Tumor necrosis factor-α–induced accentuation in cryoinjury: mechanisms in vitro and in vivo

Jing Jiang,1 Raghav Goel,1 M. Arif Iftekhar,1 Rachana Visaria,2 John D. Belcher,3 Gregory M. Vercellotti,3 and John C. Bischof1,2,4

Departments of 1Biomedical Engineering and 2Mechanical Engineering, Medical School, and Departments of 3Medicine and 4Urologic Surgery, University of Minnesota, Minneapolis, Minnesota

Abstract
Cryosurgical treatment of solid cancer can be greatly assisted by further translation of our finding that a cytokine adjuvant tumor necrosis factor-α (TNF-α) can achieve complete cancer destruction out to the intraoperatively imaged iceball edge (−0.5°C) over the current clinical recommendation of reaching temperatures lower than −40°C. The present study investigates the cellular and tissue level dose dependency and molecular mechanisms of TNF-α–induced enhancement in cryosurgical cancer destruction. Microvascular endothelial MVEC and human prostate cancer LNCaP Pro 5 (LNCaP) cells were frozen as monolayers in the presence of TNF-α. Normal skin and LNCaP tumor grown in a nude mouse model were also frozen at different TNF-α doses. Molecular mechanisms were investigated by using specific inhibitors to block nuclear factor-κB–mediated inflammatory or caspase-mediated apoptosis pathways. The amount of cryoinjury increased in a dose-dependent manner with TNF-α both in vitro and in vivo. MVEC were found to be more cryosensitive than LNCaP cells in both the presence and the absence of TNF-α. The augmentation in vivo was significantly greater than that in vitro, with complete cell death up to the iceball edge in tumor tissue at local TNF-α doses greater than 200 ng. The inhibition assays showed contrasting results with caspase-mediated apoptosis as the dominant mechanism in MVEC in vitro and nuclear factor-κB–mediated inflammatory mechanisms within the microvasculatures the dominant mechanism in vivo. These results suggest the involvement of endothelial-mediated injury and inflammation as the critical mechanisms in cryoinjury and the use of vascular-targeting molecules such as TNF-α to enhance tumor killing and achieve the clinical goal of complete cell death within an iceball. [Mol Cancer Ther 2008;7(8):2547–55]

Introduction
Cryosurgery is a surgical technique that uses extreme cold temperatures to treat diseased tissues such as tumors in the body. The finding that clinical imaging (ultrasound, computed tomography, and magnetic resonance) can be used to monitor the growth of an iceball in vivo has made cryosurgery an important minimally invasive thermal therapeutic modality (1, 2). Despite advantages including ease of operation, low morbidity, and low cost, the use of cryosurgery is limited by its inability to destroy the entire tissue within an iceball as reflected in local recurrence of cancer after freezing (3). The clinical guideline to ensure complete cell death by this technique alone is −40°C, which limits the control and predictability of the procedure (Fig. 1; refs. 2, 3). Thus, whereas the edge of the iceball (−0.5°C) can be visualized using ultrasound, computed tomography, or magnetic resonance imaging, the means to enhance or predict the “kill zone” within the iceball are urgently needed. Recent research in cryosurgery has focused on the use of molecular adjuvants to increase tissue cryosensitivity at the periphery of an iceball (0 to −40°C; Fig. 1), which would otherwise remain viable (4–8). It was shown recently for the first time in an in vitro prostate cancer model that a cytokine, tumor necrosis factor-α (TNF-α), could enhance cryosensitivity and achieve tissue destruction up to the edge of an iceball (−0.5°C; ref. 8). This study focuses on understanding the dose dependency and mechanisms of TNF-α–induced cryosurgical augmentation at both cellular (in vitro) and tissue (in vivo) levels. The results are very significant as they suggest the involvement of contrasting injury mechanisms in vitro compared with in vivo.

Several theories have been put forth as to the mechanisms of injury by freezing alone and grouped mainly under direct cellular effects and vascular effects (9). Direct cell injury during freezing can occur by intracellular ice formation at higher cooling rates near the cryoprobe or solution effects injury at low cooling rates present at the periphery of the iceball. Whereas intracellular ice formation directly damages cells by mechanical interaction with large stable ice crystals, solution effects cause cellular dehydration with an increase in both intracellular and extracellular solute concentrations leading to destabilization of the cell membrane (9–11). Post-thaw analysis of injury suggests that both apoptosis and necrosis can occur (7, 10). A second theory regarding the mechanism of damage at the tissue level is vascular injury due to the shutdown of microvasculature...
Mechanisms of TNF-α-Induced Accentuation in Cryoinjury

after freezing and the resultant ischemic necrosis (12–14). Damage to the endothelium, ischemia-reperfusion injury, inflammation, and the resultant loss of microcirculatory support are considered critical in defining the edge of the cryolesion (12, 14). At the molecular level, studies have shown the activation of nuclear factor-κB (NF-κB) and the expression of cell adhesion molecules such as selectins, intercellular adhesion molecule (ICAM), and vascular cell adhesion molecule (VCAM) after endothelial insult, leading to an enhanced inflammatory response (15). Accentuation of either cellular or vascular mechanisms of injury is a goal in adjuvant-enhanced cryosurgery.

Adjuvants aiming to accentuate established cryoinjury mechanisms can be grouped into several categories: (a) thermophysical adjuvants such as antifreeze protein and chemicals (salts and some amino acids), which work directly on cells; (b) chemotherapeutics such as peplomycin, Adriamycin, 5-fluoracil, bleomycin, and navelbine, which are established cytotoxic drugs; and (c) cytokines or vascular-based agents (4, 6, 7, 16, 17). Although several adjuvants used in the first two approaches have shown some accentuation of injury at temperatures higher than -40°C, particularly in vitro, until recently none have been able to show an overlap of the iceball edge with the edge of the cryolesion in vivo.

Recent research in our laboratory has focused on the accentuation of vascular injury during freezing using a vascular targeting cytokine, TNF-α (5, 8). For the first time, it was shown that, with the proper dose and delivery of TNF-α, it is possible to achieve an overlap of the kill zone and the iceball edge, allowing imaging feedback of injury and attainment of the procedure (8). TNF-α is a well-known cytokine for its role in inflammation and immunity and for its antitumor properties (18–20). At the cellular level, TNF-α can lead to direct cell death by apoptosis or induce inflammation by the activation of NF-κB, which is important, particularly in vivo, due to the presence of immune cells (19, 21). It now remains to be determined which one (or both) of these pathways is active at the cellular and tissue levels in enhancing cryosurgical injury.

The aims of this study are to investigate and compare both in vitro and in vivo: (a) the effect of TNF-α dose on cryoinjury and (b) the mechanisms of TNF-α-induced accentuation in cryoinjury. Cryoinjury was assessed in vitro using viability and DNA fragmentation assays and in vivo by measuring perfusion defects and histology. Specific inhibitors were used to target caspase-mediated apoptosis and NF-κB-mediated inflammatory pathways to investigate the molecular mechanisms involved in TNF-α-enhanced cryoinjury. The results show a direct dose dependency of TNF-α on cryoinjury both in vitro and in vivo but contrasting cell injury mechanisms responsible for the observed augmentation.

Material and Methods

In vitro

Cell Culture. Both LNCaP Pro 5 (LNCaP) cells and MVEC released from newborn human foreskin were grown as adherent monolayers in 25 cm² T-flasks as described previously (22). All treatments were performed when flasks were 80% confluent.

TNF-α and Inhibitor Treatments. Recombinant human TNF-α (a gift from CyntImmune Sciences, Inc.) was diluted with Dulbecco’s PBS (BioWhittaker) and reconstituted to a final concentration (1, 10, 100, and 1,000 ng/mL) with fresh medium. Pan-caspase inhibitor Z-VAD-FMK (ZVAD; R&D Systems) and NF-κB inhibitor BAY 11-7085 (BAY; EMD Biosciences) were dissolved in DMSO and diluted to final concentrations of 100 and 10 μmol/L, respectively, in fresh medium before application. The cells were incubated with medium containing TNF-α for 4 h. The inhibitors were present in the medium until injury measurement.

Freeze/Thaw of Monolayer Cultures. Cell monolayers in 25 cm² flasks were frozen in an ethanol bath maintained at a constant temperature (-5°C or -10°C) and nucleated when the temperature reached -1°C. LNCaP cells were frozen until the culture temperature reached -10°C and then held on a copper block maintained at -10°C for 5 min. MVEC were frozen until they reached -5°C with zero hold time. Cells were allowed to thaw passively at room temperature for 15 min and then incubated at 37°C until injury assessment.

Injury Measurement in Monolayer Cultures

Cell Counting and Viability Assay. Cells were mixed with 9 μmol/L Hoechst 33342 (Sigma-Aldrich) and 7 μmol/L propidium iodide (Sigma-Aldrich) and assessed at ×200 by fluorescent microscopy (Olympus BX-50). At least seven representative fields and a total of 200 to 300 cells per sample were counted in each monolayer culture. Cell viability was calculated at several time points (0, 24, 48, and 72 h) after the treatments.

DNA Fragmentation Assay. Cells were collected and fixed with 70% ethanol at 4°C overnight. After

Figure 1. Improvements of the outcome and image guidance of cryosurgery by use of adjuvants. Adjuvants enlarge the cryolesion within the iceball and create an overlap of killing zone and the imaged iceball edge.
centrifugation, cells were washed twice with 1 mL Dulbecco’s PBS. Staining solution (1 mL; containing 3.8 mmol/L sodium citrate and 50 μg/mL propidium iodide in Dulbecco’s PBS) and 50 μL of 10 μg/mL RNase A (Worthington Biochemicals) were added to the pellets. The pellets were stored in dark for 1 h at 37°C and kept covered until further analysis. The propidium iodide fluorescence was measured using ~1 × 10^6 cells in a FACSCalibur E4513 flow cytometer. The fraction of the cells in various cell cycle stages was estimated from DNA content of the cells (23).

**In vivo**

**Dorsal Skin Fold Chamber Implantation.** The dorsal skin fold chamber (DSFC) allows intravital two-dimensional, controlled growth and visualization of tumors as described previously (24, 25). This model was adapted by our laboratory to perform controlled freezing and has since been used in several cryosurgery studies (5, 8, 24, 26). Briefly, the dorsal skin of each nude mouse was sandwiched between two anodized aluminum frames with 10 mm diameter viewing windows, separated by a distance of 450 μm, maintained by spacers on the screws (24, 25). The epidermis was removed from the viewing side along with excess fascia to permit better visualization of the microvasculature.

**Tumor Implantation.** LNCaP cells (1 million-2 million) were suspended in 30 μL Matrigel matrix (Matrigel diluted 3:1 in serum-free medium; BD Biosciences) and inoculated into the DSFC chamber window on both days 0 and 4 of implantation. The experiments were done on day 12 following DSFC implantation, when the tumor covered the entire chamber (5, 8).

**TNF-α and Inhibitor Treatments.** On the day of the study, the glass window was removed and soluble TNF-α (2 ng, 200 ng, 500 ng, or 1 μg) dissolved in 30 μL saline was applied topically in the DSFC. The glass window was replaced after 15 min and cryosurgical treatment was done 4 h later. Specific NF-κB inhibitors BAY and andrographolide (ANDRO) were dissolved in DMSO at concentrations of 10 and 15 mg/mL, respectively. BAY at a dose of 0.4 mg/kg was applied topically in the DSFC 15 min before

![Figure 2](https://example.com/figure2.png)

*Figure 2.* In vitro effect of TNF-α concentration on the viability of (A) LNCaP monolayers with freezing to -10°C, 5 min hold time and (B) MVEC monolayers with freezing to -5°C, 0 min hold time. Cells were treated with TNF-α at concentrations of 0.1, 1, 10, 100, and 1,000 ng/mL for 4 h before freezing. In vitro effect of TNF-α concentration on DNA fragmentation of (C) LNCaP monolayers with freezing to -10°C, 5 min hold time and (D) MVEC monolayers with freezing to -5°C, 0 min hold time. Cells were treated with TNF-α at concentrations of 10, 100, and 1,000 ng/mL for 4 h before freezing. Viability and DNA fragmentation were assessed at 48 h (LNCaP cells) or 24 h (MVEC) after FT. Mean ± SE of three independent experiments. Ctr, untreated cells. *, P < 0.05; **, P < 0.001, significant effects of TNF-α compared with control and TNF-α + FT compared with FT alone.

Mol Cancer Ther 2008;7(8). August 2008
TNF-α application, whereas ANDRO was administered twice i.p. at a dose of 30 mg/kg each at 2 h and 15 min before TNF-α application (22, 27–29). Pan-caspase inhibitor Q-VD-OPH (MP Biomedicals) was administered i.p at a dose of 90 mg/kg 15 min before TNF-α application (22, 27–29).

Freeze/Thaw of the DSFC. The normal skin control (nontumor s.c. tissue within the DSFC) was frozen 3 days after chamber implantation to allow the tissue to recover from surgery. The tumor implanted DSFC were allowed 12 days to grow in the DSFC before cryosurgery.

The freezing procedure in the DSFC has been described in detail previously (8). Briefly, a 1 mm diameter brass fin fitted to a 5 mm cryoprobe (Endocare) was inserted in the center of the DSFC and allowed to attain a temperature of -100°C for 55 s followed by a passive thaw at room temperature. The temperature was monitored throughout the procedure by the use of thermocouples placed at 2, 3, and 4 mm radius from the center respectively and by an infrared camera (FLIR). The thermocouple measured temperatures and infrared measured temperatures correlated well with each other and were also validated by a quasi steady-state mathematical model as shown in our previous publications (5, 8, 24).

Results

Viability. MVEC were found to be more sensitive than LNCaP to cryoinjury at similar freeze/thaw (FT) conditions (data not shown). Therefore, the freezing protocols were adjusted to produce similar viability (~50%) in both cell lines, thereby allowing comparison after TNF-α addition, as well as to simulate the thermal conditions present near the edge of the iceball. The effect of TNF-α-induced augmentation of cryoinjury was observed in MVEC as early as 24 h after treatment, and a similar trend continued at 48 and 72 h (data not shown). In LNCaP cells, a significant effect due to combined treatment was observed at 48 h after treatment (data not shown). Therefore, the 24 and 48 h time points were chosen for MVEC and LNCaP cells, respectively, to study the dose dependency and mechanisms of TNF-α-induced augmentation in cryoinjury.

A direct dose dependency on cell viability was observed in the cells after the application of TNF-α (0.1-1,000 ng/mL) before FT. A 4-h treatment of TNF-α at 1,000 ng/mL dose reduced the cell viability to 64.2 ± 6.1% and 72.3 ± 4.8% for MVEC and LNCaP cells, respectively (Fig. 2A and B). FT alone reduced the cell viability in MVEC to 39.3 ± 5.5% and 56.7 ± 5.5% in LNCaP cells. Pretreatment of cells with 1,000 ng/mL TNF-α for 4 h before FT showed a significant augmentation in cell injury when compared with FT alone with viability reducing to 23.7 ± 3.8% and 27.2 ± 6.6% (P < 0.05) for MVEC and LNCaP cells, respectively.

DNA Fragmentation. DNA fragmentation analysis was done to provide evidence supplemental to the viability analysis of TNF-α-enhanced cryoinjury. The data from DNA fragmentation assay are shown in Fig. 2C and D.
Propidium iodide intensity (X axis) corresponds with DNA content of sub-G1, G1, S, and G2-M cells. In this assay, sub-G1-phase cells were cells with reduced DNA content due to either apoptosis or necrosis. Typical histograms of DNA content per 10^6 cells in LNCaP cells and MVEC are shown at different TNF-α doses. In addition, the percentage of sub-G1 cells was calculated to compare percentage of DNA fragmented cells after each treatment. TNF-α at a dose of 1,000 ng/mL had a minimal effect on LNCaP cells (5% enhancement) but dramatically augmented the DNA damage in MVEC to 76.6 ± 1.6% from 26.2 ± 2.1% observed in control cells (P < 0.001). Freezing alone showed a DNA fragmentation of 14.5 ± 1.6% and 64.6 ± 8.9% for LNCaP cells and MVEC, respectively. On pretreatment with 1,000 ng/mL TNF-α before freezing, the DNA fragmentation increased by 16.7% in LNCaP cells and 17.5% in MVEC (P < 0.05) when compared with FT alone. The trends in the results suggest that the drop in the viability as seen in Fig. 2A and B is likely due to the presence of cells in sub-G1 state (apoptotic or necrotic).

**Tissue Injury In vivo**

Injury in the DSFC was assessed by intravital fluorescence of perfusion and postmortem histology (data not shown). All control unfrozen normal skin and tumor DSFC showed blood flow throughout the chamber as visualized by FITC-dextran fluorescence. Sham treatment displayed patent vasculature beyond the probe insertion site at 0.5 mm radius. The chambers frozen with or without TNF-α intervention displayed a central static region surrounded by perfused tissue in the rest of the chamber. There was increased permeability (not quantified) at the edge of the injury evident as blurriness due to the leakage of dye. WBC rolling and adhesion, indicative of enhanced inflammation, was observed just before cryotreatment in all TNF-α-pretreated animals as noted previously (5, 30). Post-treatment analysis of histology showed a centrally necrotic region surrounded by a transition region, which was composed of both viable and dead cells, inflammatory cells, and thrombosed, dilated vessels as described previously by Hoffman et al. (26). Surrounding the transition region, normal tissue morphology (untreated) could be seen. We have shown previously using a fluorescent dye DiOC6 that the boundary of blood perfusion coincided very well with the edge of necrosis on H&E-stained slides (8). Thus, the average temperature measured at the edge of stasis in the DSFC by vascular imaging represents the temperature threshold for tissue necrosis by cryosurgery for that particular animal.

Figure 3 shows the temperature threshold of necrosis as measured at the edge of stasis for different groups in both normal skin and tumor. The temperature threshold increased with the addition of TNF-α in a dose-dependent manner for both normal skin and tumor as seen in Fig. 3. In normal skin, it increased significantly from -27.7 ± 5.0°C for cryoinjury (FT) only to -13.0 ± 2.5°C, -2.6 ± 2.7°C, and -4.0 ± 3.1°C with pretreated doses of 2, 200, and 500 ng TNF-α, respectively (Fig. 3A). As expected, tumor was found to be more sensitive to cryoinjury when compared with normal skin with a temperature threshold of -14.5 ± 1.5°C by FT alone treatment (Fig. 3B). With pretreated doses of 2, 200, and 1,000 ng TNF-α, the temperature threshold increased considerably (P < 0.01) to -7.0 ± 1.6°C, 2.1 ± 2.7°C, and 5.1 ± 1.9°C, respectively (Fig. 3B). Thus, at a total dose of ≥200 ng, it was possible to obtain an overlap between the kill zone and the iceball edge in the tumor tissue.

**Apoptosis and NF-κB Inhibition In vitro**

Caspase-mediated apoptosis and NF-κB-mediated inflammatory response are the two well-documented mechanisms of TNF-α-induced injury of cells in vitro (32, 33). To elucidate the molecular mechanisms involved in TNF-α-enhanced cryoinjury, pan-caspase inhibitor ZVAD and NF-κB inhibitor BAY were added before TNF-α pretreatment. As shown in Fig. 4A, inhibition of apoptosis with ZVAD had no significant effect on LNCaP viability in any of the treatments (TNF-α, FT, or TNF-α + FT). However, inhibition of NF-κB with BAY decreased LNCaP viability by 26.1% after FT (P < 0.05). Contrastingly, in MVEC (Fig. 4B), ZVAD had a survival effect rescuing 25.3% cells after TNF-α treatment (P < 0.05), 17.2% after FT (P < 0.05), and 26.0% after TNF-α + FT (P < 0.05). Inhibition with BAY reduced MVEC viability by 16.9% after FT (P < 0.05). The combined treatment of ZVAD + BAY showed some rescue compared with BAY alone in MVEC with statistical significance only in TNF-α treatment group (P < 0.05) and significantly reduced MVEC viability compared with ZVAD alone in FT treatment group (P < 0.05). These results indicate an active NF-κB pathway after FT in both MVEC and LNCaP cells and active caspase in MVEC in the presence of TNF-α.

**Apoptosis and NF-κB Inhibition In vivo**

The inhibition experiments in vivo were done at a TNF-α dose of 200 ng, where a significant accentuation in cryoinjury was observed in both normal skin and tumor after FT (Fig. 3). ANDRO, a NF-κB inhibitor, significantly reduced the TNF-α enhancement in cryoinjury for both normal skin and tumor (Fig. 5). In normal skin, the temperature threshold was reduced from -2.6 ± 2.7°C to -18.3 ± 5.2°C (P < 0.01), closer to the injury obtained with FT alone at -27.7 ± 5.0°C (Fig. 5A). A similar reduction was seen in tumors where the temperature threshold changed considerably to -7.5 ± 2.2°C from 2.1 ± 2.7°C (P < 0.01; Fig. 5B). Pretreatment of tumor tissue with BAY, another NF-κB inhibitor, reduced the temperature threshold after TNF-α + FT to -8.0 ± 3.1°C, which was statistically different (P < 0.01) from TNF-α + FT alone (Fig. 5B).

In contrast, inhibition with Q-VD-OPH, a caspase inhibitor, had a minimal effect on the accentuation of cryoinjury by 200 ng TNF-α for both normal skin and tumor. The temperature threshold with caspase inhibitor was measured to be -7.4 ± 1.9°C and 1.0 ± 2.1°C for normal skin and tumor, respectively (Fig. 5A and B). Although caspase inhibition was statistically significant (P < 0.05) in normal skin when compared with TNF-α + FT without inhibition, the reduction was significantly less than what was obtained after NF-κB inhibition with ANDRO.

Mol Cancer Ther 2008;7(8). August 2008
null. None of the inhibitors (BAY, ANDRO, and Q-VD-OPH) had a significant effect on temperature threshold obtained by FT alone (data not shown). Therefore, in vivo inhibition studies with NF-κB and caspase inhibitors show the presence of NF-κB-mediated inflammatory pathways in TNF-α-induced accentuation of cryoinjury.

Discussion
It is evident from both in vitro and in vivo results that the administration of TNF-α before freezing increases the amount of injury significantly. This increase in injury is considerably higher in vivo, where complete cell destruction could be obtained up to the edge of the iceball (-0.5°C).

Inhibition of apoptotic or inflammatory pathways during cryosurgical enhancement with TNF-α presents contrasting results suggesting host-mediated inflammation responsible for augmentation in vivo and MVEC-mediated apoptosis as the mechanism in vitro.

The in vitro data provide compelling evidence to suggest the role of endothelial cells in TNF-α-induced accentuation in cryoinjury. MVEC have been shown previously to be sensitive to both FT injury and TNF-α-induced NF-κB activation and is considered a better model than human umbilical vein endothelial cells to study cryoinjury, because vaso-occlusion occurs primarily in the microvasculature (34, 35). In this study, MVEC were found to be more sensitive to freezing injury than LNCaP cells and therefore milder freezing conditions (-5°C and 0 min hold time) were used for this cell line compared with LNCaP cells (-10°C and 5 min hold time; ref. 35). Increasing amount of cryoinjury to both MVEC and LNCaP cells was obtained in a dose-dependent manner with the addition of TNF-α. The sensitivity of MVEC to cell death in the presence of TNF-α was greater than LNCaP cells both with and without freezing. The resistance to TNF-α-induced cell death in LNCaP cells compared with MVEC could be due to the constitutive expression of antiapoptotic proteins (inhibitor of apoptosis proteins), shown to be present in most tumor cell lines (36). The presence of phosphatidylinositol 3-kinase/Akt survival pathway could be another mechanism for the resistance of LNCaP cells to TNF-α-induced apoptotic injury (31).

Figure 4. In vitro effect of using NF-κB inhibitor BAY and caspase inhibitor ZVAD and on (A) LNCaP monolayers with freezing to -10°C, 5 min hold time, and (B) MVEC monolayers with freezing to -5°C, 0 min hold time. TNF-α treatment was at 1,000 ng/mL for 4 h before freezing. ZVAD (100 μmol/L) and BAY (10 μmol/L) were present in the medium until viability assay. Viability was assessed at 48 h (LNCaP cells) or 24 h (MVEC) after FT. Mean ± SE of three independent experiments. *, P < 0.05, significant effect of inhibitors compared with no inhibitor. **, P < 0.01, significant effects of BAY compared with ZVAD + BAY and ZVAD compared with ZVAD + BAY (shown in brackets).

Figure 5. In vivo effect of using NF-κB inhibitors BAY and ANDRO and caspase inhibitor Q-VD-OPH in normal skin (A) and tumor (B) grown in a dorsal skin fold chamber. The inhibition was done at TNF-α pretreated dose of 200 ng for both normal skin and tumor. Mean ± SD of five to eight animals in each group. *, P < 0.01, groups were statistically different than groups pretreated with TNF-α.
Increased cryosensitivity of endothelial cells (with or without TNF-α) suggests that injury to the endothelium may help govern the extent and enhancement of injury in vivo, where many cell types are present. In fact, several in vivo studies have shown endothelial injury and ensuing inflammation to be critical in governing the kill zone at the periphery of an iceball (12, 13, 26). The addition of TNF-α in vivo is suggested to cause even more endothelial cell death at higher subzero temperatures as observed in vitro, thereby increasing the extent of the vascular shutdown within the iceball.

The in vivo cryosurgical studies done in the DSFC showed a very significant dose dependency (Fig. 3). The temperature threshold of necrosis increased drastically with the addition of TNF-α for both normal skin and tumor in the DSFC. At a dose of 200 ng, the edge of injury overlapped with the edge of the iceball (-0.5°C) in tumor tissue, suggesting the ability to destroy all tumor within a cryosurgical iceball. This is clearly an augmentation that is not expected from the in vitro studies, where there was still significant viability remaining at -5°C (in MVEC) and -10°C (in LNCaP cells). It suggests the presence of other factors or mechanisms to account for the enhanced injury observed in vivo versus in vitro.

TNF-α is closely associated with in vivo vascular events and injury. Apart from endothelial cell apoptosis, the other reported actions of TNF-α in vivo comprised proinflammatory reactions such as an increase in procoagulant activity, decrease in anticoagulant activity, recruitment and adherence of inflammatory cells such as neutrophils, and production of other cytokines (20, 21, 37, 38). TNF-α-induced activation of NF-κB can promote the transcription of several genes that could increase the inflammatory/procoagulant response in the tissue by the expression of adhesion molecules such as ICAM and VCAM on the endothelium, facilitating leukocytes infiltration and enhancing tissue factor expression while down-regulating thrombomodulin (19, 21, 30). The increase in direct cell cryoinjury observed in vitro may partially be explained due to TNF-α-induced apoptosis of endothelial and tumor cells. However, the dramatic enhancement in cryoinjury observed in vivo also suggests the role of inflammatory prothrombotic events, which follow both after TNF-α and cryosurgical treatments. To investigate the significance of apoptosis and inflammation in TNF-α-induced accentuation in cryoinjury, specific inhibitors to block caspase (to inhibit apoptosis) or NF-κB (to inhibit inflammation) were used both in vitro (cellular level) and in vivo (host level).

In vitro inhibition suggests active apoptosis pathway in MVEC in the presence of TNF-α and/or FT and NF-κB pathway in both cell lines after FT. Caspase inhibition with ZVAD showed significant rescue in MVEC but no rescue in LNCaP cells (Fig. 4). This confirmed caspase-mediated
apoptosis as the injury mechanism in endothelial cells under TNF-α and FT insult. On the other hand, activation of NF-κB results in the prevention of apoptosis through several antiapoptotic proteins, including Bcl-XL (to inhibit cytochrome c leakage from the mitochondria), inhibitor of apoptosis protein (to inhibit caspase-3 and caspase-8 activation), and FLIP (to inhibit caspase-8 activation; refs. 39–41). NF-κB inhibitor BAY reduced the viability of LNCaP cells and MVEC after both cryoinjury and combined treatments (Fig. 4), confirming the activation of NF-κB as a protective mechanism after FT in vitro. The selection of inhibitors and dosage used in this work is based on previous literature reporting in vitro and in vivo models of NF-κB and caspase inhibition (22, 27, 29, 42–45).

In vivo blocking of apoptotic or inflammatory pathways suggests the role of TNF-α-mediated inflammatory response to be the mechanism of observed enhancement in cryoinjury. In the DSFC, both NF-κB inhibitors, BAY and ANDRO, produced a remarkable reduction in the TNF-α-induced increase in temperature threshold of necrosis of cryoinjury for both normal skin and tumor (Fig. 5). On the other hand, treatment with Q-VD-OPH, a caspase inhibitor, did not produce a significant change in the temperature threshold of necrosis (Fig. 5). This is in contrast to in vitro results, where the application of pan-caspase inhibitor reduced injury (in MVEC) and NF-κB inhibitor, to the contrary, enhanced cell injury (in LNCaP cells).

The results using apoptosis and inflammatory inhibitors observed both in vitro and in vivo supports our initial hypothesis of TNF-α-induced apoptosis to be the mechanism for increased cell death observed in vitro but TNF-α-induced host-mediated inflammatory response to be critical in vivo (Fig. 6). Although these in vitro and in vivo adjuvant augmentations are apparently divergent and affect two phenomena mediated by two distinct mechanisms, these contrasting results may represent two levels of the same in vivo phenomenon, ischemic necrosis, due to TNF-α effects at the cellular and tissue level.

Previous work with cryoadjuvants (particularly chemo-therapeutics) has shown augmentation of cryoinjury but fall short of obtaining an overlap of the kill zone with the edge of the iceball (4, 6, 7, 46). These studies have focused on increasing cryosurgical efficacy by using drugs to activate apoptotic-based cell death pathways specifically near the edge of the iceball. The bulk of this work has been done in vitro and none of the studies thus far has shown synergistic effects in vivo as observed in this study. Although very few studies have confirmed the presence of apoptosis in vivo after a FT treatment, the overall significance of this mechanism of cell death in a cryosurgical procedure is not well defined and still under interrogation (16, 47).

Host-mediated vascular changes such as inflammation have been observed and implicated in a large number of studies related to freezing induced injury (9, 10). This work is the first effort to investigate the underlying mechanisms of vascular targeting drugs such as TNF-α in the augmentation of cryosurgical injury. The results show that although apoptotic-based cellular level pathways may augment cryoinjury in vitro, the host-mediated vascular inflammatory pathways are more critical and produce a synergistic effect in cryoinjury augmentation in vivo. Future work is needed at the molecular level to determine the critical factors in the NF-κB pathway that produce the observed synergistic augmentation in vivo. This study advocates the use of vascular targeting drugs such as TNF-α or perhaps less toxic molecules that activate the NF-κB pathway to produce an augmentation in cryoinjury. Further work in a translational model is also needed to advance the clinical goal of complete cell death within a cryosurgical iceball.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
Tumor necrosis factor-α–induced accentuation in cryoinjury: mechanisms in vitro and in vivo

Jing Jiang, Raghav Goel, M. Arif Iftekhar, et al.

Mol Cancer Ther 2008;7:2547-2555.

Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/7/8/2547

Cited articles
This article cites 46 articles, 11 of which you can access for free at:
http://mct.aacrjournals.org/content/7/8/2547.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.