Re-implantation of tumour tissue treated by cryotreatment with liquid nitrogen induces anti-tumour activity against murine osteosarcoma

H. Nishida, H. Tsuchiya, K. Tomita

From Kanazawa University, Kanazawa, Japan

We evaluated the possible induction of a systemic immune response to increase anti-tumour activity by the re-implantation of destructive tumour tissue treated by liquid nitrogen in a murine osteosarcoma (LM8) model. The tumours were randomised to treatment by excision alone or by cryotreatment after excision. Tissue from the tumour was frozen in liquid nitrogen, thawed in distilled water and then re-implanted in the same animal. In addition, some mice received an immunological response modifier of OK-432 after treatment. We measured the levels of interferon-gamma and interleukin-12 cytokines and the cytotoxicity activity of splenocytes against murine LM8 osteosarcoma cells. The number of lung and the size of abdominal metastases were also measured.

Re-implantation of tumour tissue after cryotreatment activated immune responses and inhibited metastatic tumour growth. OK-432 synergistically enhanced the anti-tumour effect. Our results suggest that the treatment of malignant bone tumours by reconstruction using autografts containing tumours which have been treated by liquid nitrogen may be of clinical value.

Osteosarcoma is the most common primary malignant bone tumour. A standard protocol of neo-adjuvant chemotherapy, combined with wide local excision, has improved overall survival at five years to between 60% and 90%. However, some patients remain resistant to treatment, and lung metastases are often associated with a fatal outcome. Thus, more effective systemic treatment is required. Cryosurgery using liquid nitrogen has been successfully used for the ablation of tumours in the liver, prostate and kidney and in the palliative treatment of locally-advanced breast cancers and benign bone tumours.

We have had excellent results following reconstruction using an autograft containing tumour which underwent cryotreatment using liquid nitrogen. After en bloc excision of the tumour, the surrounding soft tissues were partially removed, and the specimen was immediately frozen in liquid nitrogen for 20 minutes. It was then gradually thawed and re-implanted with suitable internal fixation. The benefits were the relative simplicity of the procedure, sufficient biomechanical strength and the preservation of the osteo-inductive potential and cartilage matrix.

Hypothermia not only induces tumour cell death, but may also activate a systemic anti-tumour immune response stimulated by tumour antigens released by the cryonecrotic tissue. If cryotreatment of malignant bone tumours, especially osteosarcoma, can induce an anti-tumour immune response capable of reducing distant relapse, this approach may be better than other methods of reconstruction. In this study in a murine osteosarcoma we evaluated the possible induction of a systemic anti-tumour response by treatment with tumour tissue which had been treated by liquid nitrogen, by measuring the immune reaction and enumerating distant metastases.

Materials and Methods

We used a total of 104 (23 in each group) female C3H mice (Sankyo Labo Inc, Toyama, Japan) six to eight weeks of age. The animals were housed in a specific pathogen-free animal facility in our laboratory. All the experiments were performed under the guidelines for animal experiments as stipulated by the Kanazawa University Graduate School of Medical Science.

Osteosarcoma cell lines. A murine osteosarcoma cell line, LM8, derived from Dunn osteosarcoma was provided by the Riken Bio Resource Centre, Wako-City, Japan. These cells were maintained in complete medium consisting of RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum,
100 μg/ml of streptomycin, and 100 units/ml of penicillin, and cultured at 37°C in 5% CO₂. In order to establish local implantation of the tumour and subsequent lung metastases, the LM8 cells (1 × 10⁶) were suspended in 0.2 ml of phosphate-buffered serum and inoculated subcutaneously into the right flank of the mice. All the animals had macro- and microscopically confirmed lung metastases within four weeks.¹⁹ We checked the ability of the LM8 cells to promote tumour growth and lung metastases in 100 untreated mice inoculated with LM8 cells, and confirmed that there was tumour growth and also lung metastases in these animals.

**Operative technique.** Figure 1 summarises the protocols used for each group, and the schedule of adjuvant or saline inoculations. Two weeks after inoculation when the tumours had reached 25 mm² in size, they were excised and cryotreatment was undertaken using liquid nitrogen. The mice were placed in a sanitised laminar flow hood and anaesthetised with pentobarbital sodium by intraperitoneal injection. They were then placed in the prone position and the site of the tumour prepared with alcohol. Procedures were performed using a strict aseptic technique. Excision was carried out with grossly negative surgical margins. After control of haemorrhage, the wound was closed using interrupted nylon sutures.

Liquid nitrogen (-196°C) was used to treat destructive tumour tissue which was collected on gauze and soaked in liquid nitrogen for 20 minutes for *en bloc* freezing. The tissue was pre-thawed at room temperature (20°C) for 15 minutes, and then thawed in distilled water (20°C) for 15 minutes. It was then transplanted subcutaneously into the left flank of the same animal and the wound was closed.

**OK-432 adjuvant.** This is used in anti-tumour therapy and is a penicillin-killed and lyophilised preparation of *Streptococcus pyogenes*, which is used as an immunological response modifier.²⁰,²¹ Mice treated by surgical excision or liquid nitrogen received three intraperitoneal injections of OK-432 (1 clinical unit) (Chugai Pharmaceutical Co Ltd., Tokyo, Japan) at days 14, 21, and 28 after inoculation of the tumour (Fig. 1). For control comparisons, excision- or cryo-treated mice were given sterile saline on the same schedule.

**Murine splenocyte culture and activation.** The mice were killed at day 35 after inoculation with the tumour. The spleens were resected and splenocytes were aseptically released using gentle pressure between two frosted glass
Slides. The cells were depleted of erythrocytes by NycoPrep 1.077 A (Axis-Shield PoC AS, Oslo, Norway). To prepare the LM8 cell lysates as stimulators, LM8 cells were subjected to four freeze-thaw cycles (freezing in liquid nitrogen, followed by thawing at room temperature). We confirmed that the freeze-thawed LM8 cell lysates were completely dead before re-culturing. Splenocytes were cultured at a concentration of 2 × 10^6 cells/well in 24-well tissue-culture plates with LM8 cell lysate at 2 × 10^5 cells/well to give a responder-to-stimulator ratio of 10:1. The cells were cultured at 37°C in 5% CO₂ in complete RPMI-1640 media. As a control, the supernatants of the splenocytes alone were collected at 72 hours. However, the cytokine levels of interferon-gamma (IFN-γ) and interleukin (IL)-12 were not available. Cytokine release assay. The supernatants of splenocytes cocultured with LM8 cells were collected at 72 hours after re-stimulation. The release of murine IL-12 p70 and IFN-γ was measured by enzyme-linked immunosorbent assay (ELISA) using Quantikine (R & D Systems, Minneapolis, Minnesota) according to the manufacturer's protocol using an Easy Reader EAR340 microtest plate reader (SLT-Labinstruments, Groedig, Austria). Cytotoxicity assay. In order to examine tumour-specific cytotoxicity, we performed a cytotoxicity assay against LM8 murine osteosarcoma cells. Splenocytes were harvested five days after re-stimulation and used as activated effectors for the ^51Cr release assay. Target LM8 cells (10^5 cells/ml) were labelled with 3.7 MBq of ^51Cr (Perkin Elmer Inc., Waltham, Massachusetts) for one hour at 37°C. Next, 2 × 10^9/100 μl of target LM8 cells were added to round-bottomed 96-well microplates (Iwaki, Funabashi, Japan). Effector cells were added to 2 × 10^3 target cells in 96-well plates at various effector-to-tumour cell ratios. Spontaneous and maximum release of ^51Cr was calculated from the media plus targets, and targets plus 1% Triton-X-100, respectively. After incubation for four hours, 100 μl of supernatant were harvested and measured in a gamma counter (ARC-380; Aloka Company Ltd, Mitaka, Japan). Cytotoxicity was calculated by the following equation:

\[
\text{cytotoxicity (percentage)} = \frac{(\text{counts per minute experimental}) - (\text{counts per minute spontaneous})}{(\text{counts per minute maximum}) - (\text{counts per minute spontaneous})} \times 100
\]

The mean spontaneous cytotoxicity was 377.7 counts per minute (348 to 399) and the mean maximum cytotoxicity 2154.3 counts per minute (2133 to 2167).

Measurement of lung and abdominal metastases. We measured the number of lung metastases and the size of abdominal metastases at 35 days after inoculation of the tumour. Metastatic nodules on the surface of all lung lobes were counted macroscopically and were categorised into four groups as follows: none, few (1 to 5 lesions), medium (6 to 20 lesions) and many (over 20 lesions). Abdominal nodules on the bowel and liver were sized using a Vernier caliper (Niigata Seiki Co., Sanjou-City Niigata, Japan) and were categorised into four groups as follows: none, small (1 mm to 5 mm), medium (6 mm to 20 mm) and large (over 20 mm).

Statistical analysis. We used the unpaired Student's t-test to analyse the data from ELISA and ^51Cr release assays and a Mann-Whitney U test for the data from lung and abdominal metastases. Significance was set at p < 0.05.

Results

Release of cytokines. When the mice were killed on day 35 after inoculation, there was no evidence of local recurrence and only slight scar formation was seen after surgical excision and cryotreatment using liquid nitrogen.

Re-implantation of destructive tumour tissue treated by liquid nitrogen cryotreatment and OK-432 adjuvant therapy was associated with a significantly higher level of IFN-γ (99.92 pg/ml (SD 52.18)) compared with that seen in mice undergoing excision alone (2.54 pg/ml (SD 0.18); t-test, p = 0.043; Fig. 2). Cryotreatment using liquid nitrogen without OK-432 was also associated with a trend towards higher levels of IFN-γ (30.00 pg/ml (SD 30.00)) compared with that in mice undergoing excision alone (t-test, p = 0.19).

Splenocytes obtained from mice with re-implanted tumour tissue treated by liquid nitrogen and OK-432 adjuvant therapy also showed a significantly higher level of IL-12 (3.98 pg/ml (SD 0.53)) compared with that seen in mice which had excision and OK-432 therapy (2.28 pg/ml (SD 0.53); t-test, p = 0.031) or surgical excision without OK-432 (0.96 pg/ml (SD 0.48); t-test, p = 0.001; Fig. 3).

Cytotoxicity. Mice re-implanted with tumour tissue treated by liquid nitrogen showed significantly increased cytotoxicity at effector-to-tumour ratios of 100:1, 50:1 and 25:1 (6.49%, 4.03% and 1.65%, respectively) compared...
with mice treated by surgical excision alone (2.04%, 1.06% and 0.60%, respectively; $t$-test, $p < 0.05$; Fig. 4). Moreover, OK-432 adjuvant therapy synergistically increased cytotoxicity in mice treated by liquid nitrogen cryotreatment at effector-to-tumour ratios of 100:1 (10.28%), 50:1 (5.06%) and 25:1 (3.33%) versus surgical excision ($t$-test, $p < 0.01$, $p < 0.05$ and $p < 0.01$ respectively; Fig. 4).

**Measurements of lung and abdominal metastases.** As shown in the lung photomicrographs (Fig. 5), mice that underwent excision had numerous lung metastatic nodules (Fig. 5a), those that underwent excision and OK-432 had six nodules on one side (Fig. 5b), those that received cryotreatment had two nodules on one side (Fig. 5c), and those that received cryotreatment and OK-432 had none (Fig. 5d). The number of metastatic lung nodules in mice with re-implanted tumour tissue treated by liquid nitrogen and OK-432 was significantly lower than that seen in the excision groups either with or without OK-432 ($t$-test, $p < 0.05$; Fig. 6). In groups which did not receive OK-432, there was no statistically significant difference in the lung nodules between mice treated by liquid nitrogen or excision. However, two of the six mice treated by liquid nitrogen with or without OK-432, showed no evidence of lung metastases. The mean diameter of the abdominal metastases in mice that underwent surgical excision was 25 mm, in those that underwent excision and OK-432 it was 18 mm, in the cryotreatment group it was 7 mm in diameter and mice in that underwent cryotreatment and OK-432 had no abdominal metastases. The size of the metastatic abdominal nodules after re-implantation of tumour tissue treated by liquid nitrogen with and without OK-432, was significantly smaller than that in the excision group without OK-432 ($p < 0.05$; Fig. 7). There were no abdominal metastases in four of the seven mice which had liquid nitrogen with OK-432, and in three of six mice which had liquid nitrogen without OK-432.
Discussion

These results suggest that re-implantation of destructive tumour tissue treated by liquid nitrogen can result in activation of the immune system and inhibit metastatic tumour growth. The results of the cytokine release experiments showed that re-implantation of tumour tissue treated by liquid nitrogen and OK-432 adjuvant therapy induced significantly more cytokine production compared with that with excision alone. The tissue destruction from freezing tumour tissue is accompanied by an inflammatory response in which many different cytokines may be produced and this may influence the type of immune response generated.

In our study, we examined T-helper-1 (Th1) cytokines (IFN-γ and IL-12) in supernatants of splenocytes co-cultured with osteosarcoma LM8 cell lysate. Levels of IFN-γ and IL-12 were significantly increased after treatment by liquid nitrogen with OK-432 compared with excision. This suggested that re-implantation of frozen tumour tissue treated by liquid nitrogen and OK-432 released tumour antigens in the body and promoted the inflammatory cytokines involved in cell-mediated immunity. Cryotreatment by liquid nitrogen or OK-432 alone did not significantly increase the cytokine levels in our model indicating that the use of both liquid nitrogen and an immunological response modifier like OK-432 was necessary to increase these levels.

We also examined the cytotoxicity of splenocytes against LM8 murine osteosarcoma cells. After treatment with re-implantation of tumour tissue which had cryotreatment the activity was significantly increased compared with that after excision. Moreover, OK-432 adjuvant therapy synergistically increased cytotoxicity activity in mice treated by liquid nitrogen. It has been hypothesised that during cryotreatment the immune system of the host becomes sensitised to the tumour being destroyed by the cryotreatment. As the body resorbs the necrotic tissue, an active immunity is developed to the tumour tissue. Our results confirmed that the splenocytes from cryotreatment were sensitised to the destroyed LM8 necrotic tissue, and that sensitised LM8-specific splenocytes attacked target LM8 cells. Our values for cytotoxicity were lower than those in previous reports, possibly because the splenocytes with LM8 lysate were cultured without any additional cytokines which might have activated the immune system, such as tumour necrosis factor-α (TNF-α).

Re-implantation of destructive tumour tissue treated by liquid nitrogen and OK-432 adjuvant therapy resulted in increased protection against lung and abdominal metastases compared with that in excision alone. Additionally, of the mice treated by liquid nitrogen, approximately 30% with OK-432 and approximately 50% without OK-432 had no lung or abdominal metastases. By contrast, all of the mice treated by excision, with or without OK-432, had lung and abdominal metastases. Our findings suggest that the immune system of the host became sensitised to the tumour destroyed by the cryotreatment, and that the metastases were inhibited by the immune system after cryotreatment, while the immune system after excision was activated non-specifically and metastases were not inhibited.

Previous reports have suggested that the immune system is activated by cryosurgery. Alblih, Soanes and Gonder reported that spontaneous regression of metastases occurred after cryosurgery of a primary prostate tumour in...
clinical cases. In animal models, cryosurgery of tumour tissue resulted in rejection of tumour re-challenge and in inhibition of secondary and metastatic tumour growth. The levels of TNF-α, IFN-γ and IL-12 increased in cryosurgery models. Cytotoxicity assay and the activity of natural killer T-cells were increased after cryosurgery in lymphocytes from tumours draining lymph nodes or the spleen. These reports considered that cryosurgery not only destroyed tumour tissue directly by freezing and thawing, but also induced a specific anti-tumour effect by an immune mechanism which was stimulated by tumour antigens released by the necrotic tissue. The specific anti-tumour effect from cryosurgery enhances the systemic immune reaction and reduced the tumour growth and metastases. These reports differed from our method in that cryosurgery was performed by an in situ contact method using a cryobar or cryoprobe to the skin, liver or prostate near the site of the tumour. In our study, we performed en bloc tumour resection and en bloc freezing outside the body, which also induced an anti-tumour effect.

In order to enhance the immune effect after cryosurgery, adjuvant therapy has also been considered. Urano et al reported that cryosurgery with administration of the polysaccharide Krestin increased cytokine production and cytotoxic activity. Lubaroff et al suggested that cryosurgery combined with Bacillus Calmette-Guérin (BCG) was capable of producing an anti-tumour immunity which protected from re-challenge. Udagawa et al recently reported that cryoablation with intra-tumoural administration of BCG-cell-wall skeleton and dendritic cells induced tumour-specific T cells and reduced metastases.

In our study, we combined cryotreatment with OK-432, a non-specific immunoactivator made from a penicillin-killed and lyophilised preparation of Streptococcus pyogenes. OK-432 has been used for cancer therapy and has increased the survival rate of patients with lung cancer, oesophageal cancer, gastro-intestinal cancer, bladder cancer and peritoneal carcinomatosis. OK-432 activates neutrophils, macrophages, T cells and natural killer cells to produce various cytokines, including IL-2, IL-6, TNF and IFN. The proposed mechanism is that OK-432 activates macrophages to produce IL-12, and then induces production of IFN-γ by T cells through co-operation between IL-12, IL-2 and TNF-α. Our results suggested that OK-432 enhanced the anti-tumour effect non-specifically, but synergised with the tumour-specific immune reaction induced by cryonecrotic tissue.

In a study using the Dunn osteogenic sarcoma cell line, it was reported that in mice treated by cryosurgery there was a decreased incidence of metastasis, but natural killer cell activity and tumour-specific autologous Ig G antibodies were not increased. In our study, we confirmed activation of anti-tumour immunity and inhibition of metastases after cryotreatment using the LM8 mouse osteosarcoma cell line. In recent years, there have been reports on the use of cryosurgery with injection of dendritic cells as antigen-presenting cells. Machlenkin et al reported that intratumoural injections of dendritic cells after cryosurgery led to local tumour-induced, tumour-specific cytotoxic T lymphocyte responses as well as reduced lung metastases and a prolonged rate of survival in mouse models of lung carcinoma and melanoma. den Brok et al reported that antigen-presenting cells after cryoablation induced dendritic cell maturation and anti-tumour immunity in a mouse melanoma cell model. Udagawa et al stated that intratumoural administration of dendritic cells with a BCG cell-wall skeleton, which stimulated the dendritic cells, after cryoablation, induced tumour-specific CD8+ T cells and increased the anti-tumour effect in a mouse colon cancer model. In a clinical study, Osada et al reported that patients with unresectable liver tumours who had been treated by cryosurgery, displayed tumour necrosis not only in the treated areas, but also outside these areas, and had increased serum levels of IL-6 and TNF-α, and an increased Th1/Th2 ratio. However, patients with a localised effect who did not show reduced satellite lesions did not have increased cytokines and required another immune activation method. The determination of methods to activate the immune response is important for future studies.

In a clinical study using liquid nitrogen, we treated 28 patients (17 men, 11 women, mean age 31.1 years) with malignant bone tumours. Of these, 13 had osteosarcoma, four Ewing's sarcoma, four chondrosarcoma, three metastatic tumours, and four other tumours. At the time of the final follow-up, six patients had died at a mean of 19.8 months after the operation while 21 remained free from disease at a mean follow-up of 51.9 months. The remaining patient was alive, but with disease. Of the seven patients with metastases at the initial diagnosis, three had died at a mean follow-up of 17.7 months. However, four patients were still alive and of these, two had no evidence of disease and two continued to be free from disease at a mean follow-up of 53 months. Thus, 57.1% of the poor prognosis patients with metastases at the initial diagnosis were still alive at the time of follow-up, suggesting that immune activation by cryotreatment may influence the rate of survival. However, further studies with a larger number of patients are necessary.

Tumour-associated antigens from the surfaces of tumour cells are present in patients with malignant tumours, and T-cells specific for these tumour-associated antigens are produced in the body. However, the size of the tumour actually increases because of decreased production of tumour-associated antigens as the tumours develop, and tumour cells suppress the immune activity of the host. Cryotreatment using liquid nitrogen removes the immune suppression effect of tumour cells after excision of tumour tissue and releases a large quantity of tumour-associated antigens after re-implantation of destructive tumour tissue. We suggest that the increase of tumour-associated antigens in the body induced the anti-tumour immune activity.
We conclude that re-implantation of destructive tumour tissue treated by cryotreatment using liquid nitrogen can activate immune responses and inhibit metastatic tumour growth. OK-432 adjuvant therapy synergistically enhances this anti-tumour effect. These results may offer benefits to patients with malignant bone tumours undergoing surgical reconstruction using autografts containing tumours treated by liquid nitrogen.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


35. Townsend SE, Allison JP. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science 1993;259:368-70.