Review

A Novel Strategy by Cryoablation for Advanced Hepatoma

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Abstract. *Ablative techniques have been developed against advanced liver cancer in cases where surgical resection is impossible. Among the ablation procedures used, thermal ablation therapy is widely applied, and the safety and efficacy of this technique in controlling local progression of cancer have been well characterized. However, the principle of cryosurgery is not only to control local recurrence, but also to stimulate the immune system into initiating an antitumor response. In recent years, we have used a liquid nitrogen-based cryogenic procedure and developed a treatment method for patients with unresectable liver tumors. Following this, repeated treatment was demonstrated to induce anticancer immune reaction-related factors. In the present study, a novel strategy as a cryoablation-induced anti-cancer immune reaction is introduced.*

Hepatic resection has been the only curative option for patients with liver tumors. However, due to limited hepatic reserves, high surgical risk, or unfavorable tumor location, surgery is possible for only 10 to 20% of these patients (1, 2), therefore ablative techniques have been developed as an alternative to hepatic resection (3). With ablation therapy, tumor cell death is achieved *via* chemical (percutaneous ethanol injection), cold-based (cryotherapy), or heat-based (radiofrequency ablation, RFA; microwave coagulation therapy, MCT), or laser hyperthermia techniques. Among these procedures, thermal ablation therapy is widely used, and the safety and efficacy of this technique have been well characterized (4, 5). In addition, cryotherapy has also gained acceptance as a local ablative treatment (6, 7). According to a recent comparative study of thermal and cryoablation therapies, while similar success and complication rates were found, local recurrence is more frequent in cryotherapy (8). However, the principle of cryosurgery is not only to facilitate a low recurrence rate, but also to expand intracellular water, causing destruction of tissue (9). Therefore, intracellular antigens that are released into the circulation enable cryogenic treatment to stimulate the immune system for an antitumor response (9, 10). In recent years, we have used a liquid nitrogen-based cryogenic procedure and developed a treatment method for patients with unresectable liver tumors (10). In the present study, a novel strategy with a cryoablation-induced anticancer immune reaction is introduced.

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Evaluation of Ablation Therapy to Control Local Cancer Progression

Several strategies for the treatment of unresectable hepatic tumors have been proposed (11). Among them, RFA is one of the most convenient and reliable regional therapies used to prolong patient survival (12). A worldwide study demonstrated complication and mortality rates of 8.9% and 0.5%, respectively, from a total of 3670 patients undergoing RFA for malignant hepatic tumors (13). In addition, another large multicenter study also reported similar results (14), therefore, RFA is usually performed with safety. Post treatment imaging showed complete avascularity if the tumor diameter was smaller than 3 cm (15), or not over 5 cm (16). Tumors close to other vital adjacent structures such as the diaphragm, stomach and bowel loop require careful consideration to avoid thermal damage. In an alarming study, there was a 12.5% incidence of tumor seeding in the needle track, emphasizing the importance of prevention for needle track plantation during RFA (17). Thermal ablation therapy was also indicated as accelerating the growth of the remaining tumor by affecting local inflammatory changes (18, 19).

In contrast, the main effect of cryoablation is based on intra- and extracellular ice formation. Intracellular ice formation causes injury of intracellular structures, membrane rupture, osmotic dehydration, anoxia and finally cell death (20). The frostbite effect induced by thrombosis in vessels feeding the tumor has been indicated as providing similar conditions to transcatheter arterial embolization (21). Within

the liver, each cell has a different sensitivity to death by freezing, with the critical temperature for cell destruction reported as ranging from –5˚C to –50˚C (22). During cryoablation, the interface of the frozen/unfrozen liver can be assessed easily with intraoperative ultrasound by the appearance of an echogenic edge with posterior acoustic shadowing, and this is a major advantage of cryoablation over RFA (described later).

Morbidity and mortality rates after cryosurgery were demonstrated as being higher than those after thermal ablation, ranging from 8 to 41% and around 20%, respectively (23, 24). Among the complications, the phenomenon of cryoshock, which is a similar state to disseminated intravascular coagulation (DIC), is responsible for the majority of all mortality. Due to this rare (1%) but severe complication, cryoablation is recognized as a problematic treatment, despite it being an effective local ablative procedure. Relatively recent reports have demonstrated the trigger of cryoshock, in which the ablation of a large area immediately induced a systemic inflammatory response syndrome that was caused by releasing a high volume cytokines (25).

Larger tumor size increases the risk of complications and also of local recurrence. According to controlled studies of RFA and ethanol injections for hepatocellular carcinoma, a significant survival advantage for RFA has already been demonstrated (26). In addition, in a comparative study, a significantly higher local recurrence rate (38% *vs*. 17%) was reported for large tumors, with a diameter over 3 cm, but not for small tumor, when RFA was compared with cryotherapy (8). In contrast, there is another report demonstrating the therapeutic benefit of RFA over cryotherapy, 53% *vs*. 18% (27). The findings of Ahmad *et al*. showed that refinements in RFA probe technology significantly reduced the rates of local recurrence, by using a new probe designed for larger ablation areas (28). The use of second-generation probes is associated with a significantly lower local recurrence (5.2% *vs*. 17.4%). Therefore, RFA is now evaluated as the most favorable procedure among ablation therapies due to its safety and effectiveness, but there are still limits in its usage due to tumor size and location (29).

Immune Reaction Against Cancer Progression

Two subtypes of T-helper (Th) cells were found to have differences in cytokine secretion pattern and other functions, which indicated that Th1 and Th2 cells were important regulators of immune response. Th1 cells are hypothesized to lead the attack against intracellular pathogens such as viruses, raise the classic delayed-type hypersensitivity skin response to viral and bacterial antigens, and fight cancer cells. Th2 cells are believed to emphasize protection against extracellular pathogens such as multicellular parasites. Furthermore, the Th1 pathway has been explained as generating organ-specific autoimmune diseases, such as arthritis and multiple sclerosis (30), but more recently, a new subset of helper T-cells, called Th17, was reported as being related to these autoimmune diseases (31). Th1, Th2 and Th17 subsets are produced from a non-committed population of precursor naïve T-cells (Figure 1). The differentiation process is dependent on cytokine types being released from antigen-presenting cells (APC), which first come into contact with antigens (32). A related APC exposed to an intracellular pathogen migrates to a lymph node and begins to secrete interleukin (IL)-12 to influence naïve T-cells so that they mature into Th1 cells. Natural killer (NK) cells also respond to the IL-12 environment and proceed to release interferon (IFN)-γ, which reinforces the APC production of IL-12 and also helps drive the naïve T-cell commitment process. As they attain maturity, Th1 cells also produce IFN-γ, tumor necrosis factor (TNF)-α, and IL-2. Like the Th1 cells, the emergence of Th2 cells is also dependent on their cytokine environment, such as IL-4 from an APC. In addition, for Th17 cells, IL-6 or tumor growth factor (TGF)-β is important.

Chronic inflammation is now estimated to play a critical role in both tumor initiation and malignant progression. In this chronic inflammatory pro-tumor microenvironment, cell-mediated immunity is suppressed while the activity of hormonal immunity and angiogenesis is increased (33). The development of an immune microenvironment for tumor tolerance is achieved through a variety of immune-suppressive mechanisms, and subsequently diverts the immune system away from tumor recognition and rejection (34, 35). During the process of immune suppression, malignant tumor progression can be associated with a general shift in Th1/Th2 immune responses, particularly in the tumor microenvironment. IFN-γ and other Th1 cytokines are typically lower in patients with advanced cancer patients, while the Th2 marker IL-4 can be higher or unchanged (36). For example, nodules of non-small cell lung cancer freshly removed from patients exhibited a marked imbalance toward Th2 (37). In prostate cancer patients, IL-2 was low (Th1) and IL-10 was high, the latter being a confirmed Th1-suppressive cytokine and common factor in cancer (38). Il-10 has a variety of suppressive effects that include inhibiting Th1 cytokine production, down-regulating APC and NK cell function, and lowering overall T-cell proliferation (39). In particular, under the influence of IL-4 (Th2), tumor cells apparently up-regulate IL-10 and this in turn suppresses nearby NK cells. Tumor-derived IL-10 has been documented in lymphoma, ovarian carcinoma, melanoma, neuroblastoma, and renal cell and colon carcinoma (37). In contrast, IL-12 is another cytokine that can be up-regulated by Th1 activity and inhibited by Th2 (39). Subsequently, the IL-12/IL-10 ratio was found to be important in cervical cancer patients (40), and IL-10 can be predictive for poor prognosis in patients (38). Additionally, a more recent report has shown IL-17 to

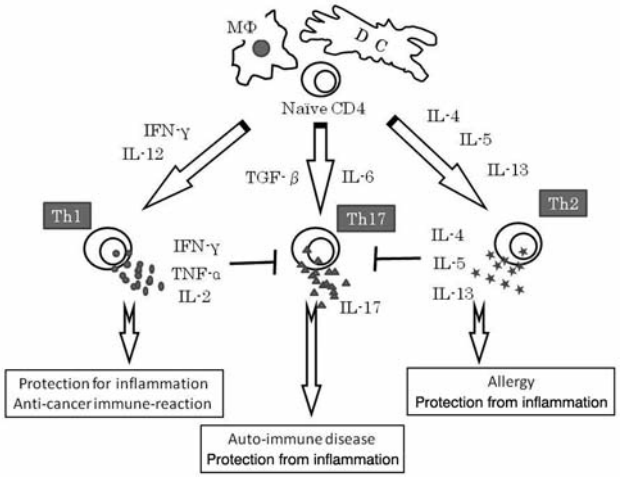

Figure 1. *Schema of immune control balance. Two subtypes of T-helper cells were found to have differences in cytokine secretion pattern and other functions, which indicated that Th1 and Th2 cells were important regulators of immune response. Th1 cells are hypothesized to lead the attack against intracellular pathogens such as viruses, raise the classic delayed-type hypersensitivity skin response to viral and bacterial antigens, and fight cancer cells. Th2 cells are believed to emphasize protection against extracellular pathogens such as multicellular parasites. Furthermore, the Th1 pathway has been explained as generating organ-specific autoimmune diseases, such as arthritis and multiple sclerosis, but more recently, a new subset of helper T-cells, called Th17, was reported as being related to these autoimmune diseases. Th1, Th2 and Th17 subsets are produced from a non-committed population of precursor naïve T-cells.*

increase in tumor tissue, and to be related to cancer vascular development (41). The macrophages associated with developing solid tumors have a phenotype that is also driven primarily by Th2 cytokines and which supports a pro-tumor microenvironment (42).

At present, new approaches to cancer therapy are currently in progress showing that cancer-specific Th1 induced cytokine works with cytotoxic T lymphocytes (CTL) (43). In fact, the treatment of tumors by *i.v.* transfer of Th1 cells combined with intratumor local injection of tumor antigen was found to induce complete tumor rejection, despite Th1 cell therapy alone not being effective against tumor therapy (44). These studies have indicated that induced inflammatory changes in tumor tissue combined with the Th1-dependent immune reaction in sentinel lymph nodes induced the appearance of cancer-specific CTL and cancer rejection. An anticancer peptide or protein to activate cancer-specific Th1 cells is essential, but the induction of the specific anticancer antigen is usually difficult. To activate an antitumor reaction for advanced liver cancer, cryosurgery has been applied in our Department to both destroy tissue by freezing and to induce production of antitumor cytokines.

Novel Development of Cryotherapy

Established method in our department. Unresectable liver tumors were identified at our institute and selected for ultrasound echography-guided percutaneous cryosurgery (PCS) under local anesthesia with a cryoablation system (Mycom cryoneedle; Mayekawa Co. Ltd., Tokyo, Japan). The size of the ice ball was evaluated by intraoperative ultrasound, and freezing was performed for 15 minutes to create a 3-cm ice ball (Figure 2). Three freeze/thaw cycles per tumor were performed per treatment. Once a week PCS combined with daily administration of polysaccharide-K (polysaccharide-Kureha; PSK) was performed, with an overnight hospital stay. PSK, also known as krestin, is a unique protein-bound polysaccharide that is used as a chemoimmunotherapy agent in the treatment of cancer (45). Immunotherapy or biological response modification induced by PSK may improve the host *versus* tumor response, thereby increasing the ability of the host to defend itself against tumor progression (46). PSK-induced production of TNF-α, in particular, was found to play a main role in inhibiting cancer growth (47). In an experimental study, PSK was also found to reduce cryotreatment-induced increases in IL-4 and IL-10 production and to mediate Th1 dominance (48). Thus, simultaneous treatment with PSK may indirectly enhance PCS-activated production of TNF-α and/or IFN-γ by blocking Th2 lymphocyte production. During the treatment, no other therapy was given. Evaluation of serum factors was performed before and after ablation therapy, and serum tumor markers were measured after every four treatments. Tumors were evaluated by abdominal computed tomography after eight treatments.

Clinical benefit and complications. In our experience for the cases with unresectable liver tumors, PCS was performed without any complications (10). In all these cases, not only did levels of serum tumor markers decrease, but local tumor necrosis was also observed. In addition to local changes of the treated area, tumor necrosis was identified away from the treated area in some cases, both in metastatic tumors and primary ones, as described in Figure 3 for a typical case. In these cases, some antitumor immune reaction was suggested, so this group of patients was classified as the immune-reaction (IR) group. By contrast, simple local change without any extra benefit was noted in the remaining cases, so these patients were classified as the local effect (LE) group. Levels of serum factors in the IR group were compared with those in the LE group.

Levels of serum factors to evaluate immune reaction. Serum amyloid A (AA) is a major acute-phase protein released into the circulation in response to inflammation (49) and has been shown to stimulate the rapid expression of TNF-α from

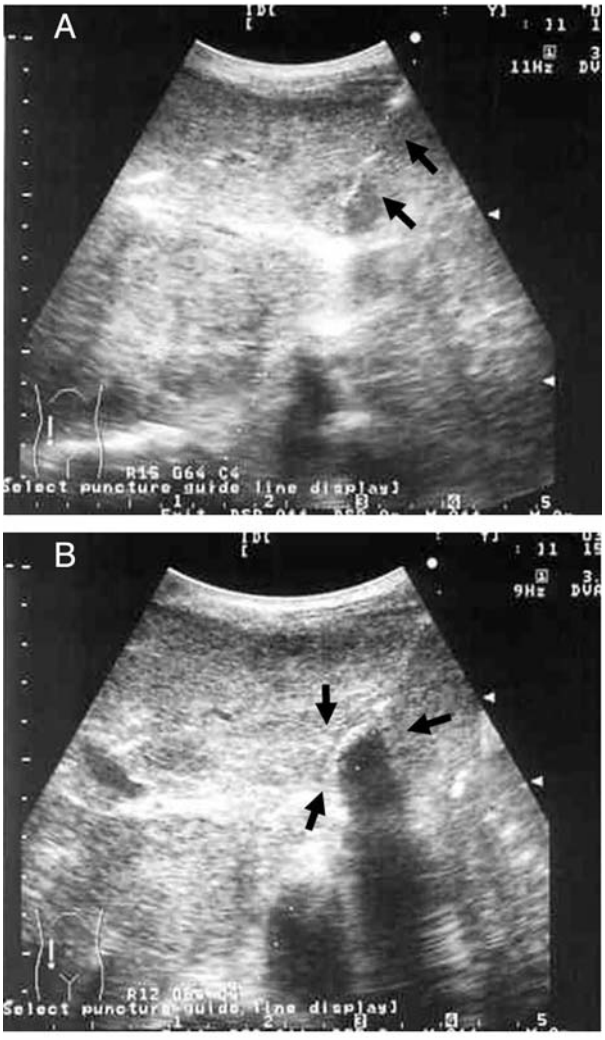

Figure 2. *The local effect of percutaneous cryosurgery. The cryosurgery probe (black arrow in A) penetrated the tumor and the size of the ice ball was evaluated (black arrow in B) by intraoperative ultrasound, and freezing was performed for 15 minutes to generate a 3-cm ice ball.*

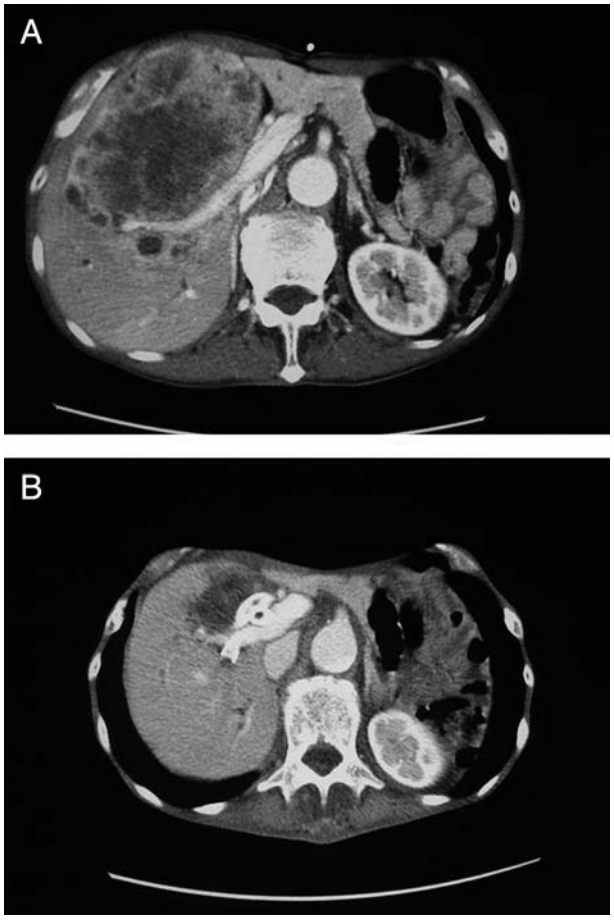

Figure 3. *Benefit of cryosurgery. In all cases, not only did levels of serum tumor markers decrease, but local tumor necrosis was also observed. In addition to local changes of the treated area, tumor necrosis was identified away (B) from the treated area before treatment (A) on computed tomography.*

cultured neutrophils (50, 51). Recently, TNF-α and IFN-γ singly were reported to mediate cell death directly by triggering apoptosis (52) and to increase significantly the effect of cryotherapy (53). Therefore, combined cryosurgery and inflammation-induced TNF-α expression may have mediated tumor necrosis in the absence of serious complications in the IR group. In animal models, Th1 cells have been shown to be critical for the induction of cellular immunity and eradication of the tumor mass (54).

Although the increased production of TNF-α and other cytokines induced by cryosurgery inhibits secondary tumor growth, high plasma levels of these factors have been associated with the occurrence of cryoshock (55, 56). Significant complications may occur when more than 35% of the liver volume is treated by cryoablation, but ablation of small areas of the liver is usually well tolerated (57). In addition, because patients with multiple liver metastases or tumors of more than 4 cm in diameter have an unfavorable prognosis (58), the numbers and sizes of metastases should be considered. In our Department, cryoablation treatment is performed for tumors less than 3 cm in diameter. Instead of small treatments for large tumors, repeated ablation is used to cut through the tumor; after approximately three treatments, increases in serum cytokine levels occur. Studies of repeated cryotreatment have not been reported. Given the lack of complications and the induction of immune reactions, repeated cryotreatment appears to be a favorable strategy.

Serum levels of AA and C-reactive protein (CRP) were increased in both the IR and LE groups after the third treatment, and the levels of IL-6 paralleled the CRP increases. No differences in the level of serum IL-2 were

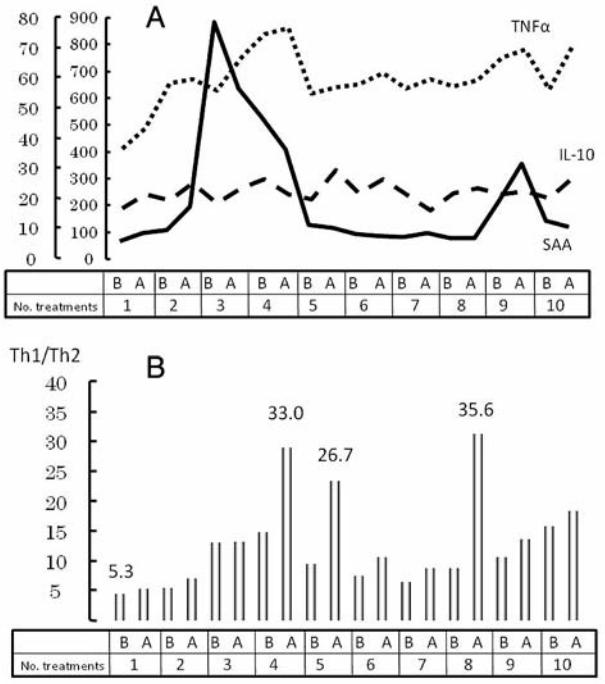

Figure 4. *The changes of serum factors. Serum level of amyloid A increased in both the IR and LE groups after the third treatment. The serum level of IL-10 was low and the level of TNFα was increased in the IR group (A). In addition, the Th1/Th2 ratio clearly increased after treatments (B), compared to the LE group. B, Before, A, after treatment on x-axis.*

observed after treatment in any of the patients. The serum level of IL-10 was low in the IR group and relatively high in the LE group, but it tended to increase with the number of treatments. In contrast, the level of TNF-α was increased in the IR group but showed no remarkable changes in the LE group. In addition, the Th1/Th2 ratio was increased in the IR group, compared to the LE group. A typical case of the IR group is shown in Figure 4. To evaluate the clinical significance of these alterations in serum cytokines, pretreatment levels, maximum levels in response to therapy, and the number of treatments necessary to induce maximum levels were compared between the two groups. Although serum levels of AA and CRP were increased in response to PCS, there was no significant difference between the two groups. Pretreatment levels of IL-10 in the LE group were significantly greater than in the IR group (p=0.0071), and the maximum value (67.9±6.3 pg/mL) was greater in the LE group than the IR group (58.4±8.1 pg/mL) but no significant difference was found between the two groups. In contrast, both pretreatment and maximum levels in response to treatment of TNF-α were significantly greater in the IR group than in the LE group. The maximum Th1/Th2 ratio was significantly greater in the IR group than in the LE group, despite the fact that pretreatment levels and treatment times to induce maximum levels were similar in the two groups. All these details were described in a previous publication from our Department (59).

We can now indicate that induction of immune responses by cryosurgery increases the usefulness of this treatment for unresectable liver tumors. Further studies will assess serum cytokine levels in order to evaluate the timing of PCS and adjustment of PSK volume as a step-up strategy.

Next Step in the Development of Cryosurgery

During the last decade, the development of the procedure has been focused on the control of local ablative treatment. From these efforts, highly effective instruments have been produced, and ablation therapy has been expanded for application to larger tumors. In addition, the study of anticancer immune reaction has pushed new boundaries that have initiated advancements through several clinical trials. However, outcomes of both these approaches are still uncertain because it is unclear how the anticancer immune reaction is associated with cancer death, despite it being clear which factors play a critical role. Cryosurgery including the ability to release anticancer proteins might be emphasized to lead immune therapy for cancer. Future observations of the mechanisms behind the induced anticancer effect of cryosurgery might lead to novel concepts, not only for locally treated tumors, but also at the systemic level as described here.

As a conclusion to this review, the next decade will be an exciting time to develop new approaches to combating cancer using immune reaction theory. Such treatments should provide better outcomes for patients with difficult-to-treat cancer such as advanced liver cancer.

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