Immunoregulatory effects of freeze injured whole tumour cells on human dendritic cells using an *in vitro* cryotherapy model

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**Abstract**

Tumour cryotherapy has been described as both immunostimulatory and immunoinhibitory in previous studies. However, previous studies have not accurately reproduced the precise conditions of current clinical cryotherapy. The objective of this study is to assess the immunological effects of cryotreated whole tumour cells on dendritic cells (DC) maturation and function using an *in vitro* model. Prostate cancer cells were cooled using Endocare cryo-system to mimic temperatures achieved during clinical cryotherapy. Human DC were prepared from cluster of differentiation (CD) 14 monocytes and matured with lipopolysaccharide (LPS). Cryotreated cancer cells were added to DC on day 3. On day 7, DC were harvested and phenotyped. Cytokine gene expression was assessed using real time quantitative polymerase chain reaction (RT-PCR). Functional activity of DC was assessed in allogenic mixed lymphocyte reaction (MLR) and the molecular changes using gene microarray technology. There was statistically significant upregulation of costimulatory molecules and maturation markers (CD86, CD83, CD80 and CL II) in DC loaded with cryotreated whole tumour cells compared to both control DC and DC matured with LPS (*P* < 0.001). There was a significant increase in stimulatory cytokines gene expression (IL-2, IL-12, IL-15, IL-18 and IFN-c). However, IL-10 and TGF-b expression reduced significantly. The effect of different freezing temperature was equal. cDNA microarray analysis showed upregulation of interleukin 1 (IL-1) and cycline dependent kinase inhibitor 1A (CDKN1A (p21) and downregulation of Caspase 8 and BCL2. Overall, our findings suggest that the effect of cryotherapy is generally stimulatory to DC which may enhance anti-tumour effects. Therefore, the combination of cryotherapy and DC vaccine may represent a novel method to increase the efficacy of cryotherapy especially at the peripheral zones of the prostate where cells are exposed to sublethal temperature.

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**Introduction**

Cryotherapy is a procedure which includes exposure to subzero freezing temperature resulting in tissue necrosis. Systemic anti-tumour immune responses have been postulated following few clinical reports observing regression of metastatic disease and symptomatic relief following prostate cryotherapy [24]. Local tumour destruction by cryotherapy results in the release of cryonecrotic tissue and tumour antigens and enhances the uptake of these antigens by local DC resulting in tumour specific immune response and tumour eradication [5]. Cryoimmune response has been studied in several animal models and both immunostimulatory and immunoinhibitory effect was noticed [19,10,6,20,13].

The precise mechanism of the immunostimulatory effect was not clear. Early cytokine mediated response was reported [6,20]. Involvement of T cell immunity and enhanced natural killer (NK) cell cytotoxicity was also described [2]. Other reports suggested the development of anti-tumour antibodies following cryotherapy [29]. On the contrary, other reports suggest suppressed immunity and enhanced tumour growth and metastases following cryotherapy [10,6].

Dendritic cells (DC) are bone marrow derived cells that are specialized in antigen processing and presentation. Immature DC reside in the peripheral tissues sensing their microenvironment for antigens. They are highly endocytic with poor antigen presenting capacity. Upon maturation, DC undergo phenotypic and functional changes. They show increased expression of major histocompatibility complex (MHC), adhesion and costimulatory molecules (CD80 and CD86). Mature DC have increased antigen presenting capacity and are more capable of migration to local lymph nodes where they act as efficient antigen presenting cells (APC) to prime
naive antigen specific T cells [1]. The ability of DC to process tumour antigens and present them to T cells to induce tumour specific immune response renders them the ideal immune cells for tumour immunotherapy. Dendritic cell immunotherapy has been shown to have significant anti-tumour effects in numerous phase I and phase II trials [3]. Recently a randomized phase III study of DC-type autologous vaccine was shown to improve overall survival in patients with metastatic prostate cancer, resulting in Food and Drug Administration (FDA) approval of this approach [9]. Ex vivo generation and loading of DC remains the preferable method in DC based vaccine therapy. Such vaccines are usually delivered intradermally, relying on efficient DC migration from injection sites to the regional lymph node [25]. Cryonecrotic tissue represents an excellent milieu for APC. den Brok et al. [5] demonstrated that in situ tumour destruction by cryotherapy was more efficient than radiofrequency in loading DC with tumour antigen. Intratumoral administration of ex vivo generated DC combined with cryotherapy of the primary tumour was shown to induce tumour specific Th1 type immune response [28,15]. The objectives of this study are to investigate the immunological effects of cryotreated whole tumour cells on DC maturation and function and to identify the mechanisms of immune modulation by cryotherapy. We also assessed the molecular changes in DC following exposure to frozen prostate cancer cells using gene microarray technology.

Materials and methods

Cell line and preparation of samples for cryotreatment

DU145 and PC-3 prostate cancer cell line were obtained from American Type culture collection (ATCC). Cells were grown in culture medium (RPMI 1640 with 10% fetal calf serum, 1% l-glutamine and 1% penicillin/streptomycin all from Sigma–Aldrich, Poole, UK) and incubated in 37 °C with 5% CO2. Cells were washed and suspended in fresh culture medium prior to treatment.

Freezing protocol

Cells were treated using the Cryocare system™ (Endocare, Inc., Irvine, CA) as described before [12]. Cells were cooled to 0, −5, −10 and −20 °C for 10 min and then thawed to room temperature. All cells groups were exposed to the same experimental conditions.

Generation of monocyte derived DC

Auffy coat was obtained from the National Blood Service in 40 ml transfusion bag. Peripheral blood mononuclear cells (PBMCs) were resuspended in culture medium (RPMI-1640 containing 10% FCS, 100 U/ml penicillin 100 μg/ml streptomycin and 50 μg 2-mercaptoethanol, all from Sigma–Aldrich, Poole, UK) and incubated in 37 °C with 5% CO2. Cells were washed and suspended in fresh culture medium prior to treatment.

Tumour lysate

DU145 and PC-3 cells were re suspended in serum free RPMI-1640 medium at a density of 1 × 106 cells/ml. Cell suspension was frozen at −80 °C, interrupted by four freeze–thaw cycles. The lysate was centrifuged at 690g for 10 min and supernatant was passed through 0.2 μm filter. Protein contents of the lysate were determined by Bio-Rad’s protein assay (Bio-Rad, Herts, UK).

Treatment protocol

Cryotreated prostate cancer cells were added to both immature DC and DC matured by LPS at day 3, at least 2 h after the addition of LPS. DC cancer cells ratio was 5:1. Two hundred microlitres of tumour lysate (= 200 μg/ml protein) was added to the remaining wells at the same time.

DC staining and phenotyping

Phenotypic analysis of DC was performed by flow cytometry using anti–CD14, CD11c, CD86, CD80, CCR7, CD1a, CL II, CD209 and CD83 antibodies. Briefly, cells were resuspended in 300 μl FACS buffer (450 ml sterile distilled water, 50 ml of 10x calcium/magnesium free PBS, 3 g bovine serum albumin, 3 g sodium azide, all from Sigma–Aldrich, Poole, UK) and stained with FITC, PE, or PECY5 conjugated mAb (all from BD Biosciences, Oxford, UK). Cells then washed with FACS buffer and fixed with 150 μl FACS FIX (450 ml sterile distilled water, 60 ml of 10x calcium/magnesium free PBS, 15 ml 38% formaldehyde solution, all from Sigma–Aldrich, Poole, UK). Stained DC were analyzed on Epics XL (Beckman-Coulter, High Wycombe, UK).

Real time quantitative RT-PCR

RT-qPCR analysis was performed using Stratagene Mx3005P qPCR system. GAPDH was used as a house keeping gene and all reactions were performed in duplicated in 96-well plate. Two four microlitres of PCR mixture contained 1 μl cDNA, 1 μl of each cytokine primer, 10.5 μl nuclease free water and 12.5 μl SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma–Aldrich, Poole, UK). Primers were designed by Primer3 software and obtained from Eurogentec group (Eurogentec Ltd., UK). RT-qPCR efficiency was calculated using standard curve. Thermal cycling conditions were as follow: 1 cycle (10 min at 95 °C) followed by 40 cycles (30 s at 95 °C, 1 min at 60 °C) and 30 s at 72 °C) followed by 1 cycle (1 min 95 °C, 30 s at 55 °C and 30 s at 95 °C). The 2−ΔΔCT method was used to calculate the difference in Ct value between the treated sample and untreated control and is expressed as a fold changes in gene expression relative to the untreated cells. The results then normalized to an endogenous reference gene (GAPDH) whose expression is constant in all groups.

\[ \Delta \Delta CT = (C_{T, target} - C_{T, reference} \text{ gene}_{treated}) - (C_{T, target} - C_{T, reference} \text{ gene}_{untreated}) \]

Cytokine assay

Supernatant was collected at different time points (2, 6, 24 and 72 h) and stored in aliquots at −80 °C. Cytokine levels secreted in the supernatant from the treated DC were quantified using Bio-Plex cytokine assay (Innogenetix, Paisley, UK) following the manufacturers’ protocol. The multiplex bead-based assay is designed to quantitate IL-2, IL-6, IL-12(P70), IL-15 and IFN-γ.

Mixed lymphocyte reaction (MLR)

Functional activity of DC was assessed in allogenic MLR. Allogenic human T cells (responder) was isolated from auffy coat, and added in triplicate in graded concentration (104–106 cells per ml) in a rounded bottomed 96-well plate. DC (1 × 103/ml) were
then added into each well. Plates were incubated at 37 °C in 5% CO₂ for 5 days. Proliferation of T cells was measured after adding ³H-thymidine for 18 h. Cells were harvested using Filtermate harvester (PerkinElmer, Massachusetts, USA) and the amount of ³H-thymidine incorporated into the DNA is measured using MicroBeta TriLux counter (PerkinElmer, Massachusetts, USA). The counts were expressed as a count per minute (cpm).

cDNA microarray analysis for gene expression profile

Total RNA was isolated using RNeasy_Plus mini kit (Qiagen, West Sussex, UK) according to the manufacturer’s protocol. cRNA for each sample was synthesized by using One-Color Low RNA Input Linear Amplification Kit PLUS (Invitrogen, Paisley, UK) and was labeled with Cy3 by direct labeling method. Following purification by RNeasy_Plus mini kit, cRNA was fragmented by incubation in the fragmentation buffer at 60 °C for 30 min. The fragmented and labeled cRNA was hybridized to the human whole genome 188 K cDNA chip (Invitrogen, Paisley, UK). After washing, the slides were scanned using Agilent’s microarray scanner (Agilent, Cheshire, UK) and gene expression profiles were analysed by Gene spring GX 7.3.1 software.

Results

Cryotreated prostate cancer cells induce upregulation of costimulatory molecules on cultured DC

On day 7, mean fluorescence intensity (MFI) of surface markers expression was measured and compared between six groups, immature DC (control), DC matured with LPS (LPS), DC loaded with untreated whole tumour cells (UT), DC loaded with tumour lysate (Lys), immature DC loaded with cryotreated whole tumour cells (F) and DC loaded with LPS and cryotreated whole tumour cells (F(LPS)).

LPS induced upregulation of costimulatory molecules and maturation markers (CD80, CD83, CD86, Class-II and CCR7) (P < 0.001) and downregulation of CD14 and CD209 molecules (Fig. 1). Exposure of DC to cryotreated tumour cells at −10 °C resulted in 8-fold increase in the expression of CD86 compared to the control (P < 0.001). Upregulation of CD86 induced by cryotreated tumour cells was equivalent to LPS response. Further increase in CD86 expression was noted when cryotreated cells were added to DC matured with LPS (Fig. 2). There was a statistically significant increase in CD86 expression in DC loaded with cryotreated cells compared to cells loaded with untreated tumour cells or tumour lysate (Fig. 2). Furthermore, DC loaded with cryotreated tumour cells expressed significant levels of CD80 and CD83 compared to other treatments in a similar trend to CD86. Cryotreated tumour cells and tumour lysate induced more than 2-fold upregulation of surface expression of Class-II molecule compared to control DC (P < 0.001). On the contrary Class-II expression was minimally upregulated by untreated whole tumour cells (P > 0.05). DC loaded with cryotreated tumour cells showed a significant upregulation of CCR7 molecule compared to the control and it was equivalent to LPS stimulated DC response (data not shown). CD209 expression was significantly downregulated following exposure to tumour lysate and cryotreated tumour cells (data not shown).

Cryotherapy results in increased stimulatory cytokine expression in prostate cancer cells

We next looked at the cytokine response to tumour cells which had been exposed to freeze injury. mRNA level of immunostimulatory (IL-1β, IL-2, IL-6, IL-12p40, IL-15, IL-18 and TNF-α) and immunoinhibitory (IL-10 and TGF-β) cytokines in response to cryotherapy were assessed using RT-qPCR.

DU145 and PC-3 cells were evaluated and showed similar results overall; representative data for DU145 is shown in Fig. 3. Both cell lines expressed IL-18, IL-16 and IL-15 at relatively high levels, while they expressed lower levels of IL-12, IL-6 and TGF-β and minimal levels of IL-2 and IL-10 (data not shown). mRNA expression for IL-2, IL-15 IL-18 and TNF-α was significantly upregulated in response to freezing injury with the highest expression being at −10 °C (P < 0.001). IL-6, and IL-12 mRNA expression were clearly upregulated at all treatment temperatures and predominantly at −5 °C (P < 0.001). In the contrary, immunoinhibitory cytokines (IL-10 and TGF-β) mRNA expression was downregulated upon freezing. The change in expression was insignificant (P > 0.05) except for IL-10 at −10 °C. IL-1β mRNA expression was the only proinflammatory cytokine tested which is reduced with freezing in DU145 cells whereas in PC-3 cells the mRNA levels increased significantly at −10 °C (data not shown).

DC exposed to cryotreated whole tumour cells express high levels of immunostimulatory cytokines

We investigated the effects of cryotreated tumour cells on DC. Pro- (IL-1β, IL-2, IL-6, IL-12, IL-15, IL-18, IFNγ, TNF-α, and CCR-7) and anti- (IL-10, TGFβ) inflammatory cytokine gene expression was quantitatively assessed in the six experimental groups using RT-PCR. DC loaded with cryotreated tumour cells expressed higher levels of IL-12p40, TNF-α, IL-1β IFN-γ and CCR7 (P < 0.001) compared to control group. IL-15 and IL-18 mRNA expression levels were statistically insignificant (Fig. 4). Upregulation of proinflammatory cytokine induced by cryotreated tumour cells was superior to LPS, tumour lysate and untreated tumour cell response (P < 0.001). Stimulation of DC with LPS followed by cryotreated tumour cells resulted in a strong upregulation of most of immunostimulatory cytokines (IL-1β, TNFα, IFNγ, IL-12p40, CCR7, IL-6 and IL-15 (P < 0.001). IL-2 and IL-6 mRNA expressions were strongly upregulated in DC loaded with tumour lysate (P < 0.001). Untreated tumour cells did not result in any significant change in the level of cytokines tested. mRNA levels of the Immunoinhibitory cytokines (IL-10 and TGF-β) showed downregulation trends nevertheless, changes were statistically insignificant (P > 0.05) (data not shown).
Cytokine secretion in supernatant from DC exposed to various stimuli

Cytokine secretion was evaluated in the supernatant collected from different groups of DC at different time intervals (Fig. 5). Five groups of DC were compared, immature DC (control), LPS stimulated DC (LPS), DC loaded with untreated tumour cells (UT), DC loaded with tumour lysate (Lys) and DC loaded with cryotreated tumour cells (F). Supernatants were collected at 2, 6, 24 and 72 h post treatment. IL-6, IL-15, IL-12p70, IL-2 and IFN-γ cytokine levels in the supernatant were assessed using Bio-Plex cytokine assay. Significant levels of IL-6 were detected in the supernatant of all groups. There was a significant reduction in IL-6 production upon exposure to cryotreated tumour cells compared to the control DC (P = 0.0028). There was a significant increase in IL-15 secretion by DC pulsed with cryotreated tumour cells compared to the control DC and DC matured by LPS (P < 0.001). IFN-γ levels were significantly increased in DC loaded with cryotreated tumour cells compared to control (P = 0.0212). There were no significant difference in IFN-γ levels in the supernatant from DC loaded with cryotreated tumour cells and LPS stimulated DC. IL-12p70 levels were significantly reduced in the F group compared to control (P = 0.0175). There was no significant change in IL-2 levels in the supernatant collected from different groups (P = 0.0517).

DC exposed to cryotreated cells acquire potent T cell stimulatory capacity

We assessed the capacity of DC exposed to cryotreated tumour cells to prime naïve T cells in allogenic MLR. Human T cells were isolated from peripheral blood and stimulated in vitro by immature
DC (control), DC mature with LPS (LPS), DC pulsed with tumour cells cooled at −10 (F), tumour lysate (Lys) or untreated tumour cells (UT) (Fig. 6). Proliferative response was then measured. A strong stimulation of allogenic T cell proliferation was observed with DC that has been pulsed with cryotreated tumour cells compared to DC pulsed with tumour lysate or untreated tumour cells (P < 0.001 and P < 0.05 respectively). The stimulatory activity was higher in DC matured with LPS (P < 0.05) (data not shown).

Gene expression profile by gene microarray in DC loaded with cryotreated tumour cells

We studied the molecular changes in DC loaded with cryotreated cells (F), Lysate (Lys), LPS (LPS) and untreated tumour cells (UT). Immature DC served as a control. Total numbers of differentially expressed genes at the mRNA level were 50, 59, 48 and 67 in LPS, UT, Lys and F groups respectively with more than twofold change in gene expression. All reported genes were statistically significant (P < 0.05). Altered genes were sorted on their biological function which showed that cryotherapy regulates important genes involved in DC maturation and activation (Table 1). Interleu-
kin 1 (IL-1) was upregulated 57-fold in DC loaded with cryotreated tumour cells. It represents a key cytokine for DC activation during early stages of inflammation. It also stimulates CD40 ligand leading to increased cytokine secretion by activated DC.

Fas was upregulated approximately 35-fold in DC loaded with cryotreated tumour cells compared to the control DC. It was previously reported that DC express Fas receptor that can induce T cell apoptosis via Fas–FasL dependent mechanism [18]. DC loaded with cryotreated cells showed 6-fold increase in cycline dependent kinase inhibitor 1A (CDKN1A (p21)) expression compared to control. CDKN1A is required for DC maturation and function [14]. Tumour necrosis factor receptor superfamily, member 11b (osteoprotegerin) was 4-fold upregulated in DC loaded with cryotreated cells. It was demonstrated that osteoprotegerin expression was controlled by NF-κB and it may play a role in regulating immune response [23]. Other genes which was upregulated in the (F) group is vascular endothelial growth factor (VEGF 3.83-fold) and CXCR4 (3.46-fold). CXCR4 is a chemokine receptor that is expressed in mature DC and is responsible for DC migration. It promotes DC survival via altering the balance between key anti- and pro-apoptotic proteins and enhances its function [8]. A number of important genes involved in the induction of apoptosis were downregulated in DC exposed to cryotreated cells such as Caspase 8 (0.426-fold) and BCL2 (0.455-fold).

Discussion

The aim of prostate cryotherapy is to destroy neoplastic tissue and preserve vital structures around the prostate. It is technically challenging to achieve lethal critical temperature in all the prostatic tissue as this will result in a high percentage of complications. Therefore complete ablation of prostate cancer tissue sometimes fails and results in local disease recurrence. In an attempt to overcome this issue, several reports described an adjunctive therapy to cryotherapy in order to increase the sensitivity of cells to cryogenic injury [11]. One approach was intratumoral administration of DC following cryo-ablation of the primary tumour or cryoimmunotherapy [28].

In this study the effect of cryotreated prostate cancer cells on DC maturation and function was addressed and the mechanism responsible for the DC stimulation by cryotherapy identified.

DC can be activated by endogenous signals such as stressed cells, virally infected, necrotic or apoptotic cells [7,22,5]. Primed with tumour freeze/thaw lysate resulted in the development of active immune response and the regression of the primary tumour in cancer patients [17]. Whole tumour cells provide many tumour specific and tumour associated antigens and is ideal for DC priming [26]. Den Brok et al. [4] demonstrated that tumour debris created by radiofrequency and cryotherapy comprises an effective antigen source for local DC which was able to induce DC maturation.

We demonstrated that prostate cancer cells cooled to −10 °C were more effective in inducing DC maturation than tumour lysate and untreated tumour cells. Flowcytometric data revealed 8-fold increase in CD86 marker expression and more than double of class-II expression. Maturation was further increased by treating DC with LPS. The nature of maturation stimuli provided by cryotreated cells is not well identified. Two cytokines are important for DC maturation, TNF-α and IL-1β, both cytokines were not detected in the supernatant of necrotic tumour cells which induced DC maturation [22]. Salio et al. [21] demonstrated that the stimulation was mainly as a consequence of mycoplasma contamination. Cell lines used in this experiment tested negative for mycoplasma infection. Matzinger [16] proposed that stressed cells release alarm signals responsible for DC activation. Todryk et al. [27] have shown that heat shock protein (HSP) 70 expression by tumour cells was associated with increased infiltration of DC into the tumour tissue and resulted in increased antigen uptake by immature DC.

In order to explore the mechanism by which cryotreated tumour cells stimulate maturation signal in DC, we evaluated cytokine gene expression by DC exposed to cryotreated cells, virally infected, necrotic or apoptotic cells [7,22,5]. Differentially expressed genes in DC loaded with LPS (LPS), untreated tumour cells (UT), cryotreated tumour cells (−10) and tumour lysate (Lys). Numbers represent fold change in gene expression compared to control DC. Empty cells means genes were not differentially expressed.

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References


