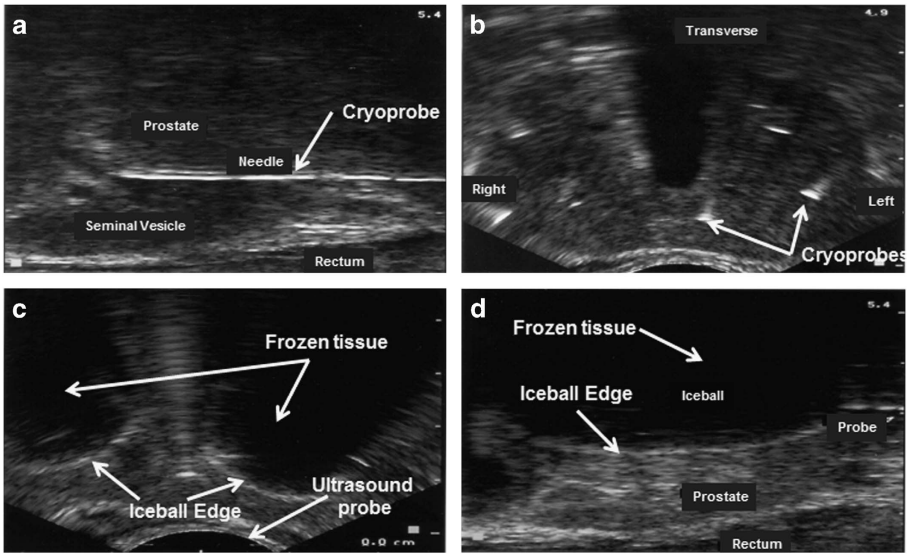

Figure 2. Ultrasound (US) images of a prostate during a cryosurgical procedure. (**a**) Sagittal US view during cryoprobe placement into the prostate. (**b**) Transverse view of the prostate following cryoprobe insertion. (**c**) US image of the prostate during freezing. Frozen tissue appears as a dark region due to the reflectance of the US waves. The edge of the frozen mass appears as a white hyperechoic rim. (**d**) Image of iceball as the frozen mass extends down towards the rectum and US probe.

the periphery makes it challenging to predict the outcome based on freeze zone imaging alone (ultrasound, computed tomography or magnetic resonance imaging).[6,50] As such, two limitations remain in the use of cryosurgery for cancer: (1) tumor recurrence where non-lethal conditions are applied and (2) morbidity associated with freezing adjacent structures.[51] Therefore, cryosurgical adjuvants are now being explored as a means of destroying a tumor more effectively during freezing to create an overlap of the iceball and effective kill zone. This would enable imaging technologies (including ultrasound, computed tomography and magnetic resonance imaging) to precisely visualize the 'lethal zone' (Figure 3). Whether or not adjunctive agents are employed, visualization of the advancing freeze zone with ultrasound is precise (±1–2 mm). This adjunctive strategy is designed to accentuate the tissue injury mechanisms known to be induced by freezing. Further, much of this research has focused on the use of sub-lethal (minimally toxic) doses of agents that when combined with freezing results in a highly lethal '1–2 punch' to the targeted region.[35,52] Four categories of cryoadjuvant have been reviewed by Goel *et al.*,[38] including (1) thermophysical adjuvants, (2) chemotherapeutics, (3) proinflammatory cytokines or vascular-based agents and (4) immunomodulators. These categories are non-exclusive. Further, other strategies, including TIA (targeted initiation or inhibition of apoptosis) and neutraceutical-based sensitization, have also been reported to be effective when combined with freezing in both *in vitro* and animal studies.[39,45,53] An overview of select cryoablative adjuvants studied is presented in Table 4 (*in vitro*) and Table 5 (*in vivo*).

Thermophysical adjuvants

Thermophysical adjuvants modify the shape of ice crystals during freezing enhancing the mechanical destruction of the cell membranes.[54–57] These adjuvants include antifreeze proteins, salts (that is, NaCl, KCl) and some amino acids (that is, glycine). These adjuvants can enhance the threshold injury temperature up to − 5 °C.[42,43,57,58] However, a major challenge is delivering high concentration to achieve the desired effect without causing toxicity.

Chemotherapeutics

The synergistic effect of CA and anticancer drugs (chemotherapeutics) was first reported using peplomycin and adriamycin in combination with freezing.[59] Synergy was due to the increase in vascular volume and permeability immediately after cryosurgery. Therefore the frozen tumor preferentially traps the anticancer drugs as compared with untreated tumor. As apoptosis has been recognized as a critical mode of cell death in CA, many studies have shown the benefits of using apoptosis inducers combined with CA to enhance cell destruction at mild temperatures (−40 to − 0.5 °C). These inducers include 5-fluorouracil, cisplatin, doxorubicin, mitomycin and bleomycin, vinorelbine, navelbine and tumor necrosis factor (TNF)-related apoptosis-inducing ligand.[60–66] As most of these studies have been performed *in vitro*, there remains a need to repeat these studies *in vivo* to establish clinical benefit. Although further studies are needed, reports by Forest *et al.*[62,63] have demonstrated the benefit and established feasibility of cryochemo combinations *in vivo*.

Pro-inflammatory cytokines and vascular agents

Several studies have shown that the pro-inflammatory cytokine TNF-α, with appropriate dose and timing, can be used with CA to destroy the tumor up to the freeze zone edge (−0.5 °C).[67,68] TNF-α is a multifunctional cytokine that can induce complex actions on the tumor cells, vasculature and immune cells by activating different intracellular pathways (apoptosis vs inflammation). Knowledge of the exact mechanisms of TNF-α enhancement following CA are being studied. In addition, as systemic toxicity induced by TNF-α is a major clinical limitation, more selective or targeted delivery of TNF-α using nanoparticles continues to be an area of active research.[69]

Immunomodulators

As antigen-presenting cells (especially dendritic cells) have a pivotal role in the induction of adaptive immune response, several studies have shown an ability to enhance cryosurgical destruction by inducing and stimulating dendritic cells in the injury site

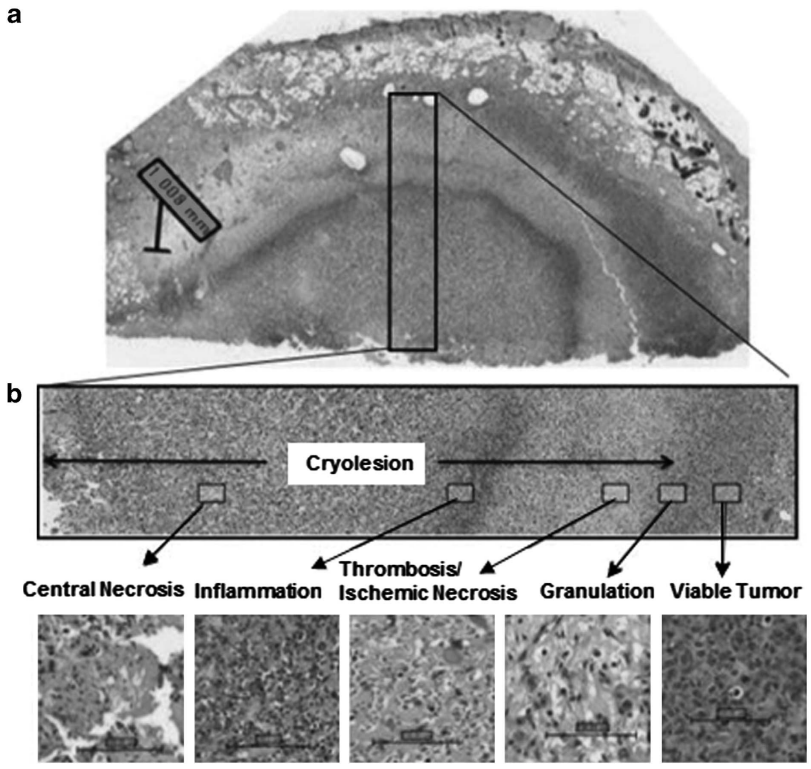

Figure 3. Identification of the zones of cell death within a tissue following cryosurgery. Five histological zones were evident after cryosurgery: central necrosis, inflammation, thrombosis/ischemic necrosis, granulation tissue influx, and viable tumor. (**a**) Demonstration of these histological zones at day 3 post cryosurgery (x20 objective; scale bar = 1 mm). (**b**) Demonstration of these histological zones at x40 objective magnification (scale bar = 100 μm (Adapted from Jiang, *et al*. Cryobiology, 2010).

Table 4. *In vitro* adjuvant for cryosurgical enhancement

Mechanism	*Model*	*Adjuvant*	*Dose*	*Time*	*Reference*
Direct cell injury					
Thermophysical adjuvant: ice crystals	Normal rat liver	ATP-1	10 mg ml^{-1}	0–5 min before FT	54
	Rat prostate	Eutectic salts	Eutectic conc.	0–5 min before FT	42
	Human breast	Glycine	10 g ml^{-1}		58
Chemotherapeutics: apoptosis, antiproliferative	Human colorectal	Cisplatin	25 μg ml^{-1}	2 days before FT	52
	Human prostate	5-FU	25 μg ml^{-1}	2–4 days before FT	60
	Human prostate	5-FU[a]	25 μg ml^{-1}	2 days before FT	37
	Human prostate	Cisplatin	25 μg ml^{-1}	2 days before FT	52
	Human prostate	TRAIL	500 ng ml^{-1}	0–5 min before FT	61
	Human renal	5-FU[a]	100 μg ml^{-1}	24 h before freeze	81
	Human hepatoma	Doxorubcin[b]	55 μg ml^{-1}	24 h before FT	66
Inflammatory cytokines: apoptosis, inflammation	Human prostate	TNF-α	1–1000 ng ml^{-1}	4 h before FT	67
	Human endothelial	TNF-α	1–1000 ng ml^{-1}	4 h before FT	67
	Human skin	Imiquimod	100 ng ml^{-1}	24 h before FT	75
	Human prostate	TNF-α	1 μg ml^{-1}	4 h before FT	68

Abbreviations: FT, freeze–thaw; 5-FU, 5-fluorouracil; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand. Adapted from Goel *et al.*[38]
[a]Enhancement was also reported with other drugs: cisplatin, etoposide, and taxotere. [b]Enhancement was also reported with other drugs: mitomycin, 5-FU, and cisplatin.

utilizing agents such as imiquimod, CpG-oligodeoxynucleotides, BCG-CWS (Bacillus Calmette–Guerin cell wall skeleton). GM-CSF (granulocyte macrophage-colony-stimulating factor) and Saponins.[70–75] This topic has been recently reviewed[46] and is being more fully explored using anti-CTLA-4 antibody blocking.[76] The future selection and targeted use of immunomodulators appears promising. However, challenges include the need to better understand and harness the immunological mechanisms stimulated by cryosurgery vs other forms of focal therapies, such as heat, irreversible electroporation or others.[77] Investment in research that addresses this will position cryosurgery as a technique to treat both local and systemic (that is, disseminated) disease.

Table 5. *In vivo* adjuvant for cryosurgical enhancement

Mechanism	*Model*	*Adjuvant*	*Dose*	*Time*	*Reference*
Direct cell injury					
Thermophysical adjuvant: ice crystals	Human prostate	AFP-1	10 mg, local	0–5 min before FT	57
	Rat prostate	Flounder AFP	0.5 mg, local	0–5 min before FT	56
Chemotherapeutics: apoptosis, antiproliferative	Human lung	Navalbine	120 μg, i.v.	Multiple time points	62
	Human lung	Vinblastine	120 μg, i.v.	20 h after FT, 15 days after FT	63
	Human prostate	5-FU	15–20 ng, local	0–5 min after FT	64
	Mouse prostate	Vitamin D_3	4.0 μg kg^{-1}, local	18 h prior to FT	45
Vascular injury inflammatory cytokines: apoptosis, inflammation	Human prostate	TNF-α	0.2–1 μg, local	4 h before FT	67,68
	Human prostate	TNF-α	5 μg, i.v.	4 h before FT	67
Immune injury	Mouse melanoma	Imiquimod	3.12 mg, local	Up to 10 days after FT	72
	Mouse melanoma	CpG-ODN	100 μg, local	1 h after FT	73
	Mouse colon	BCG-CWS	200 μg	Days 1, 4, 7 and 10 after FT	70
	Mouse prostate	Anti-CTLA-4 mAb	200 μg	Days 1, 4, 7 and 10 after FT	76
	Mouse melanoma	Saponin	40 μl of 500 μg ml^{-1} sol	30 min after FT	74

Abbreviations: AFP, antifreeze proteins; BCG-CWS, Bacillus Calmette–Guerin cell wall skeleton; CpG-ODN, CpG-oligodeoxynucleotides; FT, freeze–thaw; 5-FU, 5-fluorouracil; i.v., intravenous; mAb, monoclonal antibody; TNF, tumor necrosis factor. Adapted from Goel *et al.*[38]

Other areas of sensitization

Several other categories and agents have shown promise in increasing cancer cell's sensitivity to freezing. These include targeted initiation or inhibition of cell death or survival pathways[78] as well as exploration of natural anti-cancer compounds (neutraceuticals). These agents are designed to increase the lethality of mild freezing while having little or no negative side effects Studies investigating the impact of cell attachment and signaling (integrin) inhibition,[79] AKT modulation[80] and caspase activation[26,81] among others have shown benefit in enhancing cell death as well as have a key role in elucidating the mechanisms of action involved in cancer cell's response to freezing.

The success of cryosensitization strategies coupled with the desire to minimize side effects (both systemic and localized) support more recent efforts focused on the use of neutraceuticals such as vitamin D_3 (VD_3) and Resveratrol. *In vitro* and *in vivo* studies have demonstrated the benefits of Calcitrol (VD_3 analog) to increase prostate cancer's freeze sensitivity.[39,45,53] These studies have shown that pretreatment with VD_3 results in complete cancer destruction at ~ − 5 °C, which correlates with the outer edge of the freeze zone under ultrasound. Other studies utilizing the free radical scavenger Resveratrol have shown that pretreatment of renal and prostate cancer cells sensitizes them to freeze injury yielding complete cell death at − 10 °C *in vitro*.[61] Given the promise of these and other reports, investigations and trials incorporating these agents are ongoing.

SYNOPSIS OF CRYOSURGICAL CLINICAL OUTCOME

In 2007, the American Urological Association published guidelines for the management of clinically localized prostate cancer.[82] At that time, long-term randomized and controlled trials supporting the utility of cryosurgery compared with traditional radiation or surgery were lacking. As such, the 2007 meta-analysis of localized prostate cancer treatments did not include cryosurgery, leading to the release of a Best Practice Policy Statement on Cryosurgery for the Treatment of Localized Prostate Cancer[23] by the American Urological Association in 2008. This 2008 publication identified CA as a first-line therapy for localized prostate cancer based on numerous 5- and 7-year outcome reports and the first 10-year single-center patient follow-up report.[83]

To better understand the oncological outcomes of CA reported in the literature, one must take into account that the technology has evolved. Studies with the longest follow-up report on first-generation liquid nitrogen systems in combination with second- and third-generation argon gas devices. It should be noted, however, that treatment efficacy is dependent on target tissue exposure to nadir temperature and not necessarily the cryogen of choice. Finally, most early series grouped the various disease stages rather than separately reporting outcomes based on individual clinical stage.

Understanding indicators of success after treatment

There have not been well-established PSA level thresholds (for example, absolute PSA cut points) or kinetics to determine success/failure following prostate CA. This is reflected in a range of posttherapy nadir values reported based on criteria adapted from radiotherapy, including the ASTRO 1997 definition of three consecutive PSA rises after nadir and the 2006 Phoenix definition of PSA nadir+2 ng ml^{-1}.[84] Additionally, while posttherapy biopsy data are included in many reports, there are no established standards with some authors taking biopsies routinely at set time points while others reserve biopsy 'for cause with clinical suspicion' based on increasing PSA levels. Along with timing, the number and pattern of biopsies also varies.

Few studies have evaluated the association between biochemical relapse and biopsy-proven recurrence. Caso *et al.*[85] evaluated a number of PSA parameters that could predict biopsy-proven recurrence. Time of undetectable (< 0.2 ng ml^{-1}) PSA was an independent predictor of recurrence with a 9% decrease in the risk of a positive biopsy for each additional month of undetectable PSA.[86] Levy *et al.*[87] found that postcryo nadir PSA correlated with biochemical disease-free survival (bDFS). A PSA level of > 0.6 ng ml^{-1} correlated with a 29% biochemical failure rate at 2 years regardless of D'Amico risk stratification. Pitman *et al.*[88] evaluated a number of biochemical definitions of failure compared with a 12-core surveillance biopsy performed in a cohort of patients with clinical suspicion of prostate cancer recurrence following primary, salvage and focal CA and found the Phoenix definition to be most accurate for biochemical failure.[88]

Oncological outcomes: primary cryotherapy

Recognizing the range of definitions for biochemical recurrence used in the literature, overall biochemical progression-free survival has ranged between 56% and 92% based on patients treated with

primary prostate cryotherapy with at least 5-year outcomes.[83,89] Long *et al.*[90] reported on 975 men with 5-year follow-up and using a disease-free PSA cut point of $< 0.5\ ng\ ml^{-1}$ found that 60% of men in the low- and intermediate-risk groups were bDFS. Bahn *et al.*[91] reported on 590 men treated with CA having 7-year outcomes and using a PSA cut point of $< 0.5\ ng\ ml^{-1}$ reported bDFS rate of 64–70% in all the three D'Amico risk groups.[92] Of note, in the studies by Bahn *et al.*[91] and Long *et al.*,[90] using a more liberal PSA cut point of $< 1.0\ ng\ ml^{-1}$, bDFS ranged from 76% to 87%, 71–79% and 45–71% in the low-, intermediate- and high-risk categories, respectively. Cohen *et al.*[83] reported on 370 men with T1–T3 disease consecutively treated with primary cryotherapy with a median follow-up of 12.5 years and found an overall negative biopsy rate of 77%. Applying Phoenix, Kaplan–Meier curves identified bDFS rates of 81, 74 and 46% at 10 years for the low-, intermediate- and high-risk groups, respectively.

In an effort to overcome the paucity of single-center data, the Cryo On-Line Data (COLD) registry was created to pool clinical information. Comprising 6 academic medical centers and ~34 community urologists, the registry contains all CA outcome data from these sites. Data from the COLD registry show an average age of 70 ± 8 years for the 1198 patients treated. Pretreatment PSA averaged $9.6 \pm 8.6\ ng\ ml^{-1}$, and the median biopsy Gleason score was 7 (range: 4–10). Mean follow-up was 24 ± 26 months, although 136 men had at least 5-year outcomes. Overall bDFS was 77 ± 2 % by ASTRO and 73 ± 2% by Phoenix. Based on ASTRO, bDFS was 85 ± 4, 73 ± 4 and 75 ± 4% for the D'Amico low-, intermediate- and high-risk groups. Based on Phoenix, those same bDFS rates are 91 ± 3, 79 ± 4 and 62 ± 5%, respectively. Dhar *et al.*[93] reported on 860 men aged > 75 years in the COLD registry treated with primary prostate CA and found using ASTRO that the 5-year bDFS rates were 82 ± 8, 78 ± 6 and 78 ± 8% for the D'Amico low-, intermediate- and high-risk groups.

Salvage prostate cryotherapy outcomes

Patients who fail radiation therapy and other *in situ* primary treatments represent a challenging group. These patients have high-risk cancer, and according to surgical pathology based on a retrospective analysis of an international, multi-institutional cohort salvage radical prostatectomy series, extra-prostatic extension is present in 45%, seminal vesicle invasion in 30% and lymph node involvement in 16%.[94] It is also believed that many patients who fail radiotherapy come to the attention of the urologists very late in the course of events[95] and may have micro-metastatic disease at the time of salvage therapy. It is not surprising therefore that oncological success in the salvage setting is reduced compared with that of a primary setting. Despite this, CA is recognized as a first-line salvage therapy especially as technical improvements have further reduced the side effect profile.[95,96] In a recent review, Mouraviev *et al.*[96] report that bDFS following salvage CA is estimated to be 50–70% at 5-year follow-up in properly selected patients but noted that definitions of PSA failure vary limiting comparisons between the different salvage methodologies.[96] When salvage CA is used for radiotherapy failure, Pisters *et al.*,[97] reporting on 279 patients from the COLD Registry, demonstrated a bPFS rate of 55% and 59% using Phoenix and ASTRO, respectively. Speiss *et al.*[98,99] used the COLD registry to report on the percentage of patients undergoing salvage CA who achieved the therapeutic 'bifecta' of a postcryo nadir PSA $< 0.6\ ng\ ml^{-1}$ and who remained continent. In 183 evaluable men, the bifecta was achieved in 73%, with mean duration of follow-up being 3 years. Williams *et al.*[100] reported a single-center series of 176 patients with an average follow-up of 7.5 years, with 30% of patients evaluated > 10 years. In this study, the 10-year DFS was 39% although the more favorable patients with a presalvage PSA $< 5\ ng\ ml^{-1}$ had a 10-year DFS of 64%.

In the largest series of salvage CA patients treated to date in a single center, 328 evaluable men were followed for a mean of 48 months, with 38% and 11% of patients having had at least 5- and 10-year follow-up, respectively. Disease-specific survival (DSS) was 91% and 79%, and DFS was 63% and 35%, respectively, at 5 and 10 years. Patients who eventually died of prostate cancer had a significantly higher median PSA level prior to salvage CA (6.1, range: 0.1–112.4 $ng\ ml^{-1}$) compared with those who survived (4.0, range: 0.1–45 $ng\ ml^{-1}$). Only the PSA nadir following salvage CA was significantly associated with cancer recurrence and DSS in multivariable analyses.[101]

Renal CA outcomes

Cryotherapy has been utilized to treat kidney tumors with success for nearly two decades. This approach has included open, laparoscopic and percutaneous methods, with the latter two being more common. In general, renal CA targets smaller tumors (< 3.5 cm), and patients are generally older and in overall poorer health.[102,103] The renal cryotherapy series tend to encompass fewer patients, and the majority of reports in the literature represent short-term outcomes.

Aron *et al.*[102] report on a group of 340 patients treated with laparoscopic renal CA by a single surgeon and prospectively followed. The median follow-up was 8 years (range 5–11 years), with 80 of those patients having had a minimum of 5-year follow-up. Posttreatment surveillance included a biopsy at 6 months and an annual magnetic resonance imaging. Mean tumor size was 2.3 cm (0.9–5.0 cm) and median American Society of Anesthesiologists score was 3. The 5-year DSS and DFS was 92% and 81% and at 10 years were 83% and 78%, respectively.

Lusch *et al.*[103] reported on 5-year outcomes for a large international multi-institutional experience from eight academic medical centers. Of the 256 evaluable patients treated with either percutaneous or laparoscopic renal CA, 149 had a minimum of 5-year (range 60–106 months) follow-up. Mean tumor size was 2.6 cm (0.9–6 cm) with a median American Society of Anesthesiologists score of 3. Five-year DSS and DFS rates were 96% and 87%, respectively.

SUMMARY

The complexity of cancers' defensive strategies challenge the concept of 'cure' when the treatment options are limited to traditional monotherapies. Many emerging therapeutic strategies rely on additive, sequenced (bundled) monotherapies with the intent of targeting a second or even a third defensive cell signaling pathway. CA offers a multiplicity of damaging consequences to all cells within the tumor microenvironment thereby creating a lethal state incompatible with initiation of any defensive strategy, including growth, re-differentiating or senescence. Even before the disruptive physical effects of the freezing cascade (that is, physical damage to cell membranes and hyperosmotic shock), cells of the tumor microenvironment experience severe oxidative stress due to thermal energy depletion. Although not immediately lethal, the multiple stressors (Table 2) initiated upon thawing result in the activation of a diverse molecular response leading to the post-operative activation of cell death cascades.

A key feature of CA is the non-repetitive or single-session application denying cells within the tumor environment, including cancer cells, CSCs, tumor-associated fibroblasts and macrophages, from launching defensive strategies or further mutation. Upon thawing, structurally damaged cells release chemokines and cytokines as they undergo necrosis. For cells that may avoid a lethal level of membrane disruption (that is, in the freeze zone margin), apoptotic cascades are initiated over the initial 12–24-h postthaw period. As apoptosis is an energy-requiring process, the destruction of the tumor vasculature yields a hypoxic

environment, resulting in a transition from apoptosis to secondary necrosis. The duration of this secondary necrosis is related to tumor volume and can last days to weeks.

The molecular challenges faced by cancer cells during and following CA are sufficient to prevent the induction of survival mechanisms. Although dividing cells are likely more susceptible to the process, any cell exposed to a nadir temperature of − 20 to − 50°C will be ablated regardless of cell cycle stage. The use of 'cryo-sensitizers' enhances cell lethality to assure complete destruction throughout the freeze zone. In effect, cryoablative strategies provide a combinatorial challenge to the tumor microenvironment not attained with traditional monotherapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This report was supported in part from funding from the NIH and CPSI Biotech. We thank Ms Sara E Palmer for her diligent efforts in the preparation of this manuscript.

REFERENCES

1 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**: 259–263.
2 Cooper IS. Cryobiology as viewed by the surgeon. *Cryobiology* 1964; **1**: 44–51.
3 Cooper IS. Cryogenic surgery for cancer. *Fed Proc* 1965; **24**: S237–S240.
4 Gage AA. Current status of cryosurgery for cancer. *Compr Ther* 1978; **4**: 24–30.
5 O'Donoghue EP, Milleman LA, Flocks RH, Culp DA, Bonney WW. Cryosurgery for carcioma of prostate. *Urology* 1975; **5**: 308–316.
6 Onik GM, Cohen JK, Reyes GD, Rubinsky B, Chang Z, Baust JG. Transrectal ultrasound-guided percutaneous radical cryosurgical ablation of the prostate. *Cancer* 1993; **72**: 1291–1299.
7 Gage AA, Baust JG. Cryosurgery for tumors. *J Am Coll Surg* 2007; **205**: 342–356.
8 Baust JG, Gage AA, Klossner DP, Clarke D, Miller R, Cohen J *et al.* Issues critical to the successful application of cryosurgical ablation of the prostate. *Technol Cancer Res Treat* 2007; **6**: 97–109.
9 Chabner BA, Roberts TG Jr. Timeline: chemotherapy and the war on cancer. *Nat Rev Cancer* 2005; **5**: 65–72.
10 Ghisolfi L, Keates AC, Hu X, Lee DK, Li CJ. Ionizing radiation induces stemness in cancer cells. *PLoS One* 2012; **7**: e43628.
11 Sun Y, Campisi J, Higano C, Beer TM, Porter P, Coleman I *et al.* Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. *Nat Med* 2012; **18**: 1359–1368.
12 Argyriou AA, Kyritsis AP, Makatsoris T, Kalofonos HP. Chemotherapy-induced peripheral neuropathy in adults: a comprehensive update of the literature. *Cancer Manag Res* 2014; **6**: 135–147.
13 Gage AA, Baust JM, Baust JG. Experimental cryosurgery investigations in vivo. *Cryobiology* 2009; **59**: 229–243.
14 Howlader N, Noone A, Krapcho M, Garshell J, Neyman N, Altekruse S *et al.* National Cancer Institute: SEER Cancer Statistics Review, 1975–2010 (based on November 2012 SEER data submission, posted to the SEER web site, April 2013). Available from http://seer.cancer.gov/csr/1975_2010/. Last accessed on 13 April 2013.
15 Shackleton M, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. *Cell* 2009; **138**: 822–829.
16 Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730–737.
17 Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012; **21**: 309–322.
18 Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011; **144**: 646–674.
19 Togo S, Polanska U, Horimoto Y, Orima A. Carcinoma-associated fibroblasts are a promising therapeutic target. *Cancers* 2013; **5**: 149–169.
20 Jones JS. Radiorecurrent prostate cancer: an emerging and largely mismanaged epidemic. *Eur Urol* 2011; **60**: 411–412.
21 Corn PG. The tumor microenvironment in prostate cancer: elucidating molecular pathways for therapy development. *Cancer Manag Res* 2012; **4**: 183–193.
22 Moltzahn F, Thalmann GN. Cancer stem cells in prostate cancer. *Transl Androl Urol* 2013; **2**: 242–253.
23 Babaian RJ, Donnelly B, Bahn D, Baust JG, Dineen M, Ellis D *et al.* Best practice statement on cryosurgery for the treatment of localized prostate cancer. *J Urol* 2008; **180**: 1993–2004.
24 Bischof J, Christov K, Rubinsky B. A morphological study of cooling rate response in normal and neoplastic human liver tissue: cryosurgical implications. *Cryobiology* 1993; **30**: 482–492.
25 Bischof JC, Bastacky J, Rubinsky B. An analytical study of cryosurgery in the lung. *J Biomech Eng* 1992; **114**: 467–472.
26 Robilotto AT, Baust JM, Van Buskirk RG, Gage AA, Baust JG. Temperature-dependent activation of differential apoptotic pathways during cryoablation in a human prostate cancer model. *Prostate Cancer Prostatic Dis* 2013; **16**: 41–49.
27 Onik G, Porterfield B, Rubinsky B, Cohen J. Percutaneous transperineal prostate cryosurgery using transrectal ultrasound guidance: animal model. *Urology* 1991; **37**: 277–281.
28 Onik G, Rubinsky B, Zemel R, Weaver L, Diamond D, Cobb C *et al.* Ultrasound-guided hepatic cryosurgery in the treatment of metastatic colon carcinoma. Preliminary results. *Cancer* 1991; **67**: 901–907.
29 Baust JM, Snyder KK, Santucci KL, Robilotto AT, Smith J, McKain J *et al.* Assessment of SCN and argon cryoablation devices in an in vivo-like 3-D tissue engineered prostate and renal cancer model. *Cryobiology* 2014; **69**: 198 (abstract).
30 Robilotto AT, Van Buskirk RG, Gage AA, Baust JM, Baust JG. Development of a tissue engineered human prostate tumor equivalent: evaluation of cryoablative techniques. *Cryobiology* 2013; **66**: 354–355 (abstract).
31 Gage AA, Baust J. Mechanisms of tissue injury in cryosurgery. *Cryobiology* 1998; **37**: 171–186.
32 Klossner DP, Baust JM, Van Buskirk RG, Gage AA, Baust JG. Cryoablative response of prostate cancer cells is influenced by androgen receptor expression. *BJU Int* 2008; **101**: 1310–1316.
33 Smith DJ, Fahssi WM, Swanlund DJ, Bischof JC. A parametric study of freezing injury in AT-1 rat prostate tumor cells. *Cryobiology* 1999; **39**: 13–28.
34 Klossner DP, Robilotto AT, Clarke DM, Van Buskirk RG, Baust JM, Gage AA *et al.* Cryosurgical technique: assessment of the fundamental variables using human prostate cancer model systems. *Cryobiology* 2007; **55**: 189–199.
35 Baust JG, Gage AA. The molecular basis of cryosurgery. *BJU Int* 2005; **95**: 1187–1191.
36 Rodriguez-Bigas MA, Klippenstein D, Meropol NJ, Weber TK, Petrelli NJ. A pilot study of cryochemotherapy for hepatic metastases from colorectal cancer. *Cryobiology* 1996; **33**: 600–606.
37 Clarke DM, Baust JM, Van Buskirk RG, Baust JG. Addition of anticancer agents enhances freezing-induced prostate cancer cell death: implications of mitochondrial involvement. *Cryobiology* 2004; **49**: 45–61.
38 Goel R, Anderson K, Slaton J, Schmidlin F, Vercellotti G, Belcher J *et al.* Adjuvant approaches to enhance cryosurgery. *J Biomech Eng* 2009; **131**: 074003.
39 Santucci KL, Snyder KK, Baust JM, Van Buskirk RG, Mouraviev V, Polascik TJ *et al.* Use of 1,25alpha dihydroxyvitamin D_3 as a cryosensitizing agent in a murine prostate cancer model. *Prostate Cancer Prostatic Dis* 2011; **14**: 97–104.
40 Baust JG, Gage AA, Bjerklund Johansen TE, Baust JM. Mechanisms of cryoablation: clinical consequences on malignant tumors. *Cryobiology* 2013; **68**: 1–11.
41 Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007; **26**: 1324–1337.
42 Han B, Bischof JC. Direct cell injury associated with eutectic crystallization during freezing. *Cryobiology* 2004; **48**: 8–21.
43 Han B, Iftekhar A, Bischof JC. Improved cryosurgery by use of thermophysical and inflammatory adjuvants. *Technol Cancer Res Treat* 2004; **3**: 103–111.
44 Han B, Swanlund DJ, Bischof JC. Cryoinjury of MCF-7 human breast cancer cells and inhibition of post-thaw recovery using TNF-alpha. *Technol Cancer Res Treat* 2007; **6**: 625–634.
45 Kimura M, Rabbani Z, Mouraviev V, Tsivian M, Caso J, Satoh T *et al.* Role of vitamin D_3 as a sensitizer to cryoablation in a murine prostate cancer model: preliminary in vivo study. *Urology* 2010; **76**: 764 e714–e720.
46 Sabel MS. Cryo-immunology: a review of the literature and proposed mechanisms for stimulatory versus suppressive immune responses. *Cryobiology* 2009; **58**: 1–11.
47 Berrada MS, Bischof JC. Evaluation of freezing effects on human microvascular-endothelial cells (HMEC). *Cryo Lett* 2001; **22**: 353–366.
48 Hoffmann NE, Bischof JC. The cryobiology of cryosurgical injury. *Urology* 2002; **60**: 40–49.
49 Hollister WR, Baust JM, Van Buskirk RG, Baust JG. Cellular components of the coronary vasculature exhibit differential sensitivity to low temperature insult. *Cell Pres Technol* 2003; **1**: 269–280.
50 Gilbert JC, Rubinsky B, Roos MS, Wong ST, Brennan KM. MRI-monitored cryosurgery in the rabbit brain. *Magn Reson Imaging* 1993; **11**: 1155–1164.
51 Saliken JC, Donnelly BJ, Rewcastle JC. The evolution and state of modern technology for prostate cryosurgery. *Urology* 2002; **60**: 26–33.
52 Baust JG, Gage AA, Clarke D, Baust JM, Van Buskirk R. Cryosurgery- a putative approach to molecular-based optimization. *Cryobiology* 2004; **48**: 190–204.

53 Baust JM, Klossner DP, Robilotto A, Vanbuskirk RG, Gage AA, Mouraviev V *et al.* Vitamin D_3 cryosensitization increases prostate cancer susceptibility to cryoablation via mitochondrial-mediated apoptosis and necrosis. *BJU Int* 2012; **109**: 949–958.
54 Koushafar H, Pham L, Lee C, Rubinsky B. Chemical adjuvant cryosurgery with antifreeze proteins. *J Surg Oncol* 1997; **66**: 114–121.
55 Koushafar H, Rubinsky B. Effect of antifreeze proteins on frozen primary prostatic adenocarcinoma cells. *Urology* 1997; **49**: 421–425.
56 Muldrew K, Rewcastle J, Donnelly BJ, Saliken JC, Liang S, Goldie S *et al.* Flounder antifreeze peptides increase the efficacy of cryosurgery. *Cryobiology* 2001; **42**: 182–189.
57 Pham L, Dahiya R, Rubinsky B. An in vivo study of antifreeze protein adjuvant cryosurgery. *Cryobiology* 1999; **38**: 169–175.
58 Wang CL, Teo KY, Han B. An amino acidic adjuvant to augment cryoinjury of MCF-7 breast cancer cells. *Cryobiology* 2008; **57**: 52–59.
59 Ikekawa S, Ishihara K, Tanaka S, Ikeda S. Basic studies of cryochemotherapy in a murine tumor system. *Cryobiology* 1985; **22**: 477–483.
60 Clarke DM, Baust JM, Van Buskirk RG, Baust JG. Chemo-cryo combination therapy: an adjunctive model for the treatment of prostate cancer. *Cryobiology* 2001; **42**: 274–285.
61 Clarke DM, Robilotto AT, Van Buskirk RG, Baust JG, Gage AA, Baust JM. Targeted induction of apoptosis via TRAIL and cryoablation: a novel strategy for the treatment of prostate cancer. *Prostate Cancer Prostatic Dis* 2007; **10**: 175–184.
62 Forest V, Peoc'h M, Ardiet C, Campos L, Guyotat D, Vergnon JM. In vivo cryochemotherapy of a human lung cancer model. *Cryobiology* 2005; **51**: 92–101.
63 Forest V, Peoc'h M, Campos L, Guyotat D, Vergnon JM. Benefit of a combined treatment of cryotherapy and chemotherapy on tumour growth and late cryo-induced angiogenesis in a non-small-cell lung cancer model. *Lung Cancer* 2006; **54**: 79–86.
64 Le Pivert P, Haddad RS, Aller A, Titus K, Doulat J, Renard M *et al.* Ultrasound guided combined cryoablation and microencapsulated 5-fluorouracil inhibits growth of human prostate tumors in xenogenic mouse model assessed by luminescence imaging. *Technol Cancer Res Treat* 2004; **3**: 135–142.
65 Mir LM, Rubinsky B. Treatment of cancer with cryochemotherapy. *Br J Cancer* 2002; **86**: 1658–1660.
66 Yuan F, Zhou W, Zhang J, Zhang Z, Zou C, Huang L *et al.* Anticancer drugs are synergistic with freezing in induction of apoptosis in HCC cells. *Cryobiology* 2008; **57**: 60–65.
67 Jiang J, Goel R, Iftekhar MA, Visaria R, Belcher JD, Vercellotti GM *et al.* Tumor necrosis factor-alpha-induced accentuation in cryoinjury: mechanisms in vitro and in vivo. *Mol Cancer Ther* 2008; **7**: 2547–2555.
68 Goel R, Swanlund D, Coad J, Paciotti GF, Bischof JC. TNF-alpha-based accentuation in cryoinjury--dose, delivery, and response. *Mol Cancer Ther* 2007; **6**: 2039–2047.
69 Shenoi MM, Iltis I, Choi J, Koonce NA, Metzger GJ, Griffin RJ *et al.* Nanoparticle delivered vascular disrupting agents (VDAs): use of TNF-alpha conjugated gold nanoparticles for multimodal cancer therapy. *Mol Pharm* 2013; **10**: 1683–1694.
70 Udagawa M, Kudo-Saito C, Hasegawa G, Yano K, Yamamoto A, Yaguchi M *et al.* Enhancement of immunologic tumor regression by intratumoral administration of dendritic cells in combination with cryoablative tumor pretreatment and Bacillus Calmette-Guerin cell wall skeleton stimulation. *Clin Cancer Res* 2006; **12**: 7465–7475.
71 Gazzaniga S, Bravo A, Goldszmid SR, Maschi F, Martinelli J, Mordoh J *et al.* Inflammatory changes after cryosurgery-induced necrosis in human melanoma xenografted in nude mice. *J Invest Dermatol* 2001; **116**: 664–671.
72 Redondo P, del Olmo J, Lopez-Diaz de Cerio A, Inoges S, Marquina M, Melero I *et al.* Imiquimod enhances the systemic immunity attained by local cryosurgery destruction of melanoma lesions. *J Invest Dermatol* 2007; **127**: 1673–1680.
73 den Brok MH, Sutmuller RP, Nierkens S, Bennink EJ, Toonen LW, Figdor CG *et al.* Synergy between in situ cryoablation and TLR9 stimulation results in a highly effective in vivo dendritic cell vaccine. *Cancer Res* 2006; **66**: 7285–7292.
74 den Brok MH, Nierkens S, Wagenaars JA, Ruers TJ, Schrier CC, Rijke EO *et al.* Saponin-based adjuvants create a highly effective anti-tumor vaccine when combined with in situ tumor destruction. *Vaccine* 2012; **30**: 737–744.
75 Bassukas ID, Gamvroulia C, Zioga A, Nomikos K, Fotika C. Cryosurgery during topical imiquimod: a successful combination modality for lentigo maligna. *Int J Dermatol* 2008; **47**: 519–521.
76 Waitz R, Solomon SB, Petre EN, Trumble AE, Fasso M, Norton L *et al.* Potent induction of tumor immunity by combining tumor cryoablation with anti-CTLA-4 therapy. *Cancer Res* 2012; **72**: 430–439.
77 Chu KF, Dupuy DE. Thermal ablation of tumours: biological mechanisms and advances in therapy. *Nat Rev Cancer* 2014; **14**: 199–208.
78 Tian T, Olson S, Whitacre JM, Harding A. The origins of cancer robustness and evolvability. *Integr Biol (Camb)* 2011; **3**: 17–30.
79 Baust JG, Klossner DP, Vanbuskirk RG, Gage AA, Mouraviev V, Polascik TJ *et al.* Integrin involvement in freeze resistance of androgen-insensitive prostate cancer. *Prostate Cancer Prostatic Dis* 2010; **13**: 151–161.
80 Baust JM, Klossner D, Gage A, Buskirk RV, Baust JG. Akt signaling mediates prostate cancer response to cryoablation. *Cryobiology* 2013; **67**: 427–428.
81 Clarke DM, Robilotto AT, Rhee E, Van Buskirk RG, Baust JG, Gage AA *et al.* Cryoablation of renal cancer: variables involved in freezing-induced cell death. *Technol Cancer Res Treat* 2007; **6**: 69–79.
82 Thompson I, Thrasher JB, Aus G, Burnett AL, Canby-Hagino ED, Cookson MS *et al.* Guideline for the management of clinically localized prostate cancer: 2007 update. *J Urol* 2007; **177**: 2106–2131.
83 Cohen JK, Miller RJ Jr, Ahmed S, Lotz MJ, Baust J. Ten-year biochemical disease control for patients with prostate cancer treated with cryosurgery as primary therapy. *Urology* 2008; **71**: 515–518.
84 Roach M III, Hanks G, Thames H Jr., Schellhammer P, Shipley WU, Sokol GH *et al.* Defining biochemical failure following radiotherapy with or without hormonal therapy in men with clinically localized prostate cancer: recommendations of the RTOG-ASTRO Phoenix Consensus Conference. *Int J Radiat Oncol Biol Phys* 2006; **65**: 965–974.
85 Caso JR, Tsivian M, Mouraviev V, Kimura M, Polascik TJ. Complications and postoperative events after cryosurgery for prostate cancer. *BJU Int* 2012; **109**: 840–845.
86 Caso JR, Tsivian M, Mouraviev V, Polascik TJ. Predicting biopsy-proven prostate cancer recurrence following cryosurgery. *Urol Oncol* 2012; **30**: 391–395.
87 Levy DA, Pisters LL, Jones JS. Primary cryoablation nadir prostate specific antigen and biochemical failure. *J Urol* 2009; **182**: 931–937.
88 Pitman M, Shapiro EY, Hruby GW, Truesdale MD, Cheetham PJ, Saad S *et al.* Comparison of biochemical failure definitions for predicting local cancer recurrence following cryoablation of the prostate. *Prostate* 2012; **72**: 1802–1808.
89 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**: 554–558.
90 Long JP, Bahn D, Lee F, Shinohara K, Chinn DO, Macaluso JN Jr. Five-year retrospective, multi-institutional pooled analysis of cancer-related outcomes after cryosurgical ablation of the prostate. *Urology* 2001; **57**: 518–523.
91 Bahn DK, Lee F, Badalament R, Kumar A, Greski J, Chernick M. Targeted cryoablation of the prostate: 7-year outcomes in the primary treatment of prostate cancer. *Urology* 2002; **60**: 3–11.
92 D'Amico AV, Moul J, Carroll PR, Sun L, Lubeck D, Chen MH. Cancer-specific mortality after surgery or radiation for patients with clinically localized prostate cancer managed during the prostate-specific antigen era. *J Clin Oncol* 2003; **21**: 2163–2172.
93 Dhar N, Ward JF, Cher ML, Jones JS. Primary full-gland prostate cryoablation in older men ($>$ age of 75 years): results from 860 patients tracked with the COLD Registry. *BJU Int* 2011; **108**: 508–512.
94 Chade DC, Shariat SF, Cronin AM, Savage CJ, Karnes RJ, Blute ML *et al.* Salvage radical prostatectomy for radiation-recurrent prostate cancer: a multi-institutional collaboration. *Eur Urol* 2011; **60**: 205–210.
95 Kimura M, Mouraviev V, Tsivian M, Mayes JM, Satoh T, Polascik TJ. Current salvage methods for recurrent prostate cancer after failure of primary radiotherapy. *BJU Int* 2010; **105**: 191–201.
96 Mouraviev V, Spiess PE, Jones JS. Salvage cryoablation for locally recurrent prostate cancer following primary radiotherapy. *Eur Urol* 2012; **61**: 1204–1211.
97 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**: 559–563; discussion 563–554.
98 Spiess PE, Given RW, Jones JS. Achieving the 'bifecta' using salvage cryotherapy for locally recurrent prostate cancer: analysis of the Cryo On-Line Data (COLD) registry data. *BJU Int* 2012; **110**: 217–220.
99 Spiess PE, Levy DA, Pisters LL, Mouraviev V, Jones JS. Outcomes of salvage prostate cryotherapy stratified by pre-treatment PSA: update from the COLD registry. *World J Urol* 2013; **31**: 1321–1325.
100 Williams AK, Martinez CH, Lu C, Ng CK, Pautler SE, Chin JL. Disease-free survival following salvage cryotherapy for biopsy-proven radio-recurrent prostate cancer. *Eur Urol* 2011; **60**: 405–410.
101 Wenske S, Quarrier S, Katz AE. Salvage cryosurgery of the prostate for failure after primary radiotherapy or cryosurgery: long-term clinical, functional, and oncologic outcomes in a large cohort at a tertiary referral centre. *Eur Urol* 2013; **64**: 1–7.
102 Aron M, Kamoi K, Remer E, Berger A, Desai M, Gill I. Laparoscopic renal cryoablation: 8-year, single surgeon outcomes. *J Urol* 2010; **183**: 889–895.
103 Lusch A, Bucur P, Okhunov Z, Kavoussi L, Badani K, Derweesh I *et al.* Intermediate term oncologic outcomes of renal cryoablation: an international multi-institutional analysis (submitted) 2014.