ABSTRACT

Aim: Used as a palliative therapy for unresectable liver cancer, radiofrequency ablation (RFA) is associated with the induction of immunological responses. Here, we show strong evidence of tumor-specific peripheral blood mononuclear cells (PBMCs) 12 months after RFA. Methods: Three patients with colorectal cancer (CRC) metastases to the liver and two patients with primary hepatocellular carcinoma (HCC) were enrolled in this study. PBMC, isolated 12 months after RFA, were stimulated with normal and tumor tissue lysate. Interferon gamma secretion was evaluated by flow cytometry and indirectly, by luciferase assay for adenylate kinase activity in PBMC-stimulated lysates of target cells. Baseline data were detected before RFA and 4 weeks after treatment. Results: Two CRC patients and one HCC patient had recurrence-free survival. One patient with CRC developed secondary metastases; one patient with HCC developed a local recurrence. Recurrence-free patients showed a significantly higher cytolytic activity of PBMC against matched tumor cells 12 months after RFA treatment. Interestingly, patients with malignant recurrence showed a decreased cytolytic activity. Conclusion: RFA seems to overcome immune-tolerance toward tumor antigens and/or presents new tumor antigens. Patients seem to benefit from a prolonged increase in cytolytic activity. The immune-modulatory effects of RFA need further investigations in multimodality anticancer therapies.

Key words: Liver cancer; radiofrequency ablation; immune response; interferon gamma; peripheral blood mononuclear cells

INTRODUCTION

Surgical resection is still the gold standard for the treatment of hepatocellular carcinoma (HCC) and liver metastases of colorectal cancer (CRC). However, more than 75% of these patients are elected as unresectable due to the volume and localization of the tumor. Radiofrequency ablation (RFA) is a common therapy option for unresectable liver tumors.[1-4] It was shown that RFA and laser-induced thermotherapy (LiTT) can achieve, in selected patients, a survival prolongation time comparable to surgical resection.

An earlier study suggested that RFA has adjunctive immune-modulatory side effects.[5] By using the VX 2 hepatoma model in rabbits, we showed that RFA can induce a strong mononuclear infiltration around the implanted tumor. More recently, we demonstrated a marked tumor-specific peripheral T cell response in RFA-treated vs. untreated rabbits with the VX 2 hepatoma.[6,7] We further investigated whether this strong immune response to RFA can be observed in humans. We observed that significantly elevated levels of CD8+ T cells appeared 4 weeks after RFA, and this effect lasted at least up to 8 weeks after RFA. There was also significant cytolytic activity of isolated peripheral blood mononuclear cells (PBMCs) 8 weeks after RFA.[8]
The aim of this study was to investigate the immunological features of HCC and CRC metastases patients 12 months after RFA.

METHODS

The study was approved by the Ethical Committee of the University of Erlangen-Nuremberg (Ethikkomitee der Universität Erlangen Nürnberg) and performed according to the declaration of Helsinki. All treated HCC and CRC metastases were confirmed histologically prior to therapy.

Selection and description of participants
All enrolled patients took part in a prior trial in which baseline data before RFA treatment were recorded according to the present protocol.

Patients with up to 3 tumor nodules within the liver, with a maximum diameter of 6 cm per lesion, were enrolled in the study. Prior local ablative therapy (LiTT, RFA, ethanol injection) or prior chemo-embolization of the malignant liver tumor was an exclusion criterion. The possibility of curative treatment by resection had to be ruled out. Therefore, all cases were discussed in our tumor conference, including gastroenterologists, oncologists, surgeons, and radiologists.

Patients with at least one of the following findings were also excluded: Karnofsky index < 60, thrombocytes < 50,000/μL, prothrombin activity < 50%, partial thromboplastin time > 80 s. No transfusion of platelets or fresh frozen plasma was performed. Informed consent was obtained from every patient no later than 24 h before treatment.

The size and number of tumor nodules were determined by ultrasonography and by computed tomography (CT) (dynamic spiral CT with intravenous application of contrast medium) prior to RFA.

Five consecutive male patients with 8 tumor nodules in total (CRC patients with 2 nodules each and HCC patients with 1 nodule) who met the inclusion criteria were enrolled. Mean patient age was 64 years (range: 59-74 years). Three patients suffered from CRC metastases to the liver while 2 suffering from HCC.

Radiofrequency ablation technique
The whole procedure was performed under ultrasound guidance (Elegra Advanced®, Siemens, Erlangen, Germany) under sterile conditions. The proposed puncture site was infiltrated with a local anesthetic (2% mepivacaine hydrochloride) and the perfused radio-frequency (HF) needle (Integra, Rätingen, Germany) advanced into the tumor. Midazolam (0.5-5 mg) and/or pethidine (25-100 mg) were administered intravenously as necessary. Patients were monitored by pulse oximetry during the whole procedure.

Two mm (14 G) diameter RFA needle applicators and 15 mm active electrode with microbores were used. During HF application (40 W power output) the RFA needle was continuously perfused with isotonic saline via the bore holes. RF energy was delivered by a computer-assisted radiofrequency generator (Elektrotom 106 HF®, Integra, Rätingen, Germany) and continuous perfusion of the RFA needle was secured by a syringe pump (Pilot C, Fresenius Medical Care, Alzenau, Germany) linked to the RF generator. Perfusion was adjusted according to impedance by means of an electronic interface between generator and perfusor, automatically increasing in response to a rise in impedance (> 400 Ohm). The RF energy was applied for 10-15 min at each needle position, leading to a coagulation zone of 30-35 mm in diameter. Tumors larger than 20 mm were targeted using different applicator positions to create overlapping coagulation zones, in order to treat the entire lesion with a safety margin > 5 mm. Larger tumors were treated with up to 3 simultaneous needle insertions arranged in a triangle (2-4 cm) or square (for larger tumors).

Interferon gamma secretion assay and lymphocyte staining
Heparinized blood (LI-Heparin 10 mL) was collected 12 months after RFA. The samples were stored at 4 °C and tests performed within 12 h after sampling. A liver biopsy of normal and tumor tissue was collected from every patient directly before RFA and stored at -20 °C.

Tissue-lysates were freshly prepared in cold phosphate buffer (50 mmol/L, pH 7.2) using a glass homogenizer as described. The suspension was filtered with a filter tip (pore size 1.2 mm) to adjust the fragment size to < 1.2 mm. The protein concentration was measured spectrophotometrically using the Bradford assay, and adjusted to 1 mg/mL.

Autologous test antigens (normal and tumor lysate, 12.5 mg) were added to 250 μL heparinized blood and cultured in a 15 mL conical polypropylene tube for 16 h at 37 °C under 5% CO₂. A negative control without the addition of antigen lysate was included, while staphylococcal enterotoxin B served as positive control antigen. Thereafter, the samples were put on ice and washed with ice cold washing solution [phosphate buffer saline containing 0.5% bovine serum albumin and 2 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4] and the cell suspension centrifuged at 300 g for 10 min at 4 °C. The cell pellet was resuspended with 80 μL ice cold culture medium (RPMI1640 containing...
10% human AB serum). “Catch” reagent (20 μL) containing a bivalent CD45 capture and interferon gamma (IFNγ) binding antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added; the suspension was kept for 5 min on ice and 5 mL medium was added before incubation at 37 °C in closed roller tubes for 45 min. Thereafter, 20 μL phycoerythrin-conjugated antibody against IFNγ (1:5 dilution ratio) as well as 10 μL fluoresceine-thiocyanate conjugated anti-CD8 antibody (1:400 dilution ratio) (both from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added to the cooled and washed cell suspension and incubated for 10 min on ice. Erythrocyte lysis buffer (5 mL), containing 0.155 mol/L NH₄Cl, 10 mmol/L KHCO₃ and 0.1 mmol/L EDTA diluted 1:10, was added for 10 min and the cell suspension centrifuged at 300 g for 10 min. The cell pellet was resuspended in 500 μL ice cold washing buffer and immediately analyzed by flow cytometry (FACS Calibur, BD Biosciences, Heidelberg, Germany) after addition of 0.25 mg propidium iodide in 5 μL distilled water. FACS data were analyzed using software Win MDI Version 2.8 (Scripps Research Institute, La Jolla, CA, USA).

Cytotoxicity assay
Cytotoxic activity of T cells was measured by adenylate kinase (AK) release assay. Cells (10⁴) were incubated with 1,000 effector cells in a final volume of 200 μL growth medium with fetal calf serum in round-bottom 96-well microtiter plates. After incubation for 4 h at 37 °C, 100 μL of supernatant was harvested and stored at -20 °C for further analysis.

The human HCC cell line HepG2 and the human CRC cell line CaCO₂ served as target cells for T cells isolated from patients with HCC and CRC metastases, respectively. All cell lines were human leukocyte antigen matched (ABO-system) and tested previously. HepG2 (ACC-180) and CaCO₂ (ACC169) cells were purchased from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and were cultured with RPMI1640 and DMEM supplemented with 10% fetal bovine serum, penicillin (107 U/L) and streptomycin (10 mg/L) (Biochrom AG, Berlin, Germany) as previously described.⁹,¹⁰

Maximum AK release was obtained by incubating target and effector cells with Total-Lysis Reagent™ (Lonza, Cologne, Germany), and baseline AK release was obtained by incubating cells with medium alone. Baseline release from T cells and tumor cells were < 10% of maximum release in all experiments, and baseline value was subtracted from each value.

The activity of AK was determined by detection of auto-luminescence using a luciferase assay (ToxLight Kit, Lonza, Cologne, Germany). Supernatant (20 μL) was incubated with AK-detection reagent (Lonza, Cologne, Germany) for 5 min at room temperature. Bioluminescence was measured by a luminometer (BD Monolight 3096 Microplate Luminometer, BD Biosciences, Heidelberg, Germany) and expressed as relative luminescence units (RLU).

Statistical analysis
The stimulation index (SI) of CD8⁺ T cells was determined by the ratio of IFNγ⁺CD8⁺ vs. IFNγ⁻CD8⁺ T cells stimulated with tumor tissue lysate vs. stimulation with normal liver tissue, respectively, and calculated with Excel® 2003 software (Microsoft, Seattle, USA). Results were analyzed statistically by the SPSS® software package (Version 14, SPSS GmbH Software, Munich, Germany). SI values were divided into two groups according to the histological origin of the tumors. SI values were calculated numerically and are presented as columns. RLU were expressed numerically and are shown as columns. The significance of the enhanced SI after RFA treatment and augmented cytotoxic activity was tested with Fisher’s test for dependent samples. P < 0.05 were considered significant.

RESULTS
Of 3 patients suffering from CRC, 2 had a recurrence-free survival and 1 developed secondary metastases. One patient with HCC developed a local recurrence, and the other one had a disease-free survival after 12 months [Table 1]. All patients had a significant activation of tumor-specific T cells (SI baseline = 2.02, SD ± 0.2; SI CRC at 12 months = 12.3, SD ± 0.14; SI HCC at 12 months = 11.8, SD ± 0.23) (P < 0.05) [Figure 1].

Disease-free patients showed a significantly risen cytolytic activity of PBMC against matched tumor cells even 12 months after treatment (P < 0.05). The cytolytic activity after 12 months was comparable to baseline data (RLU before RFA = 10.4, SD ± 1.3; RLU 4 weeks = 354.7, SD ± 42.1; RLU CRC 12 months = 298.4, SD ± 23.1; RLU HCC 12 months = 317.4). In contrast, patients with recurrence of malignancy showed a significantly decreased cytolytic activity (RLU for CRC at 12 months = 76.2; RLU for HCC at 12 months = 102.5) (P < 0.05) [Figure 2].

DISCUSSION
In the last few years, local ablative therapies such as laser induced or radiofrequency induced thermal ablation have become more attractive as therapeutic options for patients with unresectable solid tumors. The results of local ablative
Therapies have shown encouraging survival rates similar to R0 resection of tumors. In addition, T cell vaccination or vaccination with dendritic cells is regarded as a promising strategy for the treatment of various tumor types such as malignant melanomas, and high expectations have been placed on cytokine-modulated immunotherapy for liver tumors.

It is well-known that RFA can induce an unspecific immune stimulation. Thus, thermal coagulation causes an inflammatory reaction with lymph-plasma-cellular infiltration that can be visualized as a hypervascular rim in contrast CT and contrast-enhanced ultrasound. This hypervascular rim can be so intense as to impede proper assessment of treatment success. We recently demonstrated a specific T cell response, after RFA application, toward the orthotopic VX 2-tumor implantation in rabbit livers. In the clinical setting, it was also shown that a tumor-specific immune response could be detected in patients after RFA. Our preliminary study showed a significant appearance of tumor-specific CD4+ and CD8+ T cells in peripheral blood up to 8 weeks after RFA. The cytolytic activity of isolated PBMC was also significantly elevated.

In this study, we investigated the presence of tumor-specific T cells and the cytolytic activity 12 months after RFA treatment. As these tissue samples were obtained by fine-needle biopsy prior to RFA, we decided to concentrate on the measurement of CD8+ cells. These cells are reported to be the most promising cell population for anti-tumor therapies. All patients still had significantly increased levels of CD8+ cells containing cytotoxic T cells, NK cell and NK T cells as well. Whether these observed cells are resting T cells or any other activated form of T cell remains unclear.

We observed no differences between patients with recurrence of malignancy or with disease-free survival. Interestingly, cytolytic activity was significantly lower in the two patients with tumor recurrence compared. Perhaps, recurrence of malignancy is caused by an ineffective cytolytic/cytotoxic activity of CD8+ cells but needs to be confirmed with further investigations.

Prior data showed an enhanced tumor growth if RFA or resection of liver metastases of CRC were not properly performed with remaining micrometastases due to hypoxia and growth factors induced by the process of wound healing. This might indicate that the immune response is not strong enough to control tumor growth or that the immune response is not tumor specific or not effective by the lack of tumor-specific cytolytic cells.

Furthermore, the potential role of a tumor-specific immune response as a target for supportive adjuvant therapy after RFA or resection of metastases has not yet been addressed. Therefore, larger groups with a follow-up for more than 6-12 months are needed to clarify whether lower cytolytic activity levels are correlated with recurrence of malignancy after local ablative therapies. This would suggest the need for an effective combination of drugs with RFA to achieve a long lasting strong cytolytic activity of the Th2 immune response.

Table 1: Summary of general data with entity of cirrhosis and entity of primary in case of metastases

<table>
<thead>
<tr>
<th>Patient</th>
<th>Entity</th>
<th>Stage of disease 12 months after RFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRC1</td>
<td>Colon descending</td>
<td>Disease free</td>
</tr>
<tr>
<td>CRC2</td>
<td>Rectum</td>
<td>Metastases to lung</td>
</tr>
<tr>
<td>CRC3</td>
<td>Rectum</td>
<td>Disease free</td>
</tr>
<tr>
<td>HCC1</td>
<td>Alcohol</td>
<td>Disease free</td>
</tr>
<tr>
<td>HCC2</td>
<td>Alcohol</td>
<td>Local recurrence</td>
</tr>
</tbody>
</table>

CRC: colorectal cancer; HCC: hepatocellular carcinoma; RFA: radiofrequency ablation
Further studies are needed to investigate the potential of the cytotoxic activity of CD8+ cells as a prognostic factor. The cytotoxic activity may also play an important role in tumor recurrence. In addition, the possible correlation between an active immune response and disease free survival needs to be clarified. The combination of toll-like receptor agonists with RFA has the potential to improve the outcome of patients with solid tumors in the liver.\textsuperscript{[20]}

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**REFERENCES**


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