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LABORATORY INVESTIGATION

Radiofrequency Ablation of Liver Tumors in Combination with Local OK-432 Injection Prolongs Survival and Suppresses Distant Tumor Growth in the Rabbit Model with Intra- and Extrahepatic VX2 Tumors

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Abstract
Purpose To evaluate survival and distant tumor growth after radiofrequency ablation (RFA) and local OK-432 injection at a single tumor site in a rabbit model with intra- and extrahepatic VX2 tumors and to examine the effect of this combination therapy, which we termed immuno-radiofrequency ablation (immunoRFA), on systemic antitumor immunity in a rechallenge test.
Methods Our institutional animal care committee approved all experiments. VX2 tumors were implanted to three sites: two in the liver and one in the left ear. Rabbits were randomized into four groups of seven to receive control, RFA alone, OK-432 alone, and immunoRFA treatments at a single liver tumor at 1 week after implantation. Untreated liver and ear tumor volumes were measured after the treatment. As the rechallenge test, tumors were reimplanted into the right ear of rabbits, which survived the 35 weeks and were followed up without additional treatment. Statistical significance was examined by log-rank test for survival and Student's *t* test for tumor volume.
Results Survival was significantly prolonged in the immunoRFA group compared to the other three groups ($P < 0.05$). Untreated liver and ear tumor sizes became significantly smaller after immunoRFA compared to controls ($P < 0.05$). In the rechallenge test, the reimplanted tumors regressed without further therapy compared to the ear tumors of the control group ($P < 0.05$).
Conclusion ImmunoRFA led to improved survival and suppression of distant untreated tumor growth. Decreases in size of the distant untreated tumors and reimplanted tumors suggested that systemic antitumor immunity was enhanced by immunoRFA.

Keywords Experiment · Liver · OK-432 · Rabbit · Radiofrequency ablation · VX2 tumor

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Introduction

Radiofrequency ablation (RFA) is an effective local treatment for unresectable hepatocellular carcinoma (HCC) and metastatic liver tumors [1–3]. Its effectiveness, however, is not systemic and is limited by the presence of a possible local residual tumor and distant tumor [1]. Intrahepatic recurrence and systemic metastasis often occur after RFA of HCC and metastatic liver tumors, and posttreatment prognosis is

improved only if good local control is achieved and if multiple metastases do not occur [1]. These facts mean that RFA alone does not appear to induce effective systemic antitumor immunity [4]. Nonetheless, we are increasingly seeing immunological evidence suggesting that RFA has the potential to induce a systemic antitumor response via tumor-specific T-cell activation [5, 6].

Biological response modifiers (BRMs) are substances that enhance the body's defenses against, e.g., tumor cells. They include various types of cytokines, interleukins and genes, bacillus Calmette-Guérin, granulocyte–macrophage colony-stimulating factor (GM-CSF), and OK-432 [7–9]. Some of them have been used to induce systemic antitumor immunity in vivo. OK-432 is a BRM derived from heat- and penicillin-treated and lyophilized preparation of a low-virulence strain Su of *Streptococcus pyogenes* that was first described by Okamoto et al. [10] in 1967. OK-432 was originally developed as an antitumor drug and exerts antitumor actions directly by suppressing tumor growth and indirectly by causing tissue inflammation, which stimulates immune cells and cytokine production [11, 12]. Previous studies demonstrated that OK-432 stimulates maturation of dendritic cells and potent antigen-presenting cells, and induces a T-cell response [13, 14]. However, OK-432 alone has not been shown to improve the survival of patients with HCC or other cancers. Currently, OK-432 is rarely used as an antitumor drug except for pleurodesis and sclerotherapy of lymphangiomas with inflammatory adhesion [15, 16].

In the present study, we hypothesized that systemic antitumor immunity could be enhanced by combining RFA with local OK-432 injection. We termed this new approach immuno-radiofrequency ablation (immunoRFA). Theoretically, because OK-432 induces tissue inflammation and stimulates the maturation process of antigen-presenting cells [12, 17], it may enhance the ability of RFA to produce systemic antitumor immunity when locally injected at the RFA site. To our knowledge, there has been no report in which RFA was performed on liver tumors to examine its effect on distant tumors. The aim of this study was to evaluate survival and distant tumor growth after immuno-RFA of a single liver tumor site in a rabbit model with intra- and extrahepatic VX2 tumors and to examine the effect of immunoRFA on systemic antitumor immunity in a rechallenge test in which tumor reimplantation was performed without additional treatment 35 weeks after therapy.

Materials and Methods

Animal Care

All experiments were approved by our institutional animal care committee and performed under proper anesthesia. Animals were 12-week-old female Japanese white rabbits weighing 2–2.5 kg. Anesthesia was induced by intramuscular injection of 1 mL of a mixture of ketamine 40 mg (0.8 mL) and xylazine 4 mg (0.2 mL) into a thigh muscle and maintained by intravenous injection of 0.1 mL of the same mixture via an auricular vein. Euthanasia was scheduled at 25 % body weight loss in a week as humane end points. Euthanasia was performed by high doses of intravenous administration of ketamine and xylazine under adequate anesthesia as previously described.

Tumor Model

Donors were 14-week-old rabbits in which VX2 tumors had been implanted into the hind limb muscles at 12 weeks of age. Tumor masses were collected from the donor and cut into 2-mm cubes for implantation into the liver [18]. The rest of the tumor masses were crushed and mixed with normal saline to prepare a VX2 tumor suspension (2.5×10^6 cells/mL) for implantation into the ear.

A rabbit model with intra- and extrahepatic tumors was created by implantation of VX2 tumors into three sites, two in the liver and one in the left ear. The liver of the recipients was exposed by laparotomy [18]. Tumors were implanted in the parenchyma of the left and right liver lobes. The liver was returned to its original position, and the abdominal wall was closed in double layers with silk suture. Subsequently, the recipients were subcutaneously injected with 0.1 mL of VX2 tumor suspension into the center of the left auricle with a 20-gauge needle.

Liver and auricular tumors were considered established when they reached a volume of $\geq$14 and $\geq$4 mm^3, respectively, at 1 week after implantation. Liver tumor volume was estimated on the basis of contrast-enhanced computed tomography (CT) images using the following formulae: 3.14 $\times$ maximum long diameter $\times$ maximum short diameter $\times$ (number of slices depicted $-$ 1) $\times$ 3 mm (slice thickness)/6 for the liver tumor [19]. Ear tumor diameter was measured by a caliper, and ear tumor volume was calculated as 3.14 $\times$ long diameter $\times$ short diameter $\times$ tumor thickness/6.

CT Protocol

CT was performed with a single-detector scanner (Pro-Speed; GE Healthcare, Milwaukee, WI) with the following parameters: voltage 120 kV; current 60 mA; collimation 3.00 mm; slice thickness 3 mm; pitch 1 and matrix 320 $\times$ 320 mm. 5 mL of contrast agent (300 mgI/mL iohexol; Omnipaque 300; Daiichi Sankyo, Tokyo, Japan) were intravenously injected from the right auricle at a rate of 1 mL/s [20]. At 25 s after starting injection, CT was performed.

Radiofrequency Ablation

A 17-gauge needle electrode (LeVeen; Boston Scientific, Natick, MA) with eight retractable hooks (diameter 2 cm) was percutaneously inserted into the liver tumor under CT guidance, and retractable hooks were fully deployed. Radiofrequency (RF2000 generator; Boston Scientific) was started at 30 W and increased by 10 W up to 50 W every 30 s after 1 min. Radiofrequency was applied until the generator automatically stopped due to increased impedance caused by tissue dehydration (roll-off) or at 5 min if not stopped automatically [21].

Local Injection of OK-432

First, 0.3 Klinische Einheit (KE) (=0.84 mg, 0.1 KE/kg) of OK-432 (Chugai Pharmaceutical, Tokyo, Japan) powder was dissolved in 0.15 mL of normal saline and mixed with 0.05 mL of lipiodol to obtain a 0.2-mL emulsion. It was injected percutaneously into the center of the tumor with a 23-gauge needle under CT guidance. When performing both RFA and local injection therapy, OK-432 was injected into the center of the ablation site immediately after RFA. Drug distribution was approximated by the distribution of lipiodol that covered the tumor or the ablation site, including the surrounding normal tissues, on nonenhanced CT.

Experimental Design

Seven rabbits were randomly assigned to each treatment: control, RFA alone, OK-432 alone, or immunoRFA (Fig. 1). Each treatment was performed at only one liver tumor site in the left lobe at 1 week after tumor implantation. In the immunoRFA group, OK-432 was locally injected immediately after RFA. Survival was followed for up to 35 weeks (245 days) to ensure a sufficient length of period for evaluation of systemic immunological effects. A previous report has shown that the median survival in rabbits with liver VX2 tumor is approximately 7 weeks [22]. For this reason, CT scan was scheduled on the day of treatment (week 0) and 3 and 7 weeks after treatment. Recurrence and residual tumor at the treated site were also assessed on CT at week 3. Left ear tumors were measured weekly during the 35 weeks. Tumor response was assessed with RECIST 1.1 by comparison of the tumor volumes from week 0 and the last measurement of each tumor [23]. Although most tumor sizes were less than 10 mm that are excluded by the RECIST criteria, an increase rate of tumor volume was calculated based on the RECIST criteria. When the tumor volume increased 73 % at least or decreased 66 % at least, tumor response was defined as progressive disease (PD) or partial response (PR), respectively. Complete response (CR) and stable disease (SD) were evaluated in accordance with the RECIST criteria.

In the rechallenge test, rabbits that survived the 35-week observation period were injected subcutaneously with 0.1 mL of VX2 tumor suspension into the right auricle (week 35). Subsequently, right ear tumors were measured weekly for 5 weeks (weeks 36–40).

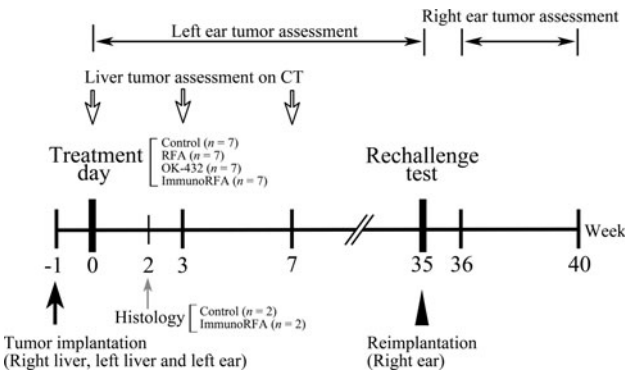

Fig. 1 Experimental schedule. Tumors were implanted at three sites a week before treatment (*black arrow*). Liver tumors in both the right and left lobes were evaluated on enhanced CT at the treatment day and 3 and 7 weeks after treatment (*white arrows*). Left ear tumors were assessed weekly for 35 weeks after treatment. In the rechallenge test, VX2 tumors were implanted to the right ear at 35 weeks after treatment (*black arrowhead*) and followed up weekly until 40 weeks after treatment. Additionally, 2 groups of 2 rabbits received control and immunoRFA treatment, respectively, for histological study at week 2 (*gray arrow*)

Histology

Apart from the above-mentioned study groups, two groups of two rabbits were implanted with tumors as described above and received control and immunoRFA treatments, respectively, for histological examination. The liver and ear tumors were collected at week 2 and examined for lymphocyte infiltration by hematoxylin–eosin staining.

Statistical Analyses

Survival curves were generated by Kaplan–Meier method. Log-rank test was used to examine differences among groups. Tumor volumes were compared between groups by Student's t test. Statistical analyses were performed using SAS (version 9.1, SAS institute, Cary, NC). The criterion for statistical significance was $P < 0.05$.

Results

Tumor Model

Thirty-six rabbits were used in this study. Four rabbits that did not develop tumors (missing liver tumors in three

rabbits and an ear tumor in one rabbit) were excluded from the study. The liver and ear tumor establishment rate was 91.7 and 97.2 %, respectively. Four rabbits were used for histology. Thus, a total of 28 rabbits were divided into the control ($n = 7$), RFA ($n = 7$), OK-432 ($n = 7$), and immunoRFA ($n = 7$) groups.

Radiofrequency Ablation

The needle electrode was inserted successfully into the tumor in all animals of the RFA group and the immunoRFA group. Roll-off was achieved in all procedures with a mean ablation time of 134 ± 70.4 s. The mean impedance at the start of ablation was 76.6 ± 34.0 Ω.

Survival

Survival curves are shown in Fig. 2. Survival after immunoRFA was significantly longer than that in the other three groups (vs. control, $P = 0.002$; vs. RFA, $P = 0.026$; vs. OK-432, $P = 0.026$). The median survival time was 65 days (range, 37–73 days) in the control group, 93 days (27–245 days) in the RFA group, 72 days (35–245 days) in the OK-432 group, and 245 days (47–245 days) in the immunoRFA group. There was also a significant difference in survival between the control group and the RFA group ($P = 0.006$). One rabbit in the RFA group, one rabbit in the OK-432 group, and five rabbits in the immunoRFA group survived the 35-week posttreatment follow-up period.

Liver Tumors

Untreated liver tumors were evaluated by CT at weeks 0, 3, and 7. By week 7, two rabbits in the control group, one rabbit in the RFA group, one rabbit in the OK-43two group, and one rabbit in the immunoRFA group were withdrawn from the study for humane reasons. At 3 weeks after treatment, untreated liver tumors became larger in all four groups, and no significant intergroup difference was observed in their sizes (Fig. 3; Table 1). At week 7, the tumor volumes were again larger in the control, RFA, and OK-432 groups, while it remained unchanged in the immunoRFA group (Fig. 4). At this point, there was a significant difference in untreated liver tumor size between the control group and the immunoRFA group ($P = 0.025$).

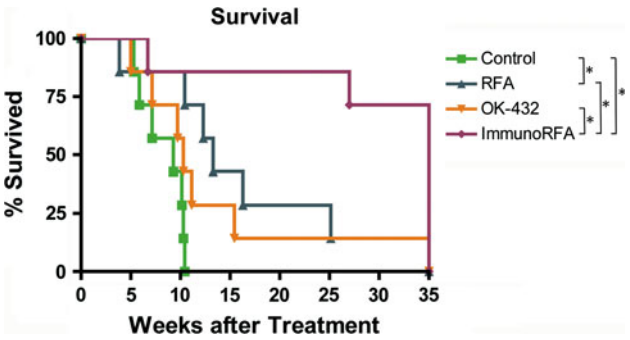

Fig. 2 Survival curves were generated for all groups by the Kaplan–Meier method. Survival was significantly longer with immunoRFA compared to that in the other groups (vs. control, $P = 0.002$; vs. RFA, $P = 0.026$; vs. OK-432, $P = 0.026$). There was also a significant difference in survival between the control group and the RFA group ($P = 0.006$), but OK-432 injection alone did not show significant improvement in survival compared to the control group ($P = 0.128$) or the RFA group ($P = 0.436$). *$P < 0.05$

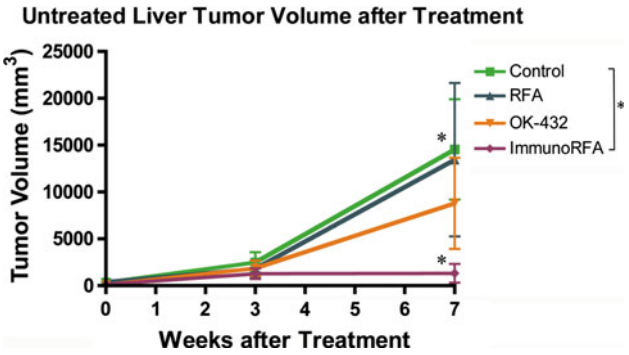

Fig. 3 Graph shows changes in untreated liver tumor volumes. In control, RFA, and OK-432 groups, sizes of untreated liver tumors increased over time. With immunoRFA, however, tumor growth was suppressed between weeks 3 and 7. At 7 weeks after treatment, there was a significant difference in tumor volume between the control group and the immunoRFA group ($P = 0.025$). *$P < 0.05$

The treated tumor volume before treatment in the control, RFA, OK-432, and immunoRFA groups was 205.5 ± 102, 256.9 ± 81.3, 186.5 ± 50.0, and 287.1 ± 72.4 mm^3 (mean tumor volume ± standard error of mean), respectively. There was no significant difference among the four groups. Local recurrence and residual tumor at the treated site were not observed in the RFA and immunoRFA group on enhanced CT at 3 weeks after implantation. With local OK-432 alone, the treated tumor increased in size in six rabbits but regressed in one rabbit at week 3. Liver tumors increased in size at both treated and untreated sites in the control group.

Ear Tumor Volume

Left ear tumors were assessed weekly up to 35 weeks after treatment. After week 4, however, it was difficult to measure the tumor size as a result of skin rupture that occurred in three rabbits in the control group, two rabbits in the RFA group, three rabbits in the OK-432, group and one rabbit in the immunoRFA group. Therefore, statistical comparison for ear tumor size was performed for the first 4 weeks. During this period, one rabbit in the RFA group was withdrawn from the study for humane reasons. In the control group, the left ear tumor volume increased steadily

Table 1 Untreated liver tumor volumes on the day of treatment and 3 and 7 weeks after treatment (mean tumor volume ± standard error of mean) and tumor response evaluation

Treatment	Mean tumor volume (mm^3)			Tumor response
	Week 0	Week 3	Week 7	CR/PR/SD/PD [CR + PR/total (%)]
Control	344.6 ± 239	2,491 ± 1,091	14,550 ± 5,349*	0/0/1/6 (0)
RFA	429.7 ± 159	1,822 ± 868	13,450 ± 8,185	0/1/1/5 (14.2)
OK-432	82.69 ± 13.5	1,863 ± 817	8,794 ± 4,859	0/0/1/6 (0)
ImmunoRFA	133.0 ± 21.5	1,282 ± 570	1,318 ± 1,000*	2/1/1/3 (42.8)

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *RFA* radiofrequency ablation

* $P < 0.05$

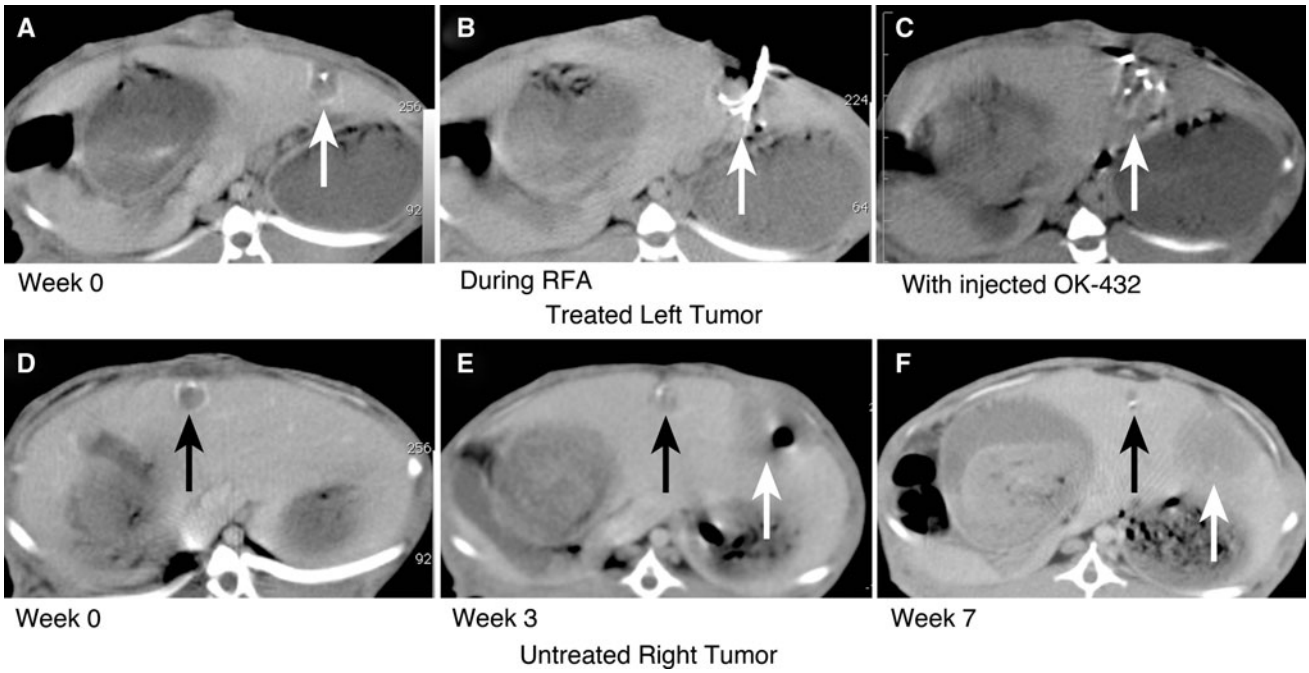

Fig. 4 CT images before and after immunoRFA. **A** A tumor (*white arrow*) in the left hepatic lobe on the day of treatment had a clear margin on enhanced CT, confirming tumor establishment. **B** RFA was performed. **C** Immediately after RFA, OK-432 was injected locally into the tumor. The high-density spots indicate lipiodol contained in the OK-432 solution. **D** In the same rabbit, the tumor in the right liver lobe (*black arrow*) also had a clear margin on the treatment day on enhanced CT. **E** At 3 weeks after immunoRFA, the margin of the untreated right-lobe tumor was less distinct (*black arrow*). Evidence of local recurrence was not identified at the site of the ablated tumor (*white arrow*). **F** 7 weeks after immunoRFA, the size of the untreated tumor decreased markedly (*black arrow*) on enhanced CT. Local recurrence was not seen at the ablated site (*white arrow*)

(Figs. 5, 6A). In the other three groups, the tumor volume increased until week 2 but remained almost unchanged after that. There were no significant differences among the four groups until week 3. At week 4, however, there was a significant difference between the control group and the immunoRFA group ($P = 0.029$) (Table 2). Left ear tumors shrank in all rabbits that survived 35 weeks after treatment.

Tumor Rechallenge Test

Seven rabbits that survived 35 weeks after treatment (one rabbit treated with RFA, one rabbit with OK-432, and five rabbits with immunoRFA) received reimplantation of VX2 tumor into the right auricle. Tumor establishment was confirmed in all of these rabbits at week 36 (1 week after reimplantation) according to the above-mentioned criteria. In all of these rabbits, the tumor shrank without further therapy during the follow-up period (Figs. 6B, 7; Table 3). There was no significant difference at 1 week after implantation between the reimplanted tumor volume of the immunoRFA group at week 36 and the ear tumor volume of the control group at week 0 ($P = 0.129$). After 2 weeks after implantation, however, there were significant differences between these two groups ($P < 0.05$) (Fig. 6B).

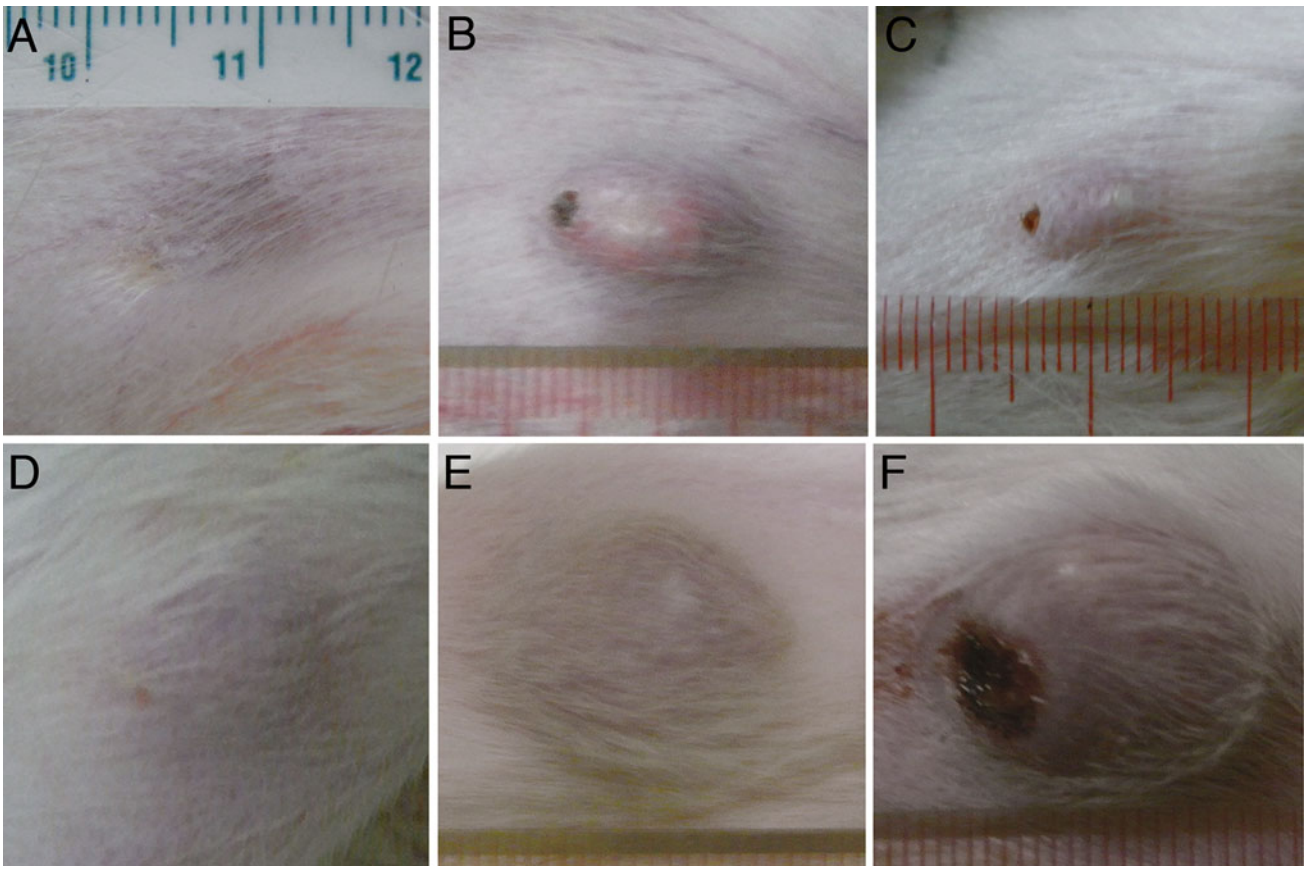

Fig. 5 Photographs of left ear tumors in the immunoRFA group and the control group. **A–C** An ear tumor in the immunoRFA group on the treatment day and at 2 and 4 weeks after treatment. From week 2 to week 4, the tumor size decreased. **D–F** An ear tumor in the control group on the treatment day and at 2 and 4 weeks after treatment. The tumor showed a tendency to increase

Histology

At 2 weeks after treatment, marked lymphocyte infiltration was observed around the untreated tumor in the liver (Fig. 8) and moderate lymphocyte infiltration around the auricular tumor in rabbits that received immunoRFA. In control animals, on the other hand, lymphocyte infiltration was minimal in both liver and auricular tumors.

Discussion

In the present study, we demonstrated that a combination of RFA and local OK-432 injection of a liver tumor improved survival and suppressed the growth of distant, untreated tumors using the rabbit model with intra- and extrahepatic VX2 tumors. We also showed that when rabbits were rechallenged by tumor reimplantation after the treatment, the reimplanted tumors spontaneously regressed without additional treatment. This study suggests that the systemic antitumor effects were enhanced by immunoRFA performed locally on liver tumors.

Marked infiltration of lymphocytes in the resected liver tumor is associated with lower recurrence and longer prognosis after liver tumor resection [24]. Activation of specific antitumor immunity against hepatic tumors may lead to lower recurrence and longer survival. In fact, RFA alone has the potential to trigger such immunity, as evidenced by tumor-specific T-cell activation shown in a rabbit VX2 liver tumor model [25] and in human clinical cases with liver cancer [5, 6]. By RFA alone, however, such antitumor immunity is weak [4], and RFA-associated spontaneous regression of distant metastasis has been reported only for lung metastases in two patients [26].

We hypothesized that two conditions were necessary to enhance potent antitumoral immunity. First, tumor cell antigens must be released from the tumor. In general, this can be achieved by thermal therapies such as RFA, hyperthermia, microwave ablation, and laser ablation [27, 28]. Thermal therapies cause tumor coagulative necrosis, thus releasing the antigens from the destroyed tumor cells [6, 29]. This heat injury caused by thermal therapies of liver tumors allows hepatic antigen-presenting cells to take up tumor antigens [30]. Although hyperthermia is

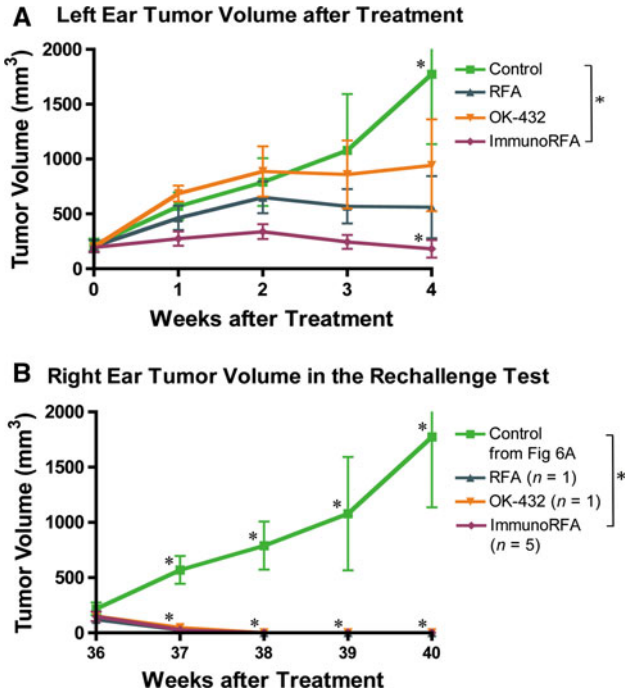

Fig. 6 Graphs showing changes in left and right ear tumor volumes. **A** The left auricular tumor volume at week 4 was significantly smaller than that of the control group only in the immunoRFA group ($P = 0.029$). *$P < 0.05$. **B** When tumors were reimplanted into the right ear of the rabbits that survived the first 35 weeks, tumors shrank rapidly. Data were not available for control animals, as none of them survived. For comparison, the data from the control group in (**A**) is overlaid. *$P < 0.05$

insufficient to create cytotoxic temperatures causing the release of antigen, thermal-induced stress is able to induce antigen release and up-regulate immunomodulating proteins, which play a role in antigen processing and presentation [31, 32]. Second, antigen-presenting cells must be matured to acquire tumor antigens and present them to T cells [33]. The liver has abundant antigen-presenting cells such as Kupffer cells and dendritic cells [34]. Previous reports demonstrated that heat shock proteins and cytokines are released by thermal therapies [28, 35, 36]. Especially heat shock proteins can induce secretion of cytokines on dendritic cells and cause the maturation of dendritic cells [37, 38]. However, the use of only thermal therapies is inadequate to cause systemic antitumor immunity to control distant tumors. The maturation of these antigen-presenting cells could be promoted by local injection of OK-432. OK-432 induces secretion of chemokines required for maturation of dendritic cells, which then become antigen-presenting cells [17]. Induction of Th1-type cytokines results in activation of lymphocyte-mediated antigen-specific tumor immunity [14].

OK-432 has been used clinically to enhance the efficacy of hepatic artery embolization [9]. In that study, dendritic cells derived from peripheral blood monocytes were matured in vitro by the use of OK-432 in the presence of GM-CSF and interleukin 4, and OK-432-stimulated dendritic cells were infused to patients with HCC during embolization. This combination therapy has shown prolonged recurrence-free survival. Conversely, intratumoral infusion of immature dendritic cells to patients with HCC during embolization did not prolong recurrence-free survival [39]. These results suggest that maturation of dendritic cells is essential to acquire antitumor effects and that local injection of OK-432 with the purpose of maturing dendritic cells in vivo is a practical approach.

As we hypothesized, the median survival time was shorter with RFA alone than with immunoRFA in the present study. RFA alone was excellent in local tumor control, but it was not sufficient to reduce the size of distant tumors. Likewise, OK-432 alone was not effective for tumor control, whether local or distant. By combining these two local treatments, however, we successfully enhanced systemic antitumor effects. Interestingly, tumor regression was very slow for the first 3 weeks after immunoRFA, and the tumor size was comparable in all of the study groups during this period. This is probably because it requires at least a couple of weeks to activate antitumor immunity as previously described [11, 40], and this result was consistent with our histological findings at 2 weeks after treatment,

Table 2 Left ear tumor volumes from the treatment day to 4 weeks after treatment (mean tumor volume ± standard error of mean) and tumor response evaluation

Treatment	Mean tumor volume (mm^3)					Tumor response
	Week 0	Week 1	Week 2	Week 3	Week 4	CR/PR/SD/PD [CR + PR/total (%)]
Control	217.6 ± 56.1	569.6 ± 126	789.6 ± 217	1,079 ± 513	1,773 ± 637*	0/1/0/6 (14.2)
RFA	205.0 ± 55.8	463.0 ± 111	650.6 ± 145	568.8 ± 156	560.5 ± 284	1/0/2/4 (14.2)
OK-432	199.8 ± 25.6	685.3 ± 72.4	887.4 ± 229	859.2 ± 309	942.6 ± 419	0/2/2/3 (28.6)
ImmunoRFA	193.3 ± 38.4	264.4 ± 68.3	338.2 ± 66.9	243.9 ± 63.7	180.8 ± 80.6*	1/3/1/2 (57.1)

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *RFA* radiofrequency ablation

* $P < 0.05$

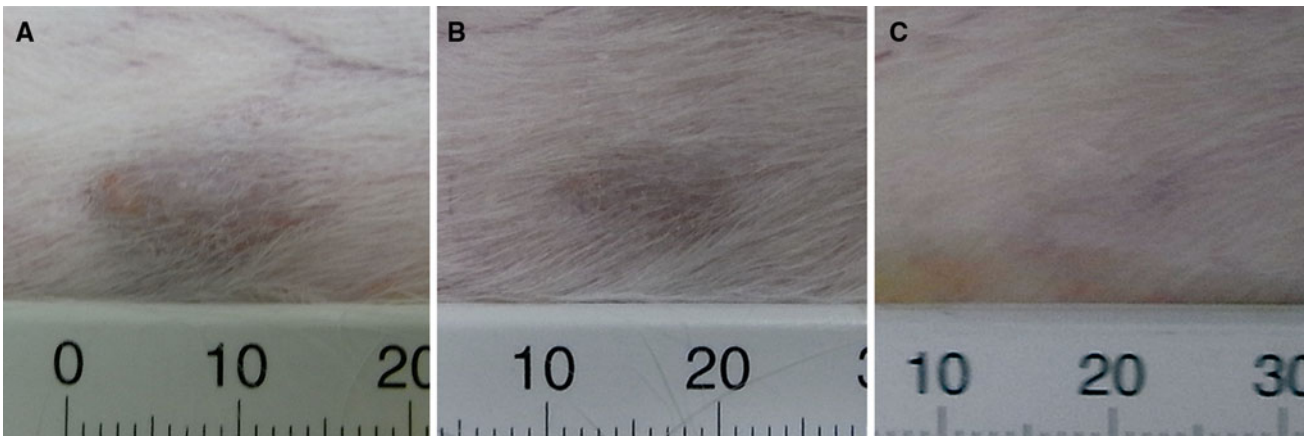

Fig. 7 Photographs of a reimplanted ear tumor in the immunoRFA group are shown. **A–C** An ear tumor in the immunoRFA group at 1, 3, and 5 weeks after reimplantation, i.e., at 36, 38, and 40 weeks after treatment. The tumor regressed during the observation period

Table 3 Right ear tumor volumes after reimplantation at week 35 (mean tumor volume ± standard error of mean)

Treatment	Mean tumor volume (mm^3)				
	Week 36	Week 37	Week 38	Week 39	Week 40
Control[a]	NA	NA	NA	NA	NA
RFA ($n = 1$)	120.84	25.12	0.52	0.52	0.52
OK-432 ($n = 1$)	150.72	47.10	1.57	0.52	0.52
ImmunoRFA ($n = 5$)	103.82 ± 37.6	23.75 ± 14.5	0.63 ± 0.1	0.63 ± 0.1	0.63 ± 0.1

NA not applicable, *RFA* radiofrequency ablation

[a] None of the control animals received tumor reimplantation because they did not survive the first 35 weeks

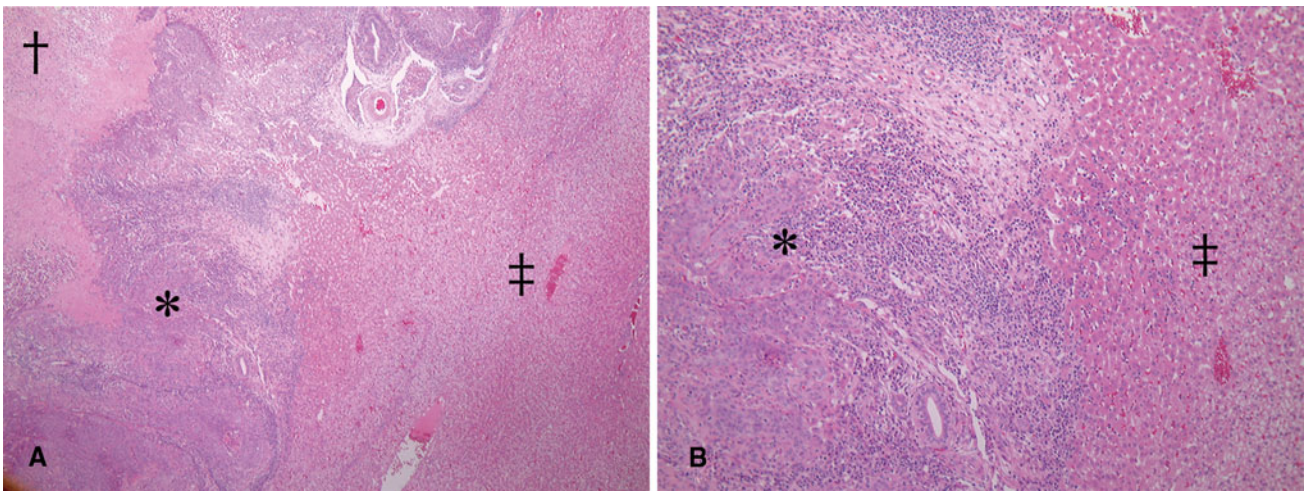

Fig. 8 Hematoxylin–eosin staining of histological specimens of untreated liver tumor in the immunoRFA group. **A** On the low-magnification (×40) image, necrosis (*dagger*) was seen within the tumor. At the tumor margin, viable tumor tissue and lymphocyte infiltration were evident (*asterisk*). On the *right side* is the normal liver parenchyma (*double dagger*). **B** On high-magnification (×100) image, the normal liver parenchyma was seen on the right side (*double dagger*). On the *left side*, there was marked infiltration of lymphocytes surrounding the tumor cells (*asterisk*)

when marked lymphocyte infiltration was observed around the tumor in the immunoRFA group. Once activated, however, antitumor immunity seemed to respond faster, similar to the in vivo vaccine mechanism [29, 41], and the reimplanted tumors started to shrink from week 36 as the rechallenge test showed in this study. Taking our findings together with those of previous reports, we propose immunoRFA as a potential clinical approach to enhance

systemic antitumor immunity in patients with unresectable multicentric HCC or multiple liver metastases.

The present study has limitations. First, it is based on an animal tumor model, which does not precisely reflect the behavior of multiple tumors in humans. Second, the optimal dose of OK-432 was not established, although a dose of 0.1 KE/kg was used based on a previous study [42]. For clinical use of BRMs, establishing an optimal dose is important, as doses too high or too low may not be effective [43]. Third, sham RFA was not performed in the control and the OK-432 groups. Last, the present study did not demonstrate how or whether systemic antitumor immunity was actually activated, because in rabbits it is difficult to demonstrate antigen–antibody reaction of systemic antitumor immunity such as T-cell receptor–CD3 complex by immunohistochemistry [25]. However, the results of our rechallenge study suggested that long-lasting systemic antitumor effects had been established in rabbits that survived the first tumors.

In conclusion, RFA of a liver VX2 tumor in combination with OK-432 local injection led to improved survival and suppression of distant untreated tumor growth in the rabbit model with intra- and extrahepatic VX2 tumors.

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Conflict of interest The authors declare that they have no conflict of interest.

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