Abstract

Purpose: Radiofrequency ablation (RFA) is a common treatment modality for surgically unresectable tumors. However, there is a high rate of both local and systemic recurrence.

Experimental Design: In this preclinical study, we sought to enhance the antitumor effect of RFA by combining it with huKS-IL2 immunocytokine [tumor-specific monoclonal antibody fused to interleukin-2 (IL2)] in mice bearing CT26-KS colon adenocarcinoma. Mice were treated with RFA, huKS-IL2 via intratumoral injection, or combination therapy.

Results: Treatment of mice bearing s.c. tumors with RFA and huKS-IL2 resulted in significantly greater tumor growth suppression and enhanced survival compared with mice treated with RFA or huKS-IL2 alone. When subtherapeutic regimens of RFA or huKS-IL2 were used, tumors progressed in all treated mice. In contrast, the combination of RFA and immunocytokine resulted in complete tumor resolution in 50% of mice. Treatment of a tumor with RFA and intratumoral huKS-IL2 also showed antitumor effects against a distant untreated tumor. Tumor-free mice after treatment with RFA and huKS-IL2 showed immunologic memory based on their ability to reject subsequent challenges of CT26-KS and the more aggressive parental CT26 tumors. Flow cytometry analysis of tumor-reactive T cells from mice with complete tumor resolution showed that treatment with RFA and huKS-IL2 resulted in a greater proportion of cytokine-producing CD4 T cells and CD8 T cells compared with mice treated with RFA or huKS-IL2 alone.

Conclusions: These results show that the addition of huKS-IL2 to RFA significantly enhances the antitumor response in this murine model, resulting in complete tumor resolution and induction of immunologic memory.

Although surgical resection remains the most effective treatment for both primary and metastatic liver tumors, only 10% to 20% of the roughly 37,000 patients who present each year with colorectal liver metastases or primary liver tumors are candidates for liver resection due to multifocal disease, tumor location near key vascular or biliary structures, or low hepatic reserve (1–7). Radiofrequency ablation (RFA) is the most common ablative cancer treatment in clinical practice for tumors that are surgically unresectable. RFA destroys tumor cells by transmitting electrical current from a radiofrequency generator to a probe tip placed directly in the tumor via image guidance or direct visualization. This high-frequency (400 kHz) alternating current causes rapid ionic vibrations within tissue, creating heat through friction and tumor cell destruction by thermal coagulative necrosis (8, 9). This treatment modality is most commonly used for primary or metastatic liver tumors, but its application has been broadened to primary tumors located in the breast, bone, brain, thyroid, parathyroid, kidney, and lung (6, 10–12). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Radiofrequency ablation is frequently used in clinical practice for both primary and metastatic tumors. However, the long-term efficacy of ablation is poor due to high rates of both local and systemic recurrence. We sought to enhance the efficacy of ablation by adding immune therapy using the huKS-IL2 immunocytokine (IC). Immunotherapy is most effective in the setting of minimal residual disease, which we used in these experiments by adding huKS-IL2 to partially ablated tumors. We show that the IC was effective in curing partially ablated tumors and had efficacy against synchronous, untreated tumors at distant sites. This simulates a clinical scenario in which detectable tumor is treated and a systemic immune response is activated against tumor antigens to resolve distant, undetectable disease. The huKS-IL2 IC has already completed phase I clinical testing, and because radiofrequency ablation is currently in use, the potential for combination therapy in a clinical setting is very promising.

Translational Relevance

This patient population has shown to induce greater local antitumor efficacy in murine models than systemic administration. Specifically, our objectives were to determine if combination therapy resulted in an antitumor immune memory response.

Materials and Methods

Mice and anesthetic agents. Six- to 8-wk-old female BALB/c mice were obtained from Harlan Sprague Dawley. All animals were housed in university-approved facilities and were handled according to NIH and University of Wisconsin-Madison Research Animal Resource Center guidelines.

Tumor cells. The CT26 colon adenocarcinoma cell line was grown in DMEM (HyClone) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), l-glutamine (2 mmol/L), all from Life Technologies, Inc.), and 10% heat-inactivated FCS (Sigma Chemical). Cells were maintained at 37°C in a humidified 5% CO2 atmosphere. The CT26-KS cell line, generated by transfection of the parental CT26 line with the gene encoding human EpCAM, was a gift from Ralph A. Reisfeld (Scripps Research Institute) and has been described previously. Constitutive expression of EpCAM in CT26-KS was maintained by growing the cells in the presence of 1 mg/mL G418 (Hyclone) in DMEM as above. Before tumor implantation, expression of the EpCAM antigen on the CT26-KS cells was verified by flow cytometry (data not shown). The murine MethA sarcoma cell line was grown in RPMI 1640 (Hyclone) with additives as described for CT26.

Tumor models. CT26-KS tumors were induced by injecting mice s.c. with 5 × 10^5 tumor cells in the center of the abdomen. The mice that resolved their initial tumor were monitored for at least 30 d for recurrence before immunologic memory was tested in vivo or in vitro. For rechallenge experiments, tumor-free mice were injected s.c. with 5 × 10^5 CT26 or CT26-KS tumor cells in the contralateral flank distant from the site of their first tumor. Some mice were separately challenged with 2 × 10^5 MethA sarcoma cells (a dose that did not result in spontaneous MethA tumor resolution). For studies of primary and distant tumor effects, mice were implanted with 5 × 10^5 CT26-KS cells s.c. on the abdomen on day 0, followed by an equivalent dose of CT26-KS tumor cells on day 3 on the flank. In all experiments, tumors were measured with a digital caliper every 3 d. Tumor volumes were calculated in cubic millimeter using the following formula: length × width × width / 2. Data shown are mean tumor volume ± SE. According to University of Wisconsin animal care guidelines, animals were euthanized with CO2 inhalation when any tumor dimension measured 15 mm or greater.

Radiofrequency tumor ablation. RFA was done using a Radio Therapeutics RF 2000 generator. The RFA probe consisted of a hollow 18-gauge needle containing a wire transducer from an Omega Type K thermocouple, which facilitated temperature monitoring at the probe tip. After anesthesia was induced, via a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in 100 μL PBS by i.p. injection, the animal was placed in prone position in the center of an 8-in² piece of aluminum foil with 5 mL of 0.9% saline to enhance conductivity. An 18-gauge metal injection needle was used as the RFA delivery probe. The probe was inserted into the center of the tumor, and an alligator clip from the RF generator was attached. A ground electrode was attached to the foil. Power (in watts) was controlled manually until target temperatures were reached. Total ablation time and time at target temperature were recorded. Previous studies in both human and animal tissue have shown that RFA works optimally when tissue temperatures are >50°C but <110°C. At temperatures >110°C, cavitation and charring severely limit thermal coagulative necrosis. Most experiments were done using one of two separate RFA dosing regimens. To simulate the clinical setting of tumor recurrence after RFA, a “partial RFA” strategy using 25 s of RFA at 90°C was done on day 10 or day 11 of tumor growth. This resulted in partial ablation (>50% necrosis) of the treated tumors, but 65% to 100% recurrence if no additional therapy was given (data not shown). To compare memory T-cell responses in animals that fully resolved their tumors after RFA treatment alone, a “complete RFA” regimen using 40 s of RFA at 90°C on day 8 was done. This resulted in
complete eradication of the local tumor, with most animals remaining tumor-free for over 30 d.

**hUKS-IL2 IC.** hUKS-IL2 IC, originally designated KS1/4-IL2, was a gift from S.D. Gillies (EMD-Serono Lexigen Research Center, Billerica, MA; refs. 20, 27). It was given as five daily i.t. injections of 15 μg (total dose of 75 μg), each in 50 μL PBS using a 30-gauge needle directed into the center of the tumor, according to the treatment schedules specified in the text. This dose of IC was previously shown to have antitumor effects when given either by i.v. or i.t. injections without toxicity (20, 25). Untreated animals served as controls. In experiments designed to investigate a more clinically adaptable i.t. injection dosing schedule, mice were injected with the same total dose of 75 μg hUKS-IL2 given as two separate injections of 37.5 μg in 50 μL on days 10 and 14.

**Surgical tumor resection.** In experiments comparing surgical resection with RFA and IC treatment, mice were implanted with 5 × 10^5 CT26-KS tumor cells on the abdomen and rendered tumor-free with either complete surgical excision on day 11, complete RFA (40 s, day 8), or partial RFA (25 s, day 11) with hUKS-IL2 via i.t. injection. Mice were anesthetized with ketamine and xylazine per the RFA protocol. S.c. tumors were completely excised under sterile conditions, and the skin was closed with absorbable suture. Mice were subsequently followed for 30 d for evidence of recurrence before tumor rechallenge or sacrifice for flow cytometry.

**Flow cytometry.** Flow cytometry was used to verify expression of EpCAM by the CT26-KS cells (data not shown). Tumor cells were collected and resuspended in PBS with 2% FCS (flow buffer) at a concentration of 5 × 10^6/mL and 50 μL of CT26-KS cells were incubated with KS-IL2, 10 μg/1 × 10^6 cells at 4°C for 40 min. Cells were washed and stained with a secondary antibody, anti-human IL2-PE (BD Biosciences), 2 μg/1 × 10^6 cells, for an additional 40 min at 4°C. Staining of cells with secondary antibody only, without IC, was used as a negative control. Cells were washed and analyzed using a FACScan cytofluorometer (Becton Dickinson). Results are presented as the percentage of CD4+ or CD8+ T cells stained positively with specific GM-CSF or IFN-γ.

Results

**hUKS-IL2 IC and RFA treatment resulted in a synergistic antitumor effect.** We have previously shown that hUKS-IL2 IC has enhanced efficacy when given by i.t. injection compared with i.v. injection (25). Here, we hypothesized that the addition of local IC treatment to partially ablated tumors would result in a greater antitumor effect than either treatment alone. We wanted this murine model to simulate the clinical setting wherein RFA provides initial local tumor control but is often followed by local recurrence. By modifying the RFA dose and timing, we developed a partial RFA strategy that created a partial ablation of the tumor followed by 65% to 100% tumor recurrence if no additional therapy was given. Specifically, treatment of day 11 CT26-KS tumors with 25 seconds of ablation at 90°C resulted in roughly 50% to 75% necrosis of the tumor. Longer treatment cycles of RFA resulted in variability in tumor growth (data not shown). To test the efficacy of IC combined with RFA, mice bearing CT26-KS tumors were treated with partial RFA, hUKS-IL2 IC by i.t. injection on days 11 to 15, or RFA and IC used in combination. On day 11, IC was given at least 60 minutes after RFA treatment in the combination group to allow the tumor bed to cool and prevent destruction of the IC protein. As shown in Fig. 1A, treatment with either RFA or hUKS-IL2 IC alone resulted in a moderate antitumor effect compared with no treatment (P = 0.001 for RFA versus no treatment and P = 0.002 for hUKS-IL2 versus no treatment). Combination therapy with RFA and hUKS-IL2 IC resulted in synergistic reduction in tumor growth compared with treatment with RFA alone or IC treatment alone (P < 0.001 on day 16 and P < 0.002 on day 19 for RFA + hUKS-IL2 versus all other groups; Fig. 1A). These results were confirmed in additional experiments using the same treatment regimens.

**Combination of hUKS-IL2 IC and RFA resulted in complete tumor resolution and enhanced survival.** Treatment with a combination of RFA and hUKS-IL2 IC not only significantly slowed tumor growth but also resulted in complete tumor resolution in some animals. Using the RFA and IC dosing schedule that resulted in reduced antitumor effects of single treatments shown in Fig. 1A, therapy with RFA plus hUKS-IL2 IC led to complete tumor resolution in 8 of 16 mice (in two combined experiments), whereas no mice had resolved tumors in any other group (Table 1, left column). The proportion of tumor-free animals was significant with combination therapy compared with all other treatment groups (P = 0.002). Using various RFA and IC dosing schedules, including complete RFA and more therapeutic IC regimens, in four different experiments (data not shown), a proportion of mice treated with either RFA alone or IC alone also had complete tumor resolution. However, the total proportion of tumor-free animals from combination therapy with both RFA and hUKS-IL2 remained significantly greater under these conditions as well (Table 1, right column).

Given that the addition of IC to RFA-treated tumors resulted in a significant augmentation of measurable antitumor effects, including complete tumor resolution in some cases, we also determined whether survival differences existed between different treatment groups. With partial RFA and a 5-day course of hUKS-IL2 IC, the combination therapy group had significantly enhanced survival compared with untreated animals (P < 0.001), RFA alone (P < 0.001), and hUKS-IL2 IC alone (P = 0.002; Fig. 1B). The survival benefit with combination therapy was reproducible in subsequent experiments. The mice that resolved their tumor were followed for a minimum of 30 additional days to monitor for tumor recurrence. These tumor-free...
animals underwent tumor rechallenge with a second s.c. injection of tumor cells to test for immunologic memory as described below.

Given the potential clinical difficulties required for five daily i.t. injections of IC to human patients (e.g., in treating hepatic metastases), we wished to determine whether this combined regimen might still be effective using fewer i.t. injections. We therefore tested the response to partial RFA when combined with two concentrated injections of huKS-IL2 IC given on days 10 and 14 (37.5 μg/injection) instead of five injections of 15 μg, thereby maintaining the same total 75 μg dose. When IC was given alone as a single treatment agent, we found that significantly greater antitumor effects occurred with the 5-day course of huKS-IL2 at smaller doses compared with two larger doses (data not shown). However, as shown in Fig. 1C, when combined with RFA, there was no significant difference between RFA with two doses of IC compared with RFA with five doses of IC. Notably, both combined treatment regimens were significantly better than RFA alone (P < 0.005 on day 25 for RFA alone versus RFA + huKS-IL2 on days 10-14 and day 10 + day 14). Thus, two concentrated doses of huKS-IL2 IC combined with RFA may prove to be an effective treatment regimen, although further studies are required.

RFA and huKS-IL2 resulted in tumor resolution of both treated and distant tumors. Given the ability of RFA, IC, or combined RFA and IC to resolve treated s.c. tumors, we wondered whether this response was strictly a local effect or whether antitumor effects might also be seen at sites distant to local combined RFA and IC treatment. We tested this using mice bearing two s.c. tumors. Mice were injected s.c. on the abdomen with CT26-KS tumor cells on day 0. A second equivalent tumor inoculation was given on the flank on day 3. Only the abdominal tumor was treated with partial RFA, huKS-IL2, or combined RFA and IC. Growth of both tumors was measured and compared with untreated animals also bearing abdominal and flank tumors. Two consecutive experiments were done, and the pooled results, showing the proportion of animals with complete tumor resolution at abdominal and flank sites, are presented in Table 2. Overall, animals treated with RFA alone had no statistically significant difference in tumor resolution of either the directly treated abdominal tumor or the untreated flank tumor compared with the no treatment group. The RFA and no treatment groups had no resolution of the distant tumors (0 of 15 mice). Seven of 15 animals treated with huKS-IL2 alone (under these conditions) resolved the treated abdominal tumor, which was significant compared with no treatment (P = 0.035) but not compared with RFA alone. However, 8 of 15 animals treated with huKS-IL2 also resolved the untreated flank tumor, which was significant compared with both the RFA and no treatment groups (P = 0.002 versus RFA or no treatment). The combination of RFA and huKS-IL2 resulted in 14 of 16 animals with complete resolution of the abdominal tumor, which was significant compared with all other groups. In addition, 13 of 16 animals resolved the untreated flank tumor after combined treatment of the abdominal tumor, which was significant compared with no treatment or RFA groups, although not statistically significant when compared with the huKS-IL2 treatment group (P = 0.10). However, the overall number of tumor-free animals, including both flank and abdominal tumor sites, in the group receiving RFA combined with huKS-IL2 was significant compared with all other groups (P < 0.044). These results indicate that i.t. administered IC can induce antitumor effects at distant sites, as we have shown previously (25), and also show that i.t. huKS-IL2 can induce a stronger systemic antitumor effect than RFA. Importantly, combination therapy resulted in a greater proportion of tumor-free mice than either treatment alone.

The addition of huKS-IL2 to RFA induced tumor-specific immune memory. Modifications in timing or regimen (higher...
Table 1. Tumor resolution after combined RFA and IC treatment

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Synergistic conditions</th>
<th>All experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>0/14</td>
<td>0/26</td>
</tr>
<tr>
<td>RFA alone</td>
<td>0/14</td>
<td>5/26*</td>
</tr>
<tr>
<td>huKS-IL2 alone</td>
<td>0/14</td>
<td>3/26</td>
</tr>
<tr>
<td>RFA + huKS-IL2</td>
<td>8/16*</td>
<td>14/28*</td>
</tr>
</tbody>
</table>

NOTE: BALB/c mice bearing CT26-KS tumors were treated using synergistic treatment conditions: partial RFA alone (25 s on day 11), suboptimal IC treatment alone (15 μg huKS-IL2 IC on days 11-15), or the combination of these same two regimens (RFA + huKS-IL2). Proportions of tumor-free mice to total mice in each treatment group from two combined experiments are shown. When data from all experiments evaluating tumor resolution were combined (right hand column), including some studies with complete RFA or with more optimal IC schedule administration (see Materials and Methods), the combined regimen continued to show enhanced tumor resolution. Proportions of tumor-free mice to total mice in each treatment group from four combined experiments are shown.

*P = 0.051 versus no treatment, P = not significant versus huKS-IL2 alone.

1P = not significant versus no treatment, P = not significant versus RFA alone.

2P = 0.002 for RFA + huKS-IL2 IC versus all other groups.

3P < 0.001 versus no treatment, P = 0.024 versus RFA alone, P = 0.003 versus huKS-IL2 alone.

Table 2. RFA and huKS-IL2 induced antitumor effects against both treated and distant CT26-KS tumors

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Proportion of mice with no tumor at specific sites</th>
<th>Proportion of tumor-free mice (all sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Treated abdominal tumor</td>
<td>Untreated flank tumor</td>
</tr>
<tr>
<td>No treatment</td>
<td>1/15</td>
<td>0/15</td>
</tr>
<tr>
<td>RFA</td>
<td>5/15*</td>
<td>0/15</td>
</tr>
<tr>
<td>huKS-IL2</td>
<td>7/15</td>
<td>8/15*</td>
</tr>
<tr>
<td>RFA + huKS-IL2</td>
<td>14/16†</td>
<td>13/16‡</td>
</tr>
</tbody>
</table>

NOTE: BALB/c mice (seven to eight per group) were implanted with 5 × 10⁵ CT26-KS cells s.c. on the abdomen on day 0. On day 3, an equivalent s.c. CT26-KS tumor cell inoculation was placed on the flank. The abdominal tumor was treated with RFA alone (day 10), 15 μg huKS-IL2 IC (days 10-14), or RFA (day 10) and huKS-IL2 IC (days 10-14). The flank tumor remained untreated and was monitored for growth or resolution. Proportions represent animals with complete resolution of either the treated abdominal (left column) or distant tumor (middle column) after pooling two consecutive experiments. Mice that were tumor-free at the conclusion of the experiment are listed in the right column. Finally, an analysis of all mice for all sites is included, identifying the proportion of mice that are completely tumor-free (right hand column).

*P = 0.17 versus no treatment, P = not significant versus huKS-IL2.

†P = 0.035 versus no treatment, P = not significant versus RFA.

‡P = 0.002 versus no treatment, P = 0.002 versus huKS-IL2.

§P = 0.006 versus no treatment, P = 0.006 versus RFA.

¶P < 0.001 versus no treatment, P = 0.023 versus huKS-IL2, P = 0.003 versus RFA.

††P < 0.001 versus no treatment, P < 0.001 versus RFA, P = 0.14 versus huKS-IL2.

**P < 0.001 versus no treatment, P = 0.001 versus RFA, P = 0.044 versus huKS-IL2. 

dose and earlier given RFA, or earlier initiation of 5 days of IC given i.t.) can enable these individual treatments to eliminate primary CT26-KS tumors in some treated animals. To test if individual or combined treatments induce long-term immunity, mice rendered tumor-free after initial therapy with partial RFA, i.e., IC, or combined treatment were rechallenged ~30 days after resolution of the primary CT26-KS tumor with both CT26-KS cells and parental CT26 cells via s.c. injection in opposite flanks. Table 3A shows the number of animals that rejected CT26-KS and CT26 delayed challenges. In all treatment groups, all animals that had previously rejected a CT26-KS tumor showed a memory response to CT26-KS on rechallenge, whereas none of the naive control animals rejected this tumor. In addition, Table 3A shows that several animals that received immunotherapy with huKS-IL2 alone or in combination with RFA also were able to reject the parental CT26 tumor challenge. The antitumor response against CT26 tumors in animals treated with both RFA and huKS-IL2 was significantly better than in animals that received RFA alone, but not huKS-IL2 alone (P = 0.018 for combined treatment versus RFA alone; P = 0.004 for combined treatment versus control; P = 0.90 for combined treatment versus huKS-IL2 alone). This suggests that animals that received IC immune therapy against their primary CT26-KS tumor have an enhanced immune response against tumor antigens found on the CT26 tumor and are not responding to the foreign human EpCAM antigen present on the CT26-KS tumor.

To test whether RFA can induce a separate antitumor response or simply acts to debulk the tumor to allow IC therapy to be more effective, a series of experiments were designed to compare RFA and IC with complete surgical excision. Mice were implanted with the standard inoculation of CT26-KS tumor cells s.c. in the center of the abdomen. Mice were then treated with complete RFA (40 seconds, day 8), complete tumor excision (day 11), or partial RFA (25 seconds, day 11) combined with huKS-IL2 on days 11 to 15. Complete resolution of the primary CT26-KS tumor occurred in all treatment groups (data not shown). Mice were monitored for 30 days to document...
absence of tumor recurrence and then rechallenged with both CT26-KS and CT26 tumor cells on opposite flanks. The results of the tumor rechallenge are presented in Table 3B. Whereas almost all animals rejected the CT26-KS tumor rechallenge, no animals in the surgical excision group rejected the parental CT26 tumor, whereas 75% of the animals treated with RFA and huKS-IL2 rejected the CT26 tumor. This showed that the combination of RFA plus huKS-IL2 induced a memory response that was stronger than that induced by surgical excision and is consistent with the results shown in Table 3A. One of three animals treated with RFA alone rejected the CT26 tumor, which was not significant compared with surgical excision. Thus, although the numbers are small, by these criteria (rejection of tumor challenges with CT26-KS and CT26), animals that resolved a primary tumor via RFA show no significant difference from animals that underwent surgical excision of their primary tumor. However, subtle immunologic differences were observed when splenocytes from these tumor-free animals were evaluated by flow cytometry (see below).

To determine if the observed immunologic memory was tumor specific, a subset of the animals that rejected both CT26-KS and CT26 in tumor rechallenge experiments and remained tumor-free for an additional 30 days was rechallenged again with CT26-KS (third exposure) and simultaneously injected s.c. with unrelated syngeneic tumor cells (MethA sarcoma) at a separate site on the opposite flank. Age-matched naive control animals received both CT26-KS and MethA cells in opposite flanks (Table 4). MethA tumors grew progressively in both immune and control animals, whereas CT26-KS tumors were rejected a third time in all immune animals. This showed that the memory response present in immune animals remained specific for the CT26 cell line, as animals that previously rejected both CT26-KS and CT26 could not mount an antitumor response against MethA.

Treatment with RFA and huKS-IL2 resulted in a stronger T-cell memory response compared with other treatments. We further evaluated the immune memory response present in animals treated with surgical excision, complete RFA alone, huKS-IL2 alone, and partial RFA combined with huKS-IL2 by measuring T-cell cytokine production by flow cytometry. CT26-KS tumor-bearing mice were treated using conditions similar to those used in the tumor rechallenge experiments presented in Table 3A and B so that all animals became tumor-free. All mice were monitored for 30 days to document absence of recurrence, and tumor-free mice were then sacrificed along with age-matched naive control animals. Splenocytes were harvested, and cells were cultured with irradiated CT26-KS or MethA sarcoma cells for 5 days. Viable cells were labeled with markers for CD4 and CD8, as well as intracellular GM-CSF and IFN-γ. A representative experiment is shown in Fig. 2A. In this experiment, the combination of RFA and huKS-IL2 IC resulted in greater T-cell responses against CT26-KS cell targets than either RFA or huKS-IL2 IC therapy alone. In the in vitro stimulated splenocytes from huKS-IL2–treated mice, the proportion of CD4 cells expressing IFN-γ was no different than naive controls (3%). IFN-γ–expressing CD4 T cells rose to 50% in the in vitro stimulated splenocytes from RFA-treated mice; however, 97% of CD4 cells secreted IFN-γ in the in vitro stimulated splenocytes from mice receiving combination therapy of RFA and huKS-IL2. A similar pattern was seen in CD4 cells with regard to GM-CSF production. The in vitro response to CT26-KS cell targets was even more striking in the CD8 cell population, with only 5% to 6% of CD8 cells expressing IFN-γ or GM-CSF after huKS-IL2 treatment, 16% to 20% of cells showing cytokine secretion after RFA treatment, and 92% to 95% of cells showing cytokine expression after RFA and KS-IL2 combination therapy. There was no significant cytokine expression in spleen cells obtained from either group when stimulated in vitro with MethA cells (data not shown).

These results were reproducible in subsequent analyses. In five flow analyses representing four separate experiments, including mice that underwent complete surgical excision, the proportion of T cells expressing IFN-γ or GM-CSF after RFA combined with huKS-IL2 continued to be much higher than all other treatment groups. The mean proportions of T cells expressing cytokines from each experiment are shown in Fig. 2B. Because means were calculated from separate individual experiments with different conditions, direct statistical comparison between means could not be calculated.

Discussion

We have shown that growth of CT26-KS colon adenocarcinoma after suboptimal, partial RFA treatment can be significantly impeded and even fully eradicated with the addition of huKS-IL2 IC therapy. Subtherapeutic doses of RFA or IC alone resulted in continued tumor growth without tumor resolution for most animals. Furthermore, there was a significant survival benefit using RFA combined with IC. Treatment with combination therapy affected both treated and untreated distant tumors and induced a tumor-specific immune memory response not limited to the tumor antigen targeted by the IC. Finally, in vitro analysis using flow cytometry showed that the strongest antitumor T-cell cytokine production occurred after combination therapy.

Our in vivo system used colorectal adenocarcinoma as the target tumor for several reasons. First, metastatic colorectal cancer to the liver is very common, and it is the most frequently treated tumor with RFA in many clinical series involving human patients (1, 3, 9, 17, 31–33). Roughly 50% of patients with a diagnosis of colon cancer will present with liver metastases or will develop them subsequently (34). Second, survival in patients with untreated metastatic disease is very poor, with a median survival of 6 to 9 months (4, 35). Although resection remains the most effective treatment, only a few patients are surgical candidates. Even with a curative resection, the majority of patients will have recurrence in the liver or other systemic sites (2, 9), and although systemic cytotoxic chemotherapy has improved, long-term survival has not been affected (35–37). Finally, EpCAM expression is very common in colorectal adenocarcinoma, which is the target of huKS-IL2 (21, 23).

RFA, without additional immune stimulation, has been previously shown in both animal and human models to induce an immune response to ablated tissue (28, 29). Using a rabbit tumor model, Wissniowski et al. (28) showed that RFA resulted in induction of a local and systemic immune response. In patients with hepatocellular carcinoma, T cells were significantly more responsive to both untreated cultured hepatocellular tumor cells and necrotic tumor 1 month after RFA (29). However, the presence of an immune response did not correlate clinically with protection from relapse. Although not a major objective of
this current study, our data comparing the immune effects of primary tumor elimination by RFA versus surgical excision are consistent with these prior reports, namely, RFA treatment seemed to induce a weak T-cell memory response as measured in vitro (Fig. 2B), not readily seen in animals treated surgically. However, RFA treatment did not result in a substantially stronger memory response to tumor rechallenge than did surgical excision (Table 3B). Augmentation of this weak immune response induced by RFA is likely required for clinical benefit.

The huKS-IL2 IC previously showed significant preclinical antitumor effects as a single agent against EpCAM-expressing tumors (19, 20, 25). As it was substantially more effective than a combination of anti-KS mAb and IL2 (20), we used huKS-IL2, rather than IL2 and antibody alone, as the immunotherapy to combine with RFA. We have previously shown that direct i.t. injection of huKS-IL2 is more effective than i.v. administration against palpable tumors. Although i.v. IC has been shown to localize to the tumor, i.t. injection facilitates higher local IC concentrations at the tumor and is present for longer periods of time (25). ICs induce both T-cell–dependent and T-cell–independent antitumor effects (19, 20). huKS-IL2 activates both natural killer cells and T cells via their IL2 receptor (39, 40), as well as Fc receptor–positive cells, such as macrophages, monocytes, neutrophils, and natural killer cells (19, 24, 41, 42). Both RFA and IC therapies have been shown to independently activate antitumor T-cell responses, and we hypothesized that these two treatments could act synergistically and potentially activate a systemic response against tumor antigens.

Because RFA is not fully curative in clinical practice, our animal model and treatment strategy were developed to simulate a clinical recurrence in patients treated with ablation alone. Therefore, we used partial RFA, which caused destruction of the bulk of each treated tumor but seemed to spare a component of the treated lesion (25-50% of the viable appearing tumor). Our first series of experiments showed that combination therapy with partial RFA and huKS-IL2 created a synergistic reduction in tumor growth. Both RFA and huKS-IL2 were moderately effective individually compared with untreated animals, but when used in combination, the antitumor response was significantly enhanced. Complete tumor resolution was also shown in some animals. The profound growth suppression resulted in a significant increase in survival in the combination group, which was not limited to the animals that completely resolved the tumor. Ultimately, this approach may be targeted for combined treatment of intrahepatic colon cancer metastases. Given the clinical complexity of five daily i.t. IC treatments to such sites, we also tested the response to a more concentrated dose of IC given in two injections (Fig. 1C). When IC was given alone, the antitumor response was significantly better with five lower dose injections. However, when combined with RFA,
tumor growth was not significantly different with two compared with five injections, although there was a trend toward a better response with five IC injections. Further work is needed to create a treatment schedule that would optimize clinical efficacy and applicability.

After observing a synergistic response in tumor growth, our next experiments addressed the ability of RFA or IC to induce immune memory after tumor resolution. The dose of both RFA and huKS-IL2 was increased to create tumor resolution in all treatment groups, including RFA and huKS-IL2 as single agents. All animals that resolved the CT26-KS tumor rejected the CT26-KS tumor on rechallenge, which is likely related to the high immunogenicity of the human KS epitope on the CT26-KS tumor in mice. However, the parental CT26 colon adenocarcinoma, which is much more aggressive and lacks any human antigens, was also rejected by a majority of animals that were free of CT26-KS due to prior IC treatment. The reactivity against CT26 after huKS-IL2 treatment of the primary CT26-KS tumor confirms prior experiments that showed T-cell activity against CT26 after i.v. treatment with huKS-IL2 (20). Additionally, the antitumor immune response noted in our experiments was tumor specific, as these immune mice did not resist a subsequent challenge with MethA sarcoma. Thus, immune memory was present after either RFA or IC, but induction of effective memory to other antigens expressed on CT26-KS that are shared with the parental CT26 tumor was only present after
IC treatment. This result may be clinically significant, as immune memory might help prevent clinical recurrence, and metastatic tumors do not always express the same antigen profile as the primary tumor, so an immune response to several tumor antigens may be important for true clinical cure (19, 43–45).

Tumor size is a significant predictor of local recurrence in most clinical RFA series, likely due to incomplete ablation of tumor cells at the tumor margin (4, 6, 8–11, 15, 16, 32). Larger tumor size has also been correlated with a higher incidence of microsatellite metastases, which result in recurrence and adversely affect survival (46). Although RFA site recurrence is a problem, a much higher percentage of patients recur at distant liver sites or in an extrahepatic location. It is unknown whether local control of hepatic disease by RFA has any effect on systemic disease in human patients (7). Our studies involving mice implanted with two tumors addressed whether the antitumor effect observed with RFA and IC was local or systemic. Interestingly, no animals in the RFA alone group resolved the distant flank tumor, whereas over 50% of mice in the huKS-IL2 group and 81% of animals in the combination group had flank tumor resolution. The previous experiments show that RFA is primarily a local therapy that can create some immune memory after tumor ablation. However, the addition of IC dramatically increases the systemic antitumor effect. At least two mechanisms may be at work on distant tumor sites when IC is given i.t. in combination with RFA. First, s.c. administered IC, given at sites distant from tumors, can act systemically to mediate IC-induced tumor destruction, similar to that observed with IC given i.v.7 Second, the presence of IC in concert with tumor destruction (either immune mediated or RFA mediated) might induce a systemic antitumor response against a range of epitopes, enabling activation of tumor-specific effector T cells to circulate systemically and help eradicate distant disease.

Prior studies have used immune therapy to augment the antitumor response after RFA. den Brok and colleagues (47, 48) treated mice bearing B16 melanoma with a complete ablation and tested tumor-free mice with rechallenge and adoptive transfer. They found that animals developed a tumor-specific immune response after RFA. Furthermore, this response was transferable to naive mice by i.v. injection of splenocytes from treated animals, whereas no immunity was transferred using splenocytes from untreated tumor-bearing animals. In addition, augmentation of the T-cell response using an anti–CTLA-4 antibody enhanced the response to the tumor on rechallenge. This work showed that not just the presence of tumor but rather RFA specifically induces a T-cell response and the combination of immune therapy with RFA can significantly enhance the antitumor effect. Our experiments build on this by showing synergy between RFA and IC-related immune therapy based on local and distant tumor control, detection of tumor-specific memory, and an enhanced response to additional tumor antigen(s) other than the antigenic target directly recognized by the mAb component of IC.

Future clinical studies may include a combination of chemotherapy, RFA, and immune therapy. Many patients included in RFA clinical series received systemic chemotherapy before ablation, and neoadjuvant therapy may downsize the tumor to facilitate ablation or resection (3, 5, 6, 15, 16). Currently, it is not known whether the combination of chemotherapy and RFA is beneficial, but a clinical trial sponsored by the European Organization for Research and Treatment of Cancer has been designed to address this question. Named the CLOCC trial (for chemotherapy with local ablation versus chemotherapy), it will investigate the effect of ablation with chemotherapy compared with chemotherapy alone (2, 6). Our study suggests that adding immune therapy to RFA merits further preclinical development to test whether combining these two treatment modalities could have clinical benefit, either alone or combined with chemotherapy.

This study shows that RFA, which is frequently used clinically, can be improved with the addition of huKS-IL2 immune therapy in this murine model. The potential clinical benefit of adding immunotherapy to RFA is 2-fold: to eradicate residual tumor cells at the site of the ablated tumor to potentially reduce local recurrence and to create a systemic tumor-specific immune response that could eradicate other small tumor deposits elsewhere in the body. The ability of IC to stimulate a polyclonal T-cell response against a variety of tumor antigens may be important in the treatment of metastatic disease. Further work is required to move this potentially effective preclinical therapy to clinical trials. This will include additional analyses of the mechanisms involved in the synergy observed (e.g., role of T cells, natural killer cells, and Fc receptor), analyses of IC plus RFA regimens in other preclinical tumor models, and testing of combined treatment at more clinically relevant sites, such as hepatic metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Radiofrequency Ablation Combined with KS-IL2 Immunocytokine (EMD 273066) Results in an Enhanced Antitumor Effect against Murine Colon Adenocarcinoma

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