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Conductive interstitial thermal therapy (CITT) inhibits recurrence and metastasis in rabbit VX2 carcinoma model

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Abstract

Purpose—To investigate the potential of conductive interstitial thermal therapy (CITT) to inhibit recurrence and metastasis in a partially resected tumour model.

Method—Fifteen New Zealand white rabbits were implanted with VX2 tumour intramuscularly in the rear thigh. Once the tumour size reached 20–25 mm in diameter, three animals were randomly selected to serve as controls, while the remaining animals were designated as the study group and treated with CITT. In the CITT group, the partially resected tumour and margins were thermally ablated. In the control group the tumour was partially resected to simulate positive margins. The animals were monitored for up to 12 weeks. At the endpoint, the animals were sacrificed, and whole-body diagnostic necropsy was conducted immediately.

Results—Recurrences and metastatic lesions were observed in iliac and popliteal lymph nodes and abdomens of all control animals. In contrast, the observed rate of recurrence and metastatic lesion was 0% among CITT-treated animals, significantly less than the $\geq 50\%$ null-hypothesis rate expected upon treatment failure (exact binomial $P = 0.0002$). Complete histopathological healing was obtained in 2 of 12 rabbits, and residual inflammation remained at the ablation site up to 12 weeks

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post-ablation in 10 of 12 rabbits. This pattern of necrosis and inflammatory response was not observed in any of the control rabbits.

Conclusions—The CITT device effectively ablated partially resected VX2 carcinoma in a rabbit model, and inhibited recurrence and metastasis in this model. CITT evoked an inflammatory response that may be linked to the mechanism involved in reduced metastatic spread.

Keywords

Conductive Interstitial Thermal Therapy (CITT); VX2 carcinoma model; Radio frequency ablation (RFA); recurrence; metastasis; breast cancer; immune

Introduction

Surgical resection remains the gold-standard treatment for solid cancerous tumours. However, surgical resection often leaves microscopic ‘positive margins’, i.e. inadequate surgical margins in which viable cancer cells are left at the surgical site. Positive margins can lead to local and possibly distant recurrence, with consequent poor prognosis for the patient [1–3]. In breast cancer studies, positive margins up to 56% have been reported following the initial lumpectomy [1,2,4,5]. In prostate cancer patients, a recurrence rate of approximately 40% after radical prostatectomy was reported [6]. Approximately 50% of patients had postoperative positive margin after radical prostatectomy [7]. Furthermore, in head and neck cancer, even when the surgical margins were diagnosed as tumour-free by histopathological examination, local recurrence rates of up to 30% have been reported [8].

Currently, there is no standardised sampling method to determine a surgical margin’s pathological status. Most non-cytological methods assess only 10–15% of the surface of a relatively rounded lesion [9]. Nor is there a standard definition of what constitutes a cancer-free region or ‘negative margin’ [3]. De Mascarel et al. calculated that going from a 90% to a 99% probability of cancer detection required tripling the number of histological sections that needed to be examined, and an additional doubling in order to approach 100% probability [10]. Carter reported that to fully evaluate the pathological status of a 2-cm biopsy specimen, 3,000 6 µm-thick sections would be needed, a formidable and impractical task [9].

Recently, we have reported on the development of a pre-clinical conductive interstitial thermal therapy (CITT) device to obtain negative margins by thermal ablation at very high temperatures [11,12]. During CITT ablation, the heat is delivered to the target tissue by conduction [11, 12]. Heat dissipation by conduction follows well-understood heat transfer principles, and the changes in heat-conduction coefficients in tissue as a function of temperature are linear and known [13,14]. Thus, in CITT, we can accurately predict the extent of the ablation zone via mathematical simulations [11,12]. The reliability, uniformity and controllability of the CITT device were previously demonstrated by ablating, in vivo, soft breast tissue in a swine model and VX2 carcinoma in the New Zealand White (NZW) rabbit model [11,12]. In the rabbit VX2 carcinoma model, our CITT device effectively ablated partially resected VX2 carcinoma in live rabbits. A uniform ablation zone of 10 mm width was created via CITT ablation at maximum probe temperature of 120° to 150°C for 20 to 10 min, respectively, immediately following ablation. Based on this data, we hypothesised that CITT ablation may induce coagulation necrosis and local inflammation that may result in inhibition of local and possibly distant recurrence of VX2 tumours.

To test this hypothesis, we performed CITT ablation on partially resected VX2 carcinoma, and examined metastasis and recurrence rates up to 12 weeks post treatment, sufficient time to observe recurrences and pulmonary metastases of VX2 in live NZW rabbits [15,16].

Materials and methods

CITT and animal model

The principles of CITT were described in detail by Shafirstein et al. in 2007 [11,12]. Briefly, the CITT device consists of a thermal probe with deployable pins and a computer-controlled heater that allows delivery of high thermal doses to the target tissue via heat conduction.

All animal experiments in this study were approved by the UAMS Institutional Animal Care and Use Committee and were conducted according to the guidelines in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication No. 86–23.

NZW rabbits weighing from 2 to 2.5 kg were obtained from Myrtle's Rabbitry Inc. (Thompsons Station, TN). The rabbits were maintained in cages in the animal care facility. The rabbits were subjected to alternating 12-hour periods of dark/light cycle, with access to standard rabbit chow (Harlan Teklad, Indianapolis, IN) and water ad libitum. At VX2 tumour implantation, a rabbit was randomly selected to be the donor, and anaesthetised by intramuscular injection of a mixture of ketamine (50 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg, Fort Dodge Animal Health). A piece of cryopreserved VX2 tumour ($2 \times 2 \times 2$ mm, National Cancer Institute, Frederick Cancer Center, Frederick, MD) was transplanted into the thigh muscle of the donor rabbit. The rabbit was allowed to recover and returned to the cage until the tumour size reached 20–25 mm in diameter.

Study procedure

When the tumour on the donor rabbit reached 20–25 mm in diameter (Figure 1), it was excised, diced to approximately $2 \times 2 \times 2$ mm sections under sterile conditions, and then implanted into the thigh of experimental rabbits. Within 3–4 weeks the tumours reached 20–25 mm in diameter. Three experimental rabbits were randomly selected to serve as controls by undergoing partial resection only, to demonstrate that the VX2 tumour tissue retained virulence after cryopreservation and restoration in the donor animal. The remaining twelve rabbits underwent CITT.

In the CITT group, the rabbits were anaesthetised with intramuscular injection of a mixture of 50 mg/kg ketamine hydrochloride and 10 mg/kg xylazine, followed by inhalation of 2% isoflurane with 2L/min oxygen flow. A 30–40 mm subcutaneous incision was made; the tumour was partially resected, by removing about $5 \times 5 \times 5$ mm cancerous tissue, to create a cavity that accommodated the CITT probe (Figure 2A). Prior to ablation, the cavity was drained of liquid and blood as much as possible. The sterilised CITT probe was then inserted into the cavity (Figure 2B). The temperature was increased at a constant rate of $0.5^\circ\text{C}/\text{sec}$, using a temperature controller, until maximal temperature was reached. Then the ablation was conducted for 10 minutes at the maximum probe temperature of 150°C , the preferred thermal dose suggested by the mathematical model to yield maximum ablation depth in minimum time [11, 12]. After the ablation phase was completed, the probe was removed from the cavity (Figure 2C). The wound was drained, and additional ablated tissue was excised near the skin surface, to improve wound healing. The incision was then aseptically closed with sutures. The rabbits were monitored every 2–3 days for a maximum time period of 12 weeks. The animals were euthanized at the end of the study or when significant pain and distress were observed. After euthanasia, the ablation site, lymph nodes, liver, and lungs were visually examined for the presence of local and distant metastatic lesions and submitted for histopathological examination.

In the control group the rabbits were anaesthetised and prepared for surgery as described above. A 30–40 mm subcutaneous incision was made and the tumour was partially resected by removing cancerous tissue visible to the naked eye. The surgical site was aseptically drained

and closed with sutures. The recovery and follow-up procedures were the same as described above for the CITT group.

Histopathology

The excised ablated region was sectioned into $4 \times 10 \times 4$ mm slices. The tissue blocks were processed and embedded into paraffin and sectioned at 5 mm. Tissue slides were routinely stained with haematoxylin and eosin (H&E) and examined under a light microscope by a veterinary pathologist for presence of recurrent tumour and evaluation of healing in ablated regions. Representative sections of lymph nodes, liver and lungs and gross lesions were similarly examined.

Statistical analysis

The recurrence rate in the CITT group was estimated as the proportion of recurrences among the 12 animals. A one-sided upper 95% confidence limit on the recurrence rate was calculated using the binomial distribution. Finally, the one-sided exact binomial test of a single proportion was used at 5% alpha to compare recurrence in the CITT group against a null-hypothesis recurrence rate of 50%. The recurrence rate among control group was estimated as the proportion of recurrences among the three animals. The statistical analyses were conducted using Excel 2003 (Microsoft Corporation, Redmond, WA).

Results

Metastasis and recurrence

All tumours were treated at a similar size of about 10–15 mm. Over the study period, all the control rabbits (100%) developed histologically verified recurrences and metastatic lesions (Figure 3), a result that is consistent with >50% chance of creating positive margins that tend to lead to recurrence.

Enlarged lymph and carcinoma mass were seen in iliac and popliteal lymph nodes and the abdomens of these animals. The tumours recurred within 2 weeks, and the animals survived 2.5–5 weeks before being euthanized to minimise pain and distress. In contrast, none (0%) of the twelve CITT-treated rabbits developed metastasis or recurrence. This result was significantly lower than the null-hypothesis recurrence rate of 50% ($P=0.0002$), and gives us 95% confidence that the CITT procedure yielded a true recurrence rate of 22.1% or less. The average follow-up time of the rabbits treated with CITT was 10 ± 1.5 weeks and was not associated with tumour recurrence or systemic toxicity. During the study, three animals (from the CITT group) exhibited distress at 6 weeks post therapy, and were euthanized at that time. All the animals undergoing the CITT procedure gained weight and had normal food intake. The ablation site healed within 3 weeks after treatment, and minimal scarring with minimal damage to muscle was observed at the end of the study (Figure 4A, B and C).

Histopathology evaluation

Complete histopathological healing was obtained in 2 of 12 rabbits. Complete healing is defined as presence of surgical scar with no evidence of ongoing inflammation or residual necrotic tissue (Figure 5A). In 10 of 12 rabbits, residual inflammation remained at the ablation site up to 12 weeks post-ablation. This inflammation was characterised by necrotic cellular debris surrounded by granulation tissue or mature fibrosis (Figure 5B). Often, the necrotic debris contained fragments of skeletal muscle fibres with well-preserved morphology (Figure 5C). These fragments were surrounded by intense eosinophilic and heterophilic cellular infiltrate (Figure 5D). Femoral lymph nodes in all ablated rabbits were mildly to moderately enlarged with varying degrees of lymphoid hyperplasia, oedema, and haemorrhage.

Heterophilic and eosinophilic lymphadenitis was present in one animal, and necrosis with fibrosis was present in a second animal (data not shown) in the CITT group.

Discussion

The efficiency and predictability of the CITT was demonstrated by our group previously [11, 12]. In these studies, histopathology and viability staining were used to validate our mathematical simulations in predicting the size of the ablation site. Approximately 60 histopathological slides were required to test our ability to predict the ablation size. However, to determine that there are no viable cancer cells within 8000 mm³, i.e. within 20 mm³ of treated tumour and margins, approximately 3000 slides must be examined via histopathology [9]. This is an extremely laborious and inefficient task to be completed. Therefore, in the present study, we elected to examine the rates of metastasis and recurrence for as long as 12 weeks following CITT, to determine the efficacy of the CITT in ablating partially resected cancerous tumour and creating negative margins in vivo. Spundzhiev et al. observed regional metastasis in 100% of the animals within 6 weeks following wide margin resection of an auricular VX2 tumour [17]. In our study, it took about 2 weeks to observe local and distant recurrence and metastasis following partial resection of the VX2 tumour in our control group. We attribute the relatively short time of recurrence in this case to grossly positive margins following the partial resection of the VX2 tumour, which was intended in this study to simulate positive margins.

In the group of animals treated with the CITT, no local recurrence and no distant metastases were observed up to 12 weeks after CITT treatment. Since the same VX2 tumour source and type were used in the control and the CITT study group, we conclude that the CITT ablation inhibited local recurrence and distant metastasis in this animal model. In comparison to the 35% local recurrence rate seen in other studies with radiofrequency ablation (RFA) of the VX2 tumour [16,18], the 0% outcome obtained in this study of observed recurrences (upper 95% confidence limit of 22.1%) suggests that CITT is more efficacious than RFA in ablating VX2 tumours. During RFA, temperature control within the tissue is obtained by thermocouples at the ends of the deployable electrodes or tines. This control can provide the desired temperature in the vicinity of the tines, but not the temperature of the tissue between the tines. Because most of the RF energy is preferentially absorbed by water and fat [19], if the tissue near the deployable tines has higher water or fat content than the tissue between the tines, the temperature of the tissue between the tines could be significantly lower than the temperature measured by the thermocouples at the tines. Wren et al. found temperature deviations of 3–12°C during RFA of brain lesions [20]. Chang stated that the errors in target temperatures during RFA would not depend on the power, but rather on the temperature at which RFA would operate [21]. Therefore, it can be assumed that during RFA of the VX2 tumours, the target temperatures within tumour and margins were not achieved (i.e. positive margins) and viable cancer cells were able to migrate to undamaged nearby tissue to induce recurrence of the VX2 tumour.

The high efficacy of the CITT in inhibiting local recurrence and distant metastasis of the partially resected VX2 tumour might be attributed to the ability of the CITT to seal the margins by thermal coagulation. With CITT, heat is delivered to the tissue via conduction from the probe's surface, which is heated to as high as 150°C. Upon heating, the tissue sears immediately [11,12]. These high temperatures allowed rapid treatment (10 minutes) of the partially resected tumour and margins while adjacent tissues remain intact, as we demonstrated previously [11, 12]. The 0% local recurrence observed in this study demonstrated that no viable cells were left within the partially resected tumour and margins treated with CITT device. These results also suggest the possibility that the ablation was able to disrupt the transit of tumour cells from the primary tumour to other parts of the body, the bone marrow, and back again to the site of the primary, leading to metastatic and recurrent lesions. We have no definitive evidence for this, yet many other groups have reported intriguing new data to support the idea that cancer may

be a disease of self-seeding [22–27]. Therefore, we hypothesise that thermal fixation of the margin and the long-term inflammatory response to ablation may disrupt a cyclic transit of tumour cells from the primary site and back again.

Lymphoid hyperplasia was observed in local lymph nodes of all CITT-ablated rabbits, suggesting on-going immune stimulation even at 12 weeks post-ablation. The presence of active eosinophilic and heterophilic inflammation in ablated regions, surrounding fragments of skeletal muscle fibres, suggests that skeletal muscle subjected to the high temperatures of CITT may release immunomodulators including chemoattractants. The pattern of this inflammation is reminiscent of eosinophilic myositis, a condition involving intense eosinophilic infiltrate into skeletal muscle tissue with resulting muscle necrosis. This pattern of necrosis and inflammatory response was not observed in any of the control rabbits, and is therefore interpreted to be the result of CITT treatment rather than tumour-related necrosis of skeletal muscle. A few studies suggested that thermal ablation induces anti-tumour immunity, in about 6 weeks following ablation [28,29]. Others have found that radiofrequency ablation (RFA) generated local necrosis and inflammation resulted in infiltration of T cells [30]. However, it was also demonstrated that anti-tumour immune response induced by RFA is not sufficient to cause complete tumour regression [29,30]. In this study we observed complete tumour regression after a single treatment. This observation can be explained by the level of thermal injury induced during CITT. In comparison to RFA, the higher temperature during CITT ablation may result in substantially more intense, but local, thermal trauma to the tumour and adjacent tissue (margins). This local thermal trauma can amplify the immune response. It has been suggested that massive cell death and disruption of tumour architecture may further stimulate the immune response by amplifying the ‘danger signal’, which will significantly increase the generation of cytotoxic T cells [31]. Thus, we can speculate that the thermal damage caused by CITT amplified the anti-tumour immune response. The mechanisms that dictate thermal-induced immune response are not well understood and are still being investigated by several groups. At this time, it is unclear if, how, and to what degree the CITT-induced inflammatory response we observed may contribute to the inhibition of tumour recurrences and metastasis. Further research is underway to determine the mechanisms by which the CITT inhibits recurrence and metastasis of solid tumours.

Clinical relevance

The 1990 Consensus Conference on Breast Cancer from the NIH stated that breast conservation surgery followed by radiation is the preferred method of treatment for stages I and II breast cancer [32]. Since 2002, completed studies on 20- and 25-year follow-ups of randomised trials comparing mastectomy with lumpectomy followed by radiation concluded that survival rates were similar for both therapeutic modalities for women with stage I or II breast cancer when the tumour was <4 cm and the resection margins of the specimen were negative [33–35]. Furthermore, a recent meta-analysis of 78 clinical trials involving 42,000 women with breast cancer concluded that, over a 15-year time period, local recurrence accounts for 25% of cancer-related deaths [36]. A direct link between positive margins and recurrence was suggested in several retrospective studies [1,2]. In a recent clinical study that was conducted at our institute, Klimberg et al. reported on the use of radiofrequency ablation to assist surgery in assuring negative margin [37]. In that study, 24 of 41 patients did not have postoperative radiation therapy, and no in-site local recurrences occurred during a median follow-up of 24 months. The results of this small pilot study suggest that margin ablation could benefit patients with stage I and II breast cancer.

In this study we demonstrate that CITT can create negative margins that inhibit local recurrence and metastasis in animal model. Although the VX2 carcinoma have a different response to heat treatment than human tumours in various ways, the NZW rabbit with VX2 carcinoma is an

established model for use in translating thermal ablation techniques into the clinic [11,38–45].

Thus, CITT has the potential to reduce recurrence rates following surgical resection of the primary tumour. The mechanisms by which CITT disrupts metastatic and recurrent lesions remain to be further elucidated. However, the results of the current study have already suggested some possible mechanisms that include inhibiting tumour-cell transit, and stimulating host immune responses.

Conclusion

The CITT device can effectively ablate partially resected VX2 carcinoma in a rabbit model, and inhibited recurrence and metastasis of VX2 tumour under the conditions of our study. In addition, CITT tumour ablation evoked an inflammatory response which might be linked to the reduced metastatic spread observed. Following surgical resection of the primary tumour, the CITT procedure could be used to reduce the chance of recurrence and metastasis, by inducing coagulation necrosis and local inflammation at the margins of the surgical cavity. Further studies are being planned to translate use of the CITT device into clinical settings.

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Figure 1.
Image of the VX2 carcinoma on a donor rabbit.

Figure 2.
The cavity created for accommodating the CITT probe prior to placement of the device (A),
after placing the CITT device (B) and at the end of the CITT ablation (C).

Figure 3.
A carcinoma mass developed after partial resection of the primary tumour in control group.

Figure 4.
The ablation site at 10 weeks post-CITT treatment showing no signs of scarring. The skin (A) and muscle (B) healed.

Figure 5.

H&E staining of histological slides that were taken from tissue collected at the ablation site at 10 weeks post CITT treatment. Complete dermal healing (A). Dense mature fibrous tissue (arrow) is present in healed dermis. Adjacent dermis (star) is normal. Residual inflammation was present in 10/12 animals (B, C, D). (B) Cellular debris and inflammatory cells (star) are walled off by a capsule of fibrous connective tissue (arrow). (C) Fragments of skeletal myofibers (arrow) are surrounded by necrotic cellular debris and inflammatory cells. (D) Inflammatory infiltrate surrounding myofibre. H&E, magnification 20× (A), 40× (B), 200× (C) and 400× (D).