Human Prostate Cancer Cells Secrete Neuro-Protective Factors in Response to Cryotherapy

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Abstract

Cryoablation is one of the established treatment modalities for prostate cancer management. Although, it is target specific, it may still lead to damage to the nerve fibers around the prostate tumor. In this study, by directly exposing the co-cultures of prostate cancer cells, PC-3 and Schwann cell-Dorsal Root Ganglion neuron (SC-DRG) to cryo-shock and by exposing SC-DRG to cryo-shock conditioned media (CSCM) obtained from PC-3 cells, robust neuro-protective effects were observed. Since this neuro-protective effect originated from cryotherapy-treated PC-3 cells, the presence of putative factors secreted by PC-3 cells in the medium following cryo-shock was analyzed. Using human cytokine antibody array analysis, differential release of cytokines in CSCM was observed with induced release of cytokines involved in neuro-protection like IL-1α, MIP-4, MIP-5, Leptin, IL-15 and ICAM-1 with simultaneous inhibition of TNFRI and TNFRII that are implicated in killing of nerve cells. Further, using Matrix Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) sequencing, two proteins were identified namely, CypA (cyclophilin A) and NM23 (nonmetastatic protein 23) in the CSCM. CypA functions as a mediator of intracellular as well as extracellular neuro-protective mechanisms and NM23 has been implicated as a potential suppressor protein of tumor metastasis. Thus, this study revealed the presence of factors in CSCM that has the potential to protect normal neuronal cells and suppress metastasis.

Keywords: Bystander effect; Cryo-shock; Cytokines; Myelination; Neuro-protection; Prostate cancer

Introduction

Currently, cryo-therapy is a standard mode of treatment for prostate cancer, however little is known about the molecular mechanisms that govern the adaptation or treatment-induced effects. The current understanding in mammalian cells suggest that the cold shock response involves a coordinated series of events involving modulation of transcription, translation, the cell cytoskeleton, the cell cycle and metabolic processes (1). In response to the cold shock generally both transcription and translation are suppressed except for a select number of cold shock proteins (CSPs) whose synthesis continues or is upregulated. The only two well characterized mammalian CSPs reported to date are Cip and Rbm3, glycine-rich RNA binding proteins that prevent secondary mRNA structures and counteract microRNAs that inhibit translation respectively.

At extremely low temperatures, mammalian cells die via necrosis due to the formation of the ice crystals. However, it has been reported that cryo-therapy can result in both apoptosis and necrosis of cancer cells (2). Furthermore, exposure of cells to low temperature (cold shock) results in the changes to the lipid bilayer and composition of the membrane (1). Hence, it is conceivable that such cold shock in cells can trigger a bystander phenomenon leading to an immune-modulation effect.

One of the problems with cryo-therapy of the prostate cancer is the damage to the normal cells as well as to nearby nerve bundles. Highly targeted cryo-treatment only to the cancer tissue is not possible, so damage to normal prostate and nerve cell bundles is inevitable. Understanding this issue will be of pivotal importance in designing optimal prostate cancer treatments with minimal side effects.
In this study, we investigated the effects of freezing on nerve tissue damage. There are no reports to implicate that cryo-therapy mediated bystander effects may potentially cause neuro-protective effects with simultaneous cancer cell death. Results from the present study demonstrated release of chemokines in response to cryo-therapy. Furthermore, we found that the cryo-conditioned media protected the nerve cells.

**Material and Methods**

**Cell culture**

Primary cultures of sensory neurons and Schwann cells were prepared according to Chernousov et al. (3). Briefly, primary cultures of sensory neurons were prepared from dorsal root ganglia of late gestation rat embryos and cultured on collagen-coated cover slips. Schwann cells were prepared from sciatic nerves of postnatal day 2 rats and cultured on poly-L-lysine (Sigma Chemical Co.) coated dishes. Neuron-Schwann cell co-cultures were prepared by adding trypsin-dissociated glial (Schwann) cells (100,000 per well) to 3 week old nerve cell cultures. Schwann cell differentiation was induced by incubating the cultures in medium that contains ascorbic acid (50 μg/ml). By one week after ascorbate addition, the Schwann cells produce myelin segments around large caliber axons and unmyelinated ensheathment of small caliber axons. In these cultures both the neurons and Schwann cells undergo normal differentiation, including the elaboration of myelin segments. Thus, the model closely resembles in vivo conditions.

Human prostate cancer line, PC-3 (p53 null; androgen-independent) cells were obtained from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C and 5% CO₂.

**Cryo-treatment**

For cryo-treatment, flasks containing PC-3 cells or dishes containing Schwann cell-Dorsal Root Ganglion neuron co-cultures (SC-DRG) with or without PC-3 cells were sealed and immersed in methanol containing cold circulating bath. To reach the target temperature, dry ice was used and cells were exposed to the target temperature for 20 minutes. For PC-3 cells, the cells were incubated at 37°C for 24 h and cryo-shock conditioned media (CSCM) was collected at different times after removing cell debriss by centrifugation. For SC-DRG cultures with or without PC-3 cells after direct cryo-treatment, cells were allowed to recover at 37°C for 48 h and then media was changed. SC-DRG cultures were also exposed to CSCM for studying the indirect effects of cryo-treatment.

**Immunocytochemistry**

Following the various treatments, demyelination was assayed by immunofluorescent staining of the cultures with anti-myelin basic protein (MBP; Sternberger Monoclonals, Exeter, UK) and axonal degeneration was assayed by fluorescent staining with anti-neurofilament antibody (Sigma, St. Louis, MO) using confocal microscopy according to Chernousov et al. (3). For confocal microscopy, images were collected using a Leica (Nussloch, Germany) DM IRE2 laser scanning microscope equipped with a Leica TCS SP2 scanner (3).

**Human cytokine antibody array**

Following cryotreatment, CSCM was subjected to TranSignal™ human cytokine antibody array 3.0 (Panomics, Inc., Fremont, CA) according to the manufacturer’s instructions. Expression of 36 cytokines can be determined using the antibody array.

**Gel electrophoresis**

At the time of treatment, media was changed to serum free media and cells were exposed to either -10 or -20°C temperatures. CSCM from these groups was collected at 0 or 6h and subjected to protein precipitation by trichloroacetic acid (TCA) for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis coupled with Matrix Assisted Laser Desorption /Ionization- Time Of Flight (MALDI-TOF) mass spectrometry sequencing. Electrophoresis was at 10mA until the bromophenol blue tracking dye had reached within lcm of the bottom of the gel. Gels were stained for 25min in 0.1% coomassie brilliant blue (in 50% methanol and 10% glacial acetic acid). After de-staining with de-staining solution (7% glacial acetic acid and 12% methanol with half-hourly changes), gels were washed in deionized water with half hourly changes for 6h. The bands that were unique to treated groups were identified and bands containing stained protein were excised from washed gels. Duplicate bands, containing no protein, were cut from the gel as close as possible to the excised, protein-containing band. The excised bands were then sent to Applied Biomics, Inc. for MALDI-TOF analysis.
Results

Effect of direct cryo-shock or CSCM on Schwann/Nerve cells co-culture with PC-3 cells

The effects of freezing on peripheral nerve cells were investigated by means of a primary co-culture model of sensory neurons and Schwann cells that exhibits normal peripheral nerve differentiation, including myelination of axons with or without prostate cancer cells, PC-3 (Figure-1). Exposure of SC-DRG to the conditioned medium obtained from untreated PC-3 cells tended to disrupt myelin and axonal integrity compared to the conditioned medium obtained from cryo-treated (-20°C) PC-3 cells (Figure-1). Freezing of SC-DRG neuron co-cultures at -20°C resulted in extensive demyelination as well as neuronal degeneration. These effects were delayed, and were not detected until 48 hours after freeze-thaw. Interestingly, there was little apparent effect of direct freezing on the glial cells. This could facilitate subsequent nerve repair, since Schwann cells have been shown to promote nerve fiber regeneration. There was a modest protective effect of the presence of prostate cancer cells on neuronal survival (Figure-2). This suggests the cryo-shock treated prostate cancer cells might release factors that inhibit neuronal cell death pathways.

Identification of novel proteins and cytokines induced in response to cryo-therapy

Based on the co-culture results suggesting that CSCM might harbor factors that play role in neuroprotective effect, we designed experiments to identify putative factors released in response to cold-shock temperatures that may have potential role in bystander and abscopal effects. Serum-free CSCM was generated from confluent cultures of PC-3 cells that were subjected to temperatures of 4°C, 0°C, -5°C, -10°C, and -20°C for 20 minutes. CSCM was collected at 0 hr, 6hrs and 24 hrs after the treatment. CSCM from these groups were subjected to (1) TranSignal Human Cytokine Antibody Array analysis; and (2) protein precipitation by TCA for SDS-PAGE analysis coupled with MALDI-TOF sequencing.

Cytokine expression array indicated significant changes in the release of various cytokines following cryo-treatment (Table 1). Specifically, significant induction of IL-1α, MIP-4, MIP-5, Leptin, and IL-15 was observed following exposure of PC-3 cells to various freezing temperatures (Table 1). Further, TNFRI and TNFRII release was repressed compared to untreated group following exposure of cells to -5°C, -10°C and -20°C. Additionally, following -20°C treatment, a moderate induction in release of intercellular adhesion molecule-1 (ICAM-1) with very significant induction of IL-1α was observed (Figure-3 and Table 1).

SDS-PAGE analysis of precipitated proteins in CSCM revealed a unique protein band at the 18 kD range for cells exposed to -20°C as compared with control and other treatments (Figure-4). Sequencing of this band by MALDI-TOF revealed the presence of CypA (peptidylprolyl isomerase A or cyclophilin A) and NM23 (metastasis suppressor gene). CypA (17.999 kDa) is also known as cyclophilin A and peptidylprolyl isomerase (PPIase) A and NM23B (17.286 kDa) is also known as nonmetastatic protein 23 or metastasis inhibition factor or nucleoside diphosphatase kinase-B. Particularly of these two factors, CypA has been reported to be involved in neuro-protective signaling pathways (4) and hence, this factor may be pivotal in inhibiting cryo-shock mediated nerve damage.

Discussion

Prostate cryoablation is an established local therapy for prostate cancer confined to the prostate gland. Prostate tumor cryoablation like other treatments can be associated with undesirable side effects of incontinence, bladder dysfunction, rectal...
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Figure 2. SC-DRG cells were co-cultured with or without PC-3 cells and were cryo-treated (-20°C). Effects on myelination and axonal integrity were assessed and analyzed by confocal microscopy.

Injury and erectile dysfunction. However, the ability of the cryoablation procedure to target specific and often localized portions of cancerous prostate with the percutaneous placement of cryo-probes represents the most flexible and customizable of all therapeutic options. However, cryoablation may damage the nerve bundles around the prostate tumor during therapy. This study provides interesting results from co-culture experiments with nerve and prostate cancer cells that suggest that the prostate cancer cells might release factors that inhibit neuronal cell death pathways caused by direct cryo-freezing of these nerve cells. These observations strongly support the hypothesis that cryo-therapy induces bystander effects and may increase the tumor cell kill with simultaneous reduction in the normal tissue toxicity and metastasis. Further, these results demonstrate the utility of the peripheral nerve tissue culture model to investigate the effects of freezing on neuronal and glial cell function and survival.

Cryo-treatment showed differential release of cytokines that are involved in anti-tumor effects and tumor regression such as IL1-α (5, 6) and inflammatory response proteins such as MIP-4 and MIP-5 (7) with simultaneous inhibition of cytokines involved in cell migration and proliferation. In addition, IL-15 that was induced in CSCM, has been shown to play a key role in regulating immune cell activation and glial reactivity after central nervous system (CNS) injury (8). Using the model of chronic constriction of the sciatic nerve (CCI), it was demonstrated that IL-15 is essential for the development of the early inflammatory events in the spinal cord after a peripheral lesion that generates neuropathic pain. IL-15 expression in the spinal cord was identified in both astroglial and microglial cells (8) and thus IL-15 has a potential role in the reduction of neuroinflammation in response to cryotherapy. Leptin, another cytokine that was differentially induced following cryo-treatment has been shown to play a compensatory role in the protection of neurons following glucose/oxygen/serum deprivation (GOSD) (9). Decline of Leptin with other molecules was reported to lead to an irreversible neuronal death (9). An
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Figure 3. CSCM samples collected at 0h, 6h and 24h from various temperature groups from 4°C to -20°C were subjected to Human Cytokine antibody array analysis. Bound antigen-antibody spots were visualized by chemiluminescence method. A representative autoradiograph is shown in which IL-1α is found to be increased in -20°C exposed PC-3 cells at both 0h and 6h time points.

induction of ICAM-1 following exposure of cells to -20°C was also observed. Glial cell line-derived neurotrophic factor (GDNF) that protects neurons from neurotoxic insults in the CNS has been reported to increase the gene expression of ICAM-1 (10) thus implicating ICAM-1 role in neuroprotection.

Further, in addition to induction of release of cytokines in CSCM that are involved in neuroprotection, a simultaneous repression in release of cytokines like TNFRI and TNFRII that are involved in killing of neurons by TNF-α (11) was observed.

In this study, cryo-treatment of prostate cancer cells resulted in the release of CypA and NM23B. CypA was originally described as binding protein for immunosuppressant agent cyclosporin A (12). Cyclophilins are intracellular or secreted proteins that help in the proper folding of the newly synthesized proteins and are also involved in repairing damaged proteins due to environmental stresses that is reviewed by Yao et. al. (12). It has been shown that CypA may be involved in apoptosis by activating caspases. CypA also has nuclease activity in addition to the PPIase activity (13). Further, CypA functions as a mediator of intracellular and extracellular neuro-protective mechanisms (4).

The most important function of secreted CypA is in chemotaxis as it is a potent chemoattractant to neutrophils, eosinophils and T cells (14). CypA binds to CD147 and transmits a signal to trigger chemotaxis. Levels of CypA and CD147 correlate

Figure 4. Identification of two bands in the medium collected after exposure of cells to -20°C. Proteins were precipitated and run on SDS-PAGE, the bands were visualized by staining and de-staining of the gels.
with disease severity in rheumatoid arthritis. CypA is over-expressed in non small cell lung cancer and in pancreatic adenocarcinoma (12). CypA also interacts with GAGs on HIV-1 to facilitate virion entry (15). Hence, CypA presence in CSCM might enhance the chemotaxis at the site of cryo-treatment and further render a neuro-protective effect. The other protein identified by MALDI-TOF was NM23B that has nucleoside diphosphate (NDP) kinase activity, regulates signal transduction by complexing with G proteins and also acts as a transcriptional regulator of the c-myc gene (16). This has been implicated as a potential suppressor protein in tumor metastasis including prostate cancer independent of its NDP kinase activity (17). The release of NM23 in CSCM may help in suppressing metastatic deposits.

Together, this study revealed for the first time the presence of novel factors in CSCM that has the potential to protect normal neuronal cells, inhibit nerve cell killing and suppress metastasis, thus contributing to effective prostate tumor management by cryotherapy.

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**Table 1. Differential release of cytokines in the medium following cryotreatment**

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<th>UT</th>
<th>4°C</th>
<th>0°C</th>
<th>-5°C</th>
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<tr>
<td>0 h</td>
<td>Apol/Fas, CTLA, MMP3, TNFRI, TNFRII, IL-4, IL-6, IL-8, IL-12 (p40)</td>
<td>Apol/Fas, CTLA, MMP3, TNFRI, TNFRII, IL-4, IL-6, IL-8, IL-12 (p40)</td>
<td>Apol/Fas, CTLA, Leptin, MMP3, TNFRI, TNFRII, IL-4, IL-6, IL-8, IL-12 (p40)</td>
<td>Apol/Fas, CTLA, Leptin, MMP3, TNFRI, TNFRII, IL-4, IL-6, IL-8, IL-12 (p40)</td>
<td>Apol/Fas, CTLA, Leptin, MMP3, TNFRI, TNFRII, IL-4, IL-6, IL-8, IL-12 (p40)</td>
<td>Apol/Fas, CTLA, Leptin, MMP3, TNFRI, TNFRII, IL-4, IL-6, IL-8, IL-12 (p40)</td>
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<tr>
<td>6 h</td>
<td>Apol/Fas, CTLA, MMP3, TNFRI, TNFRII, IL-4, IL-6, IL-8, IL-12 (p40) Leptin</td>
<td>Apol/Fas, CTLA, MMP3, TNFRI, TNFRII, IL-4, IL-6, IL-8, IL-12 (p40) Leptin</td>
<td>Apol/Fas, CTLA, Leptin, MMP3, TNFRI, TNFRII, VEGF, IL-1α, IL-4, IL-6, IL-8, IL-12 (p40) Leptin</td>
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<td>Apol/Fas, CTLA, Leptin, MMP3, TNFRI, TNFRII, VEGF, IL-1α, IL-4, IL-6, IL-8, IL-12 (p40) Leptin</td>
<td>Apol/Fas, CTLA, Leptin, MMP3, TNFRI, TNFRII, VEGF, IL-1α, IL-4, IL-6, IL-8, IL-12 (p40) Leptin</td>
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**Bold and italics:** decreased, **Bold:** increased, **Bold and underlined:** induced, **Italics:** repressed
Conflicts of Interest
No potential conflicts of interest to disclose.

References