
Antitumor effects of heating on human renal cancer cells *in vivo*

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Original article

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Abstract

Objective: Reported to be a useful and minimally invasive treatment for small renal cancers, radiofrequency ablation (RFA) typically induces tissue necrosis via heat coagulation at 100°C. Some reports, however, indicate that the propagation of heat to surrounding organs generates complications. With this in mind, we examined the antitumor effects of heat treatment *in vivo* at temperatures below those typically used in RFA.

Materials and Methods: We injected Caki-1 (human renal cell carcinoma cell line) subcutaneously into the backs of 3-week-old female BALB/c-nu nude mice. The resulting tumors were heated at 45°C, 55°C, or 65°C for 5 or 10 minutes with heat therapeutic equipment designed for research use and heat needles. We subsequently measured tumor volumes at 1, 2, 4, 7, 14, 21, and 28 days after heat treatment. Additionally, the resected tumor was histologically evaluated.

Results: Tumor volumes in the nonheated group (tumors subjected only to needle-puncture) gradually increased, reaching approximately 3.5 times the baseline volume on day 28 after heat treatment. In the groups subjected to heat treatment at 45°C for 5 and 10 minutes, the treatment inhibited tumor volume by day 7, but tumors increased rapidly thereafter. In contrast, tumors shrank in the groups subjected to heat treatment at 55°C and 65°C. In the group subjected to heat treatment at 65°C for 10 minutes, maximum reductions in tumor volume—to approximately 15% of the volume observed immediately after the puncture—were obtained on day 28. We examined the constitutive expression of nicotinamide adenine dinucleotide phosphate (NADPH) to evaluate cell function. Heat treatment at 45°C had minimal effect on the constitutive expression of NADPH in cells. While the area of the site exhibiting diminished cell function after heat treatment at 55°C or 65°C increased on day 1, this function was subsequently restored. Heat treatment resulted in a slight increase in apoptosis in the focal area surrounding the tumor compared to the control group. In the groups subjected to heat treatment at 45°C, hematoxylin and eosin (H&E) staining showed inflammatory cell infiltration on day 4 and subsequent narrow range of necrosis. In the groups subjected to heat treatment at 55°C and 65°C, inflammatory cell infiltration occurred earlier and necrosis was more extensive than in the 45°C-heated group.

Conclusion: Heat treatment at temperatures below those used in RFA as currently implemented in clinical settings has antitumor effects on renal cancer cells. In addition to direct coagulation necrosis induced by heat treatment, secondary inflammatory cell infiltration may be involved in the enhancement of these antitumor effects. Our results should lead to the development of local treatments that feature minimal complications.

Key words: renal cancer, heat treatment, antitumor effects

Introduction

Although heat treatment has long been recognized to have antitumor effects, significant actual research on heat treatment did not begin until the 1980s. Much of this research has focused on superficial tumors, exploring in particular applications to breast cancer, head and neck cancer, and skin cancer. Technological advances in equipment currently allow heat treatment at higher temperatures. Radiofrequency ablation (RFA), a method wherein radiofrequency electric energy is delivered to coagulate and necrotize a tumor mass, is an established and representative heat treatment. Since the 1997 report by Zlotta et al.¹⁾ on RFA therapy for renal cancer, RFA therapy has come to be widely recognized as a local treatment for small renal cancers. RFA therapy is currently not covered by health insurance in Japan. However, clinical practice guidelines for renal cell carcinoma²⁾ recommend RFA therapy “for patients not considered suitable candidates for radical treatment due to poor performance status or complications.” In this context, based on reports that heat coagulation may (although rarely) lead to serious complications in normal tissue, in the urinary tract, and other areas,³⁾ heat treatment at

relatively low temperatures offers a potentially useful approach to minimizing complication risks.

In this study, to identify more effective heat treatment conditions, we examined the antitumor effects of heat treatment on Caki-1 (human renal cell carcinoma cell line) *in vivo* using a strictly temperature-controlled system.

Materials and Methods

Experimental animals and cell line

We purchased 3-week-old female BALB/c-nu nude mice from CLEA Japan, Inc. (Tokyo, Japan). The mice were housed for one week before use in the experiment. The experiment was performed in accordance with rules for animal experimentation established at Ehime University. We purchased human renal cell carcinoma cell line Caki-1 from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). The Caki-1 cells were passaged several times in McCoy's 5A Medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), and frozen and stored. The experiment examined cells passaged 6–8 times after thawing. Once cells were grown to 70% confluence in a 10 cm

culture dish (Greiner Bio-One, Frickenhausen, Germany), they were treated with trypsin (Wako, Osaka, Japan) to obtain a 3×10^6 100 μ L medium. An injection solution was prepared by adding an equal amount of Matrigel (BD Biosciences, Bedford, MA, USA). Caki-1 solution was subcutaneously injected in 200 μ L increments into the back on both sides of the nude mice under inhalation anesthesia with isoflurane (Mylan, Tokyo, Japan). The animals were housed for the approximately 3 weeks required for the puncture (i.e., to a time when the tumor had grown to approximately 10–20 mm in diameter) (Fig. 1A).

Heat treatment of the tumor

Heat therapeutic equipment designed for research use (AMTC300: AdMeTech, Ehime, Japan) (Fig. 1B) and heat needles (SUS304: 22G; length, 10 mm for the heat treatment area) were used to heat the tumors. This equipment subjects internal tissue to treatment with a heat needle that punctures the tumor area, allowing heat to be conducted to the body from the needle, without using the radiofrequency electric energy used in RFA. A temperature-monitoring sensor was added to the heat needle. Tumors were punctured with a heat needle along the longitudinal axis after subjecting mice to inhalation anesthesia with isoflurane (Fig. 1D). Heat was delivered at temperatures of 45°C, 55°C, or 65°C for 5 or 10 minutes. Mice in which needle puncture alone was performed under inhalation

anesthesia served as controls.

Measurement of tumor volume

To estimate tumor volumes ($0.5 \times \text{long axis} \times \text{short axis} \times \text{height}$), we measured the long and short axes of the tumor and tumor height at 1, 2, 4, 7, 14, 21, and 28 days after puncturing and heat treatment. Based on data from five tumors in each group, we expressed tumor volumes as mean values \pm standard deviation. The mice were killed by cervical dislocation and the tumors removed and sectioned into two parts. One part was fixed for 48 hours in 10% neutral formalin (pH 7.4) and embedded in paraffin; the other was embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek: Sakura Finetek Japan, Tokyo) and frozen at -80°C.

Nicotinamide adenine dinucleotide phosphate (NADPH) staining

We sliced frozen tissue samples embedded in OCT compound to a thickness of 10 μ m and stained the samples with NADPH. The thin slice sections were allowed to react with 1 mM NADPH (Sigma-Aldrich), 0.3 mM Nitro Blue Tetrazolium (NBT; Sigma-Aldrich), and 50 mM Tris (hydroxymethyl) aminomethane (TRIS; Nacalai Tesque, Kyoto, Japan) (pH 8.2) at 37°C for 3 hours, then observed under a light microscope (BX63: OLYMPUS, Tokyo, Japan). The sections allowed to react in a solution lacking NADPH were used as negative

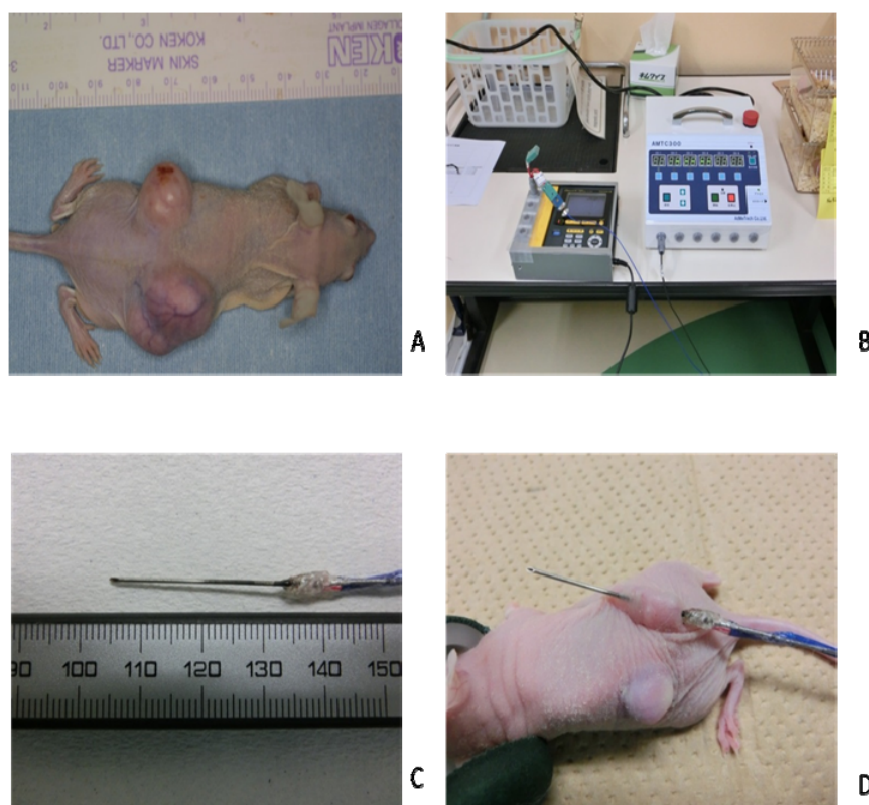


Fig. 1 (A) The Caki-1 human renal cell carcinoma cell line was subcutaneously injected into the back of the mouse. The resulting tumors grew to a diameter of approximately 10–20 mm in about 3 weeks. (B) Heat therapeutic equipment designed for research use (AMTC300) (C) Heat needle (SUS304: 22G; length, 10 mm for heat treatment area) (D) Needle puncture followed by heat treatment

controls. We measured the area in which heat treatment removed staining using image analysis software (cellSens Standard; OLYMPUS, Tokyo, Japan), expressing this area as mean \pm standard deviation.

Hematoxylin and eosin (H&E) staining and apoptosis staining

To prepare serial slice sections, we sliced frozen tissue samples embedded in OCT compound to a thickness of 5 μ m. One section was stained with H&E. Additionally, serial slice sections were stained according to the specified protocol using an In Situ Cell Death Detection Kit (Roche Diagnostics, Tokyo, Japan). The sections allowed to react in a solution lacking TdT were used as negative controls. Counterstaining was performed with Methyl Green (Dako Japan, Tokyo, Japan).

Immunohistologic study

For the immunohistologic studies, we sliced tissue samples embedded in paraffin to a thickness of 5 μ m. These samples were first treated with 20 μ g/ml of Proteinase K (Sigma-Aldrich) for 10 minutes. After the endogenous peroxidase was blocked, the tissue sample was blocked with normal goat serum at room temperature for 30 minutes. Anti-mouse CD45 rat monoclonal antibody (R&D Systems, Minneapolis, MN, USA) was allowed to react overnight at 4°C as a primary antibody, then washed with phosphate buffered saline (PBS); allowed to react with Histofine Simple Stain MAX-PO (Rat) (Nichirei Biosciences, Tokyo, Japan) at room temperature for 30 minutes; then visualized with 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich). Immunoglobulin G (IgG) from non-immunized rats was used instead of the primary antibody as negative controls for staining. Counterstaining was performed with methyl green followed by light microscopic observation.

Results

Changes in tumor volume

Fig. 2 shows the tumor condition on day 28 after heat treatment for 10 minutes at 65°C. The subcutaneously implanted tumor is markedly reduced and almost flat. Fig. 3 shows the subsequent changes in tumor volume. (Tumor volumes immediately after puncturing with a heat needle are defined as 100%.) In the group punctured with a needle without heat treatment, tumor volumes gradually increased, reaching 339.6% \pm 72.6% on day 28. In contrast, tumor growth appeared inhibited by day 7 in the groups subjected to heat treatment at 45°C for 5 and 10 minutes. However, in these cases, tumor growth subsequently resumed, and this growth was rapid. On day 28, tumor volumes in these groups subjected to heat treatment at 45°C for 5 minutes and 10 minutes remained at 154.3% \pm 16.2% and 187.0% \pm 51.7%, respectively, compared to values observed immediately after heat treatment. In the groups subjected to heat treatment at 55°C and 65°C, tumor growth was briefly observed on day 1 but was thereafter inhibited, or the tumor was somewhat reduced. In the group subjected to heat treatment for 10 minutes at 65°C, the most pronounced tumor-reducing effects were observed on day 28;



Fig. 2 External appearance of subcutaneously implanted tumor on day 28 after heat treatment (left, heat treatment at 65°C for 10 minutes; right, heat treatment at 65°C for 5 minutes)

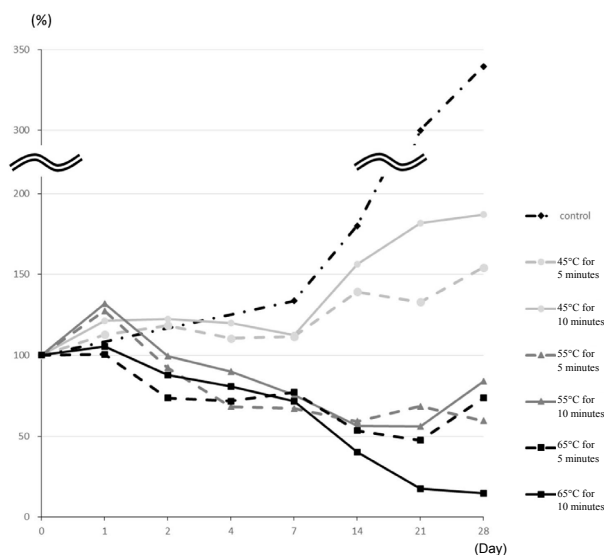


Fig. 3 Changes in tumor volume by heat treatment temperature and time

Tumor volume was determined by the following equation: $0.5 \times \text{diameter} \times \text{short axis} \times \text{height}$. The value shown is the mean tumor volume obtained from 5 mice.

tumor volumes had shrunk to 14.9% \pm 18.7% of the tumor volume immediately after heat treatment.

NADPH staining

We examined the constitutive expression of NADPH to evaluate cell function. We determined the effects of heat treatment on cell function by measuring the area in which heat treatment eliminated staining (Fig. 4). Figs. 4A and 4B, respectively, are representative photos on day 7 for the control group (tumors subjected only to needle-puncture) and for the group subjected to heat treatment at 55°C for 10 minutes. No increase in negatively-stained areas was observed ($0.6 \pm 0.19 \text{ mm}^2$) after heat treatment at 45°C, even for 10 minutes. In contrast, negatively-stained areas increased on day 1 in the groups subjected to heat treatment at 55°C and 65°C. When tumors were heated at 55°C for 5 and 10 minutes and at 65°C

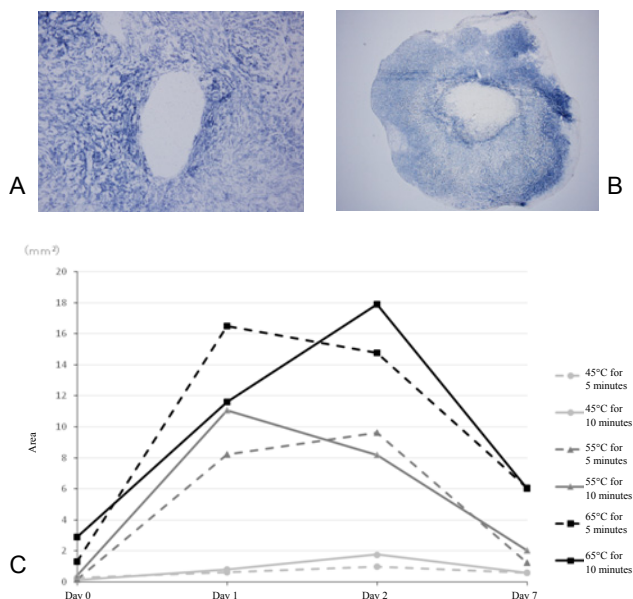


Fig. 4 NADPH staining

- (A) Control group (needle puncture only) on day 0, $\times 100$
 (B) Group subjected to heat treatment at 55°C for 10 minutes on day 7, $\times 20$
 (C) Changes in non-staining area by heat treatment temperature and time
 Each plot shows mean measurement values obtained from 4 to 10 sections.

for 5 minutes, the negatively-stained areas reached peaks on day 1 ($8.2 \pm 4.5 \text{ mm}^2$ for heat treatment at 55°C for 5 minutes; $11.0 \pm 2.4 \text{ mm}^2$ for heat treatment at 55°C for 10 minutes; $16.5 \pm 3.6 \text{ mm}^2$ for heat treatment at 65°C for 5 minutes). In the group subjected to heat treatment at 65°C for 10 minutes, the area reached the peak value ($17.9 \pm 4.6 \text{ mm}^2$) on day 2 and decreased thereafter (Fig. 4C).

Apoptosis staining

We observed sporadic apoptosis in the control group and confirmed focal apoptosis in the external area of the site that may have been affected by heat treatment and in the area surrounding the tumor (Fig. 5). The number of apoptotic cells gradually increased after heat treatment at 55°C and 65°C, reaching a peak on day 4. However, the increase in apoptotic cells was not marked, and the number was only slightly greater in these heat treatment groups than in the control group.

Immunohistologic study of H&E staining and CD45

We applied H&E staining to morphologically evaluate inflammatory cell infiltration and necrosis. We used immunohistologic staining, whereby the majority of white blood cells are responsive to the anti-CD45 antibody, to detect inflammatory cell infiltration. Although no inflammatory cell infiltration was observed in the control group (Fig. 6A), we observed various degrees of inflammatory cell infiltration after heat treatment. In the groups subjected to heat treatment at 45°C, inflammatory cell infiltration was observed on day 4. A narrow range of necrosis was detected in the area surrounding

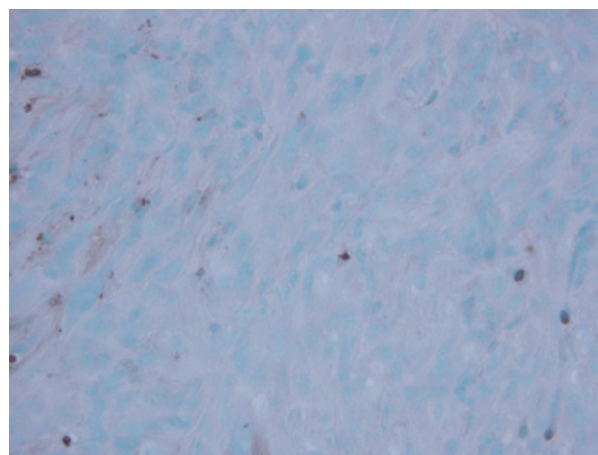


Fig. 5 Apoptosis staining
Focal apoptosis is visible in the area surrounding the site affected by heat treatment. $\times 400$

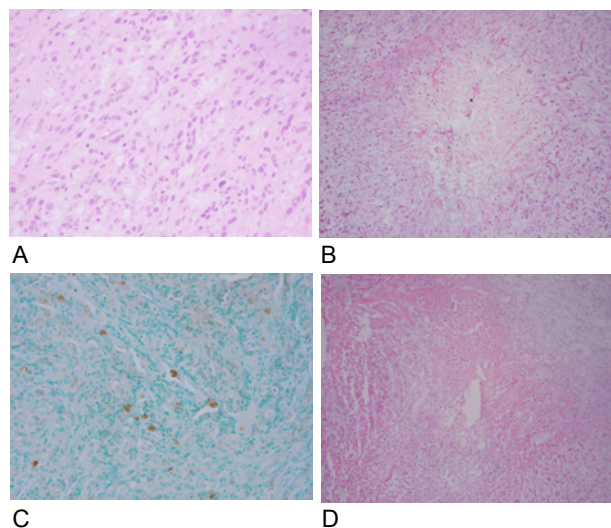


Fig. 6 H&E staining and CD45 staining

- (A) Control group: No inflammatory cell infiltration is visible. (H&E staining, $\times 200$)
 (B) Groups subjected to heat treatment at 65°C for 10 minutes: Necrosis and surrounding inflammatory cell infiltration are visible on day 1. (H&E staining, $\times 100$)
 (C) CD45 staining shows white blood cell infiltration. (CD45 staining, $\times 200$)
 (D) Group subjected to heat treatment at 65°C for 10 minutes: More extensive necrosis is visible on day 2. (H&E staining, $\times 100$)

the puncture site on day 7. In the groups subjected to heat treatment at 55°C, we observed inflammatory cell infiltration on day 1 and marked necrosis mainly at the puncture site on day 4. In the groups subjected to heat treatment at 65°C, we observed inflammatory cell infiltration on day 1 (Figs. 6B and 6C) and extensive necrosis centered mainly at the puncture site on day 2 (Fig. 6D).

Discussion

In this study, heat treatment at temperatures lower than those used in RFA resulted in inflammatory cell infiltration in the area surrounding the puncture site, ultimately leading to necrosis. The human renal cancer cells that were subcutaneously injected had shrunk markedly on day 28 after heat treatment at 65°C for 10 minutes.

In 2010, approximately 2,700 men and 1,300 women died of renal cell carcinoma in Japan, accounting for 1% of all cancer deaths among both men and women.⁴⁾ While pain, hematuria, and an abdominal mass are understood to be a triad of renal cancer symptoms, these signs in many cases do not manifest simultaneously. Small renal cancers are often discovered in imaging studies during routine health checkups or in follow-up for other diseases. Various therapeutic options are available, particularly in cases of T1a (tumor size, ≤ 4 cm). Nephron-sparing surgery targeting the treatment of lesions alone is the mainstay option. Although partial nephrectomy is generally selected, reports indicate RFA (not covered by health insurance) is also performed as a local treatment. The clinical practice guidelines for renal cell carcinoma recommend RFA therapy for patients who are not suitable candidates for surgery, whether due to advanced age or complications.²⁾ However, the potential effects of RFA on the surrounding normal tissue are a concern: Percutaneous RFA is reportedly associated with complications at rates of 8–13%. Reported complications include bleeding, abscess, urinary stricture, and hematuria, in addition to serious renal or duodenal fistulae.^{5)–7)}

RFA is performed at target temperatures of $\geq 60^\circ\text{C}$, usually around 100°C , with the goal of producing irreversible heat degeneration of tissue—i.e., coagulation necrosis. The necrotic tissue is cleared by macrophages 3–7 days after heat treatment, and the organized renal cellular architecture disappears or forms scar tissue in approximately one month.⁶⁾ The results of the present study are significant because we observed heat treatment antitumor effects at temperatures lower than the high temperatures commonly used in RFA. If heat treatment is effective at lower temperatures than is typical of RFA, it may be possible to minimize complication risks. Heat treatment at the high temperatures associated with RFA is expected to produce direct and extensive coagulation necrosis; however, if heat treatment at lower temperatures impairs cell function sufficiently, immediate inflammatory cell infiltration results, enhancing the antitumor effects of this heat treatment.

Temperatures of 40°C or lower are generally reported to result only in reversible heat degeneration. Cancer cells are regarded to be sensitive to temperatures of 42.5°C or higher. For this reason, this study set the first temperature at 45°C , then increased temperatures in 10°C increments, resulting in three temperatures: 45°C , 55°C , and 65°C . According to Sterrett et al., heat treatment at lower temperatures (45°C to 55°C) does not result in marked change, but does lead to edema formation through certain biochemical changes, including changes affecting cell oxygen and cell-membrane transporters.⁸⁾ The present study also evaluated cell function based on observations of the constitutive expression of NADPH. These evaluations showed that heat treatment at 55°C or 65°C

temporarily removes NADPH staining, indicating that heat treatment at these temperatures impairs cell function temporarily. Furthermore, tumor volumes increased temporarily on day 1 after heat treatment at 55°C or 65°C , presumably reflecting edema in the local area. In contrast, heat treatment at 45°C had minimal effects on NADPH staining, and only a narrow and insignificant range of staining was lost on day 2 after heat treatment for 10 minutes. Heat treatment at 45°C was found to impair cell function minimally in terms of the constitutive expression of NADPH. However, even heat treatment at 45°C for 5 minutes inhibited growth in tumor volumes for about one week, suggesting that heat treatment, even at 45°C , may impair other cell functions. In fact, we observed inflammatory cell infiltration and a narrow range of necrosis on days 4 and 7, respectively, after heat treatment at 45°C . However, after heat treatment at 45°C for 5 or 10 minutes, tumor growth resumed after one week. Thus, heat treatment at 45°C is inadequate for tumor control. However, tumors heated to 55°C or 65°C gradually shrank from day 2. Histological evaluations of the specimens heated to these temperatures identified necrosis but no marked increase in apoptosis in the areas surrounding the puncture sites. Since we observed inflammatory cell infiltration before the emergence of extensive necrosis, this infiltration may be involved in the extensive necrosis that follows the transient cell apoptosis induced by heat treatment, and ultimately in tumor volume reductions. This observation raises the possibility that heat treatment at 55°C or 65°C has few direct effect on coagulation necrosis but induces subsequent secondary cell damage. A report on an RFA study involving hepatocellular carcinoma indicates that necrosis results in the appearance of signals activating immunocompetent cells, including dendritic cells.⁹⁾ Furthermore, the study suggested that because cross-reactions between heat treatment and non-heat treatment tumor areas occur in immune recognition, while minimal cross-reaction of heat treatment tumor areas with normal tissue is observed, cell infiltration led by direct coagulation necrosis may play a role in this cell damage. In the present study, heat treatment at 65°C for 10 minutes resulted in a gradual decrease in tumor volume, leading, on day 28, to shrinkage to approximately 15% of the volume immediately after heat puncture. This supports the beneficial effects of heat treatment of renal cancer cells at 65°C . Additionally, despite insufficient evidence supporting heat treatment at 55°C in this study, several sessions of heat treatment at 55°C or heat treatment in combination with chemotherapy may prove beneficial. This issue requires further study.

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