Activation of Tumor-specific T Lymphocytes by Radio-Frequency Ablation of the VX2 Hepatoma in Rabbits

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ABSTRACT

Radio-frequency ablation (RFA) is used as a minimally invasive treatment for inoperable hepatic tumors. Immunological reactions secondary to RFA may play a role in the observed tumor control. In our study, the VX2 carcinoma was implanted into the liver of rabbits. After 3 weeks, tumors were treated with RFA or were left untreated. Peripheral blood lymphocytes were harvested before tumor implantation, 2 weeks postoperatively and at 2-week intervals thereafter. T cells were stimulated with lysates of either tumor tissue or nontumorous liver loaded on autologous antigen-presenting cells and their stimulation index was determined by [3H]thymidine incorporation. A 3-fold increase over background or controls was considered significant. Stimulation with phytohemagglutinin served as a positive control. The animals were necropsied, and liver and tumor tissue were analyzed immunohistologically for T-cell infiltration. T cells from tumor-bearing (n = 9) and RFA-treated (n = 11) animals were investigated in a follow-up study. The mean postoperative observation was 45 days. All of the 11 RFA-treated animals exhibited circulating T cells activated specifically toward tumor antigens throughout the observation period, which was accompanied by dense T-cell infiltration. In contrast, T cells of untreated tumor-bearing rabbits showed no reaction and only sparse T-cell infiltration. We concluded that RFA induces a tumor-specific T-cell reaction in the otherwise unreactive tumor-bearing host, apparently overcoming immune tolerance and leading to the presentation of otherwise cryptic tumor antigens. Therefore, in addition to destroying tumor tissue, RFA induces an immune response against tumor antigens that may be exploited in multimodal antitumor strategies.

INTRODUCTION

The presence of tumor-specific lymphocytes has been demonstrated in various experimental tumor models in animals (1, 2) as well as in tumor patients (3–9). Experiments performed in mice with P815-mastocytoma, EL4-thymoma, or Lewis lung carcinoma have shown that these tumor-specific lymphocytes can be activated by the addition of tumor antigens and IL-2 (1), as well as by mere addition of IL-2 with subsequent reinfusion (5). This method has also been applied successfully to tumor infiltrating lymphocytes (TILs) derived from biopsy material by adding tumor antigen and IL-2 (3). Accordingly, the VX2 tumor that is derived from a squamous cell carcinoma originating from papillomavirus infection in rabbits (10), when implanted s.c., has been shown to recede under IL-2 treatment (11), an effect that these tumor-specific lymphocytes can be activated by the addition of tumor antigen and IL-2 (3). Accordingly, the VX2 tumor cell line (10) was used for tumor implantation. This is an allogeneically transplantable epithelial rabbit tumor originating from a virus-induced papilloma. The required tumor tissue was taken from tumor-bearing passing animals. Rabbits (New Zealand White; Charles River, Kijssel, Germany) were laparatomized, a tumor fragment measuring 1 × 1 × 1 mm was implanted under Glisson’s capsule, and the wound was then closed in double layers. For ethical reasons moribund animals that refused both solid and fluid intake for more than 4 days with concomitant apathy and weight loss of more than 20% were sacrificed in accordance with the German Ordinance on Animal Experiments by using i.v. meperidine (Sövret; Lilly Deutschland GmbH, Homburg, Germany) administered i.v.

Experimental Design. A preliminary trial was performed on 24 animals to test and standardize procedures, determine optimal RFA device settings and application times and to investigate early histological immune reaction after RFA. Nine of these 24 animals were sacrificed 1 day and 7 animals 2 weeks after RFA, to define the histological immune reaction after RFA. A definitive study involving 20 animals was then performed. The animals were randomly assigned either to the treatment group (n = 11) or to the control group (n = 9). The weight of the animals in both groups was comparable with a mean of 2.8 kg (range, 2.6–3.1 kg).

After a maturation period of 21 days leading to a tumor size of >10 mm the animals of the treatment group (n = 11) were treated with ultrasound-guided percutaneous RFA. Samples of peripheral blood were taken from the ear vein before implantation, 2 weeks after tumor implantation and every 2 weeks thereafter until the animals reached the defined end point of 110 days or until they were sacrificed and tested for T-cell activation. Tumor-bearing animals not receiving RFA (n = 9) served as the control group.

RFA Application. RFA treatment was performed using a RF generator (Elektrotom HF 106; Berechtold, Tuttingen, Germany) equipped with a perfused RF needle applicator of 1.1 mm outer diameter and a 10-mm active electrode. The self-adhesive neutral electrode was applied on the animal’s shaven back. The needle applicator was advanced into the focus of the tumor under ultrasound guidance. Isotonic saline solution was continuously instilled into the coagulation zone via microbore in the needle tip at a flow rate of 40 ml/h. Treatments were performed at a power output of 20 W for 4 min.

Cell Separation and Culture. PBMCs containing T cells as proliferating cell fraction were isolated in leukosep separation tubes after density-gradient

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4 The abbreviations used are: IL, interleukin; RFA, radio-frequency ablation; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; SI, stimulation index.

MATERIALS AND METHODS

Animals and Tumor Model. Permission to carry out the animal experiments was obtained from the administration of the state of Franconia and the ethical commission of University of Erlangen-Nuremberg. All of the procedures were performed under anesthesia using weight-adapted doses of i.m. xylazine hydrochloride (Rompun 2%, 0.3 ml/kg; Bayer AG, Leverkusen, Germany), and ketamine hydrochloride (Ketavet, 30 mg/kg; Pharmacia-Upjohn, Erlangen, Germany). If necessary, an additional dose of xylazine hydrochloride was administered i.v. via a permanent vein catheter. An established VX2 tumor cell line (10) was used for tumor implantation. This is an allogeneically transplantable epithelial rabbit tumor originating from a virus-induced papilloma. The required tumor tissue was taken from tumor-bearing passing animals. Rabbits (New Zealand White; Charles River, Kijssel, Germany) were laparatomized, a tumor fragment measuring 1 × 1 × 1 mm was implanted under Glisson’s capsule, and the wound was then closed in double layers. For ethical reasons moribund animals that refused both solid and fluid intake for more than 4 days with concomitant apathy and weight loss of more than 20% were sacrificed in accordance with the German Ordinance on Animal Experiments by using i.v. meperidine (Sövret; Lilly Deutschland GmbH, Homburg, Germany) administered i.v.

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Cell Separation and Culture. PBMCs containing T cells as proliferating cell fraction were isolated in Leukosep separation tubes after density-gradient
centrifugation (PAA Laboratories GmbH, Vienna, Austria). After repeated washing with PBS (Biochrom, Berlin, Germany) containing 50,000 IU/liter heparin (Liquemin N 25000; Roche, Grenzach-Wyhlen, Germany), cells were adjusted to a concentration of 10^6 cells/ml and seeded as triplicates in 96-well round-bottomed plates at a ratio of 100,000 PBMC feeder cells (preirradiated with 30 Gy) to 50,000 PBMCs. RPMI 1640 (Biochrom) containing 10% rabbit serum (heat-inactivated and sterilized at 600 Gy), 5% HEPES buffer, and 1 µg/ml penicillin/streptomycin was used as culture medium. Incubation was performed in a humidified incubator at 37°C under 5% CO₂.

PBMC feeder cells were loaded either with lysate of the VX2 tumor or of normal liver tissue from healthy controls at a final concentration of 50 µg/ml. The mitogen PHA (10 ng/ml) served as a positive control.

The tissue-lysates were freshly prepared in cold phosphate buffer (50 mM) to adjust the fragment size to less than 1.2 µm. Protein concentration was measured photometrically according to Bradford and was adjusted to 1 mg/ml, followed by sterilization at 600 Gy.

**Stimulation of PBMC and T-Cell Proliferation Assay.** After irradiation, the loaded feeder cells were added to the native PBMCs for T-cell stimulation. At day 5 after coculture, the positive controls received 10 ng/ml PHA and at day 6, 10 µCi methyl-[3H]thymidine/well (Amersham, Buckinghamshire, United Kingdom) were added followed by cell harvest after 16 h with a Cell-Harvester (Inotek, Dottikon, Switzerland) and measurement of tritium incorporation into DNA using a β imager (BAS reader 5148; Fuji, Duesseldorf, Germany) and AIDA software (Raytest, Berlin, Germany).

The resulting data were used to calculate the SI for each experimental group considering [3H]thymidine incorporation (SI) ratios of test antigen: normal liver lysate >3.0 as proof of specific activation (17). Data conforming to SI_tumor lysate > 0 and SI_PHA > 3.0 were considered as true results; occurrence of activation by tumor lysate before tumor implantation was defined as an exclusion criterion.

**Statistical Evaluation.** Statistical evaluation was done with Microsoft Excel2000 (Microsoft Corporation, Seattle, WA) and SPSS v. 11.0 (SPSS Inc., Chicago, IL) software packages. Survival was analyzed with the Kaplan-Meyer method comparing survival curves by log-rank test. The significance of all of the data was proven using the t-test, and P < 0.05 was considered significant.

**Histology/Immunohistology.** All of the animals of the treatment and control groups reaching the defined end point 12 weeks after tumor implantation were sacrificed and completely necropsied (with the exception of the brain). Animals that died prematurely were dissected within 8 h. To evaluate histologically the immunological reactions after RFA treatment, the liver bearing the tumor was completely dissected. Furthermore, several tissue samples of heart, lungs, liver, and metastases (lymph nodes/pleura/peritoneum, if any) were taken from each animal. The samples were conserved in 10% buffered formalin, and 5-µm-thick sections were prepared from paraffin-embedded samples for H&E staining, to evaluate the basic histomorphology of the specimens.

To identify specific infiltrations of T-lymphocytes, immunohistochemistry was performed using a polyclonal rat CD3ε antibody (kind gift of Dr. E. Kremmer, GSF-National Research Center for Environment and Health, Munich, Germany), which shows a wide range of species cross-reactivity (18), using the streptavidin-biotin technique (Biogenex, San Ramon, CA) and alkaline phosphatase, as described previously (19). As negative control for immunohistochemical stainings, the primary antibody was replaced by nonimmune rat serum (Antigen Site, San Diego, CA) as well as mouse or rabbit serum (BioGenex, San Ramon, CA) or Tris-buffered saline (pH 7.2).

**RESULTS**

Twenty tumor-bearing animals were observed for a mean duration of 50 days (range, 20–119 days). Eleven animals were from the RFA treatment group, with a mean observation period after tumor implantation of 66 days (range, 41–110 days). The nine tumor-bearing animals of the control group were not treated with RFA. Their mean observation period after tumor implantation was 32 days (range, 20–58 days).

Survival analysis revealed a clear advantage for the treatment group (mean survival, 76.9 days; range, 50–110 days) compared with the controls (mean survival, 32.7 days; range, 20–58 days; P < 0.0003). Only two animals from the control group survived longer than 50 days (Fig. 1).

All of the animals of the control group developed a solid tumor with metastatic spread to the peritoneum, mediastinum, and lungs.

Two of 11 animals of the treatment group showed complete remission; 5 exhibited peritoneal metastases on dissection; and 4 showed both local recurrence and multiple metastases (peritoneum, lungs).

Never during the observation period did the tumor-bearing control group show specific activation of peripheral T cells against presented tumor lysate (P = 0.405; SI = 1.11), not even in the two animals with the longest survival times of 50 and 58 days (Table 1; Fig. 2A).

None of the examined animals, either from the treatment or from the control group, showed a reaction to tumor lysate presented before tumor implantation or before RFA application (SI [treatment group] = 1.18, SD = 0.33; SI [control group] = 1.10, SD = 0.25).

Two weeks after RFA, a marked activation of peripheral T cells against tumor lysate was found in the treatment group (SI [before treatment] = 0.93, SD = 0.24; SI [2 weeks after treatment] = 54.13, SD = 43.1; P < 0.001). This effect was seen throughout the observation period (SI = 60.68, SD = 42.30; Table 1; Fig. 2B).

Histologically, the tumors in the treatment group were surrounded by complete coagulation necrosis with a mixed inflammatory reaction consisting of neutrophils and a lymphoplasmacellular infiltrate that increased with time after RFA (data not shown). Focally a demarcating chronic fibrotic inflammation was found. Metastases were up to 50% necrotic and showed also perifocal, mostly chronic inflammatory reactions.

Immunohistologically the untreated tumor of the control group showed only sparse peritumorous CD3-positive lymphocytes (Fig. 3A). Within the native tumor, nearly no lymphocytes were found (Fig. 3B). One day after RFA, a hemorrhagic margin in the periphery of the tumor with a highly elevated amount of CD3-positive lymphocytes
could be observed (Fig. 3C). In the center of the tumors, tumor cells showed typical signs of cytoplasmic and nuclear thermic alterations of RF treatment. Here only a sparse infiltrate of CD3 positive lymphocytes was found, which, nonetheless, was more pronounced than in the untreated controls (Fig. 3D). Two weeks after treatment many CD3 positive T-lymphocytes were observed not only at the margin between thermically altered normal liver and tumor (Fig. 3E) but also along the fibrotic septa within the center of the tumor (Fig. 3F).

**DISCUSSION**

Immunotherapy through T-cell vaccination or vaccination with dendritic cells is regarded as a promising strategy for the treatment of various tumor entities such as malignant melanomas (7–9, 20, 21), and high expectations have been placed on cytokine-modulated immunotherapies for hepatic tumors (22). Although immunological phenomena in the form of lymphoplasmacellular infiltration were observed histologically after RFA (16), these findings had not been further analyzed.

The purpose of our study was to determine whether RFA is capable of inducing an “in situ” vaccination in the VX2 tumor model of liver metastases in rabbits.

None of the test animals in either the treatment or the control group spontaneously developed a specific T-cell response against the implanted tumor. However, only two weeks after RFA, all of the treated animals raised a significant T-cell response directed to tumor antigens in the peripheral blood and in the RFA-treated tumor tissue. This effect was observed throughout the entire observation period. Thus, we could show that RFA resulted in increased levels of specific T cells against the implanted tumor in the peripheral blood. In accordance with earlier results RFA was associated histologically with a peritumoral lymphoplasmacellular infiltration 2 weeks after treatment, including a large number of CD3-positive lymphocytes. Two weeks after RFA treatment, T cells were detectable not only in the periphery of the tumor but also in the center. These inflammatory infiltrates are suspected to be the area in which tumor antigens are presented to T cells.

It appears that the coagulation of tumor tissue through radiofrequency treatment leads to the enhanced release, exposure, and/or denaturation of tumor antigens. Thermally altered tumor antigens are likely to be phagocytozed by professional antigen-presenting cells like dendritic cells. In conjunction with the release of thermally altered tumor antigens, an unspecific inflammatory stimulus induced by RFA may overcome immune-tolerance toward the transplanted tumor. RFA, thus, appears to create an in situ environment resembling T-cell vaccination ex vivo.

Fig. 2. Lymphocyte SI of the control (A) and the RFA-treated (B) group. The Box-Whisker-Blots shows the SI determined by [3H]thymidine incorporation before and after tumor implantation (Impl.) relative to the tumor-bearing control group (see also Table 1). SI > 3.0 (dotted line) was taken as an indicator of T-cell activation. Outliers are marked with o.

Fig. 3. Immunohistochemical analysis. Immunostaining for CD3 in native tumor implants with and without RFA. Nuclei were counterstained with hematoxylin. Margin and center of an untreated tumor (A/B); and of a tumor one day after RFA (C/D) as well as 2 weeks after RFA (E/F) showing marked increase of CD3-positive T-lymphocytes at the margin (A/C/E) and in the center (B/D/F) of the tumor (arrows) (magnification 200×; bar: 100 μm).
The animals of the RFA group displayed significantly longer survival times than the control animals. This is all the more remarkable because only two of the treated animals were in complete remission on completing the trial schedule after more than 8 weeks (Table 1). The observed highly prolonged survival in the RFA-treated tumorbearing animals, compared with the controls, is in agreement with results obtained by Miao et al. (23). Working along the same lines, Burgener and Barbaric (24) showed that instillation of 100°C hot saline solution into VX2 tumors prolonged survival in rabbits. However, these groups could not explain their findings, whereas the present study points to a significant immunological effect on tumor growth, because survival in the treatment group was considerably prolonged despite the high recurrence rate. Whether this survival prolongation is caused by T-cell activation, tumor mass reaction, or both remains unclear. According to the present knowledge, T-cell activation is likely to contribute crucially to the observed effect. Also, several studies report tumor mass reduction not to be beneficial in terms of survival (25, 26).

Our study does not permit conclusions as to whether the proliferation of tumor-specific T cells was attributable solely to creation of a thermal necrosis, to the instillation of hot saline solution, or to the application of a high-frequency current. Although the procedure is not sufficient to prevent secondary growth or local recurrence, the immunological effect does appear to prolong survival. To what extent the insights gained in the present animal model with a transplanted tumor are transferable to humans remains to be explored.

ACKNOWLEDGMENTS

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REFERENCES


Announcements

(Requests for announcements must be received at least three months before publication.)

FUTURE ANNUAL MEETINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH

2004 March 27–31, Orlando, FL
2005 April 16–20, Anaheim, CA

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A number of meetings are now being organized in the AACR’s series of smaller scientific meetings. Following are the topics, dates, locations, and program committees for these meetings. When full details of each meeting are available, AACR members will be the first to receive complete brochures and application forms for participation in these important conferences. Nonmembers may receive this information by sending their names and addresses to Meetings Mailing List, American Association for Cancer Research, 615 Chestnut Street, 17th Floor, Philadelphia, PA 19106-4404. Up-to-date program information is also available via the Internet at the AACR’s website (http://www.aacr.org).

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January 25–29, 2004
Hilton Wai Koloa Village, Wai Koloa, Hawaii

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CALENDAR OF EVENTS


10th Hong Kong International Cancer Congress, November 19–21, 2003, Faculty of Medicine Building, The University of Hong Kong, Hong Kong. Contact: 10th HKICC Congress Secretariat, Department of Surgery, University of Hong Kong Medical Centre, Queen Mary Hospital, Hong Kong. Phone: 852.2818.0232 or 852.2855.4235; Fax: 852.2818.1186; E-mail: mededcon@hku.hk; Website: www.hkicc.org.

Third International Conference and 9th Annual Meeting of the International Society of Cancer Chemoprevention (ISCaC): Controversies in Tumor Prevention and Genetics, February 12–14, 2004, University of St. Gallen, Switzerland. E-mail: info@oncoconferences.ch; Website: www.oncoconferences.ch.


11th Conference on Advances in Neuroblastoma Research, June 16–19, 2004, Genoa, Italy. E-mail: anr2004@neuroblastoma.org; Website: www.anr2004.org.

6th International Conference on Head and Neck Cancer, August 7–11, 2004, Marriott Wardman Park, Washington, DC. Contact: Concepts in Meeting & Events, 1805 Ardmore Boulevard, Pittsburgh, PA 15221. Phone: 412.243.5156; Fax: 412.243.5160; E-mail: ssteighnercme@aol.com.

Molecular Targets for Cancer Therapy: 3rd Biennial Meeting, October 1–5, 2004, Don Cesar Beach Resort & Spa, St. Petersburg Beach, FL. Contact: Ann Gordon. Phone: 813.903.4975; E-mail: gordonac@moffitt.usf.edu.
Corrections

In the article by T. Wissniowski et al., titled “Activation of Tumor-specific T Lymphocytes by Radio-Frequency Ablation of the VX2 Hepatoma in Rabbits,” which appeared in the October 1, 2003 issue of Cancer Research (pp. 6496–6500), the names of the first two authors were misspelled. The correct author list is as follows: Thaddäus Till Wissniowski, Johannes Hansler, Daniel Neureiter, Markus Frieser, Stefan Schaber, Birgit Esslinger, Reinhard Voll, Deike Strobel, Eckhart Georg Hahn, and Detlef Schuppan.

In the article by B. Ruggeri et al., titled “CEP-7055: A Novel, Orally Active Pan Inhibitor of Vascular Endothelial Growth Factor Receptor Tyrosine Kinases with Potent Antiangiogenic Activity and Antitumor Efficacy in Preclinical Models,” which appeared in the September 15, 2003 issue of Cancer Research (pp. 5978–5991), two co-authors, Shi Yang (Department of Biochemistry, Cephalon, Inc.) and Sonya Pritchard (Department of Oncology, Cephalon, Inc.), were inadvertently omitted from the list of authors. The correct list is as follows: Bruce Ruggeri, Jasbir Singh, Diane Gingrich, Thelma Angeles, Mark Albom, Shi Yang, Hong Chang, Candy Robinson, Kathryn Hunter, Pawel Dobrzenski, Susan Jones-Bolin, Sonya Pritchard, Lisa Aimone, Andres Klein-Szanto, Jean-Marc Herbert, Francoise Bono, Paul Schaeffer, Pierre Casellas, Bernard Bourie, Roberto Pili, John Isacs, Mark Ator, Robert Hudkins, Jeffrey Vaught, John Mallamo, and Craig Dionne.

In the article by Y-T Tai et al., titled “Insulin-like Growth Factor-1 Induces Adhesion and Migration in Human Multiple Myeloma Cells via Activation of β1-Integrin and Phosphatidylinositol 3'-Kinase/AKT Signaling,” which appeared in the September 15, 2003 issue of Cancer Research (pp. 5850–5858), the Western blotting in the bottom “IGF-1R” panel of figure 3B is incorrect. The correct figure appears below:

Fig. 3. IGF-I stimulates association of IGF-IR and β1 integrin, as well as activation of PI3-K/AKT and ERK pathways. A, serum-starved MM.1S or OPM6 cells were treated with IGF-I for the indicated times. Cell lysate (500 μg) was immunoprecipitated with anti-IGF-IR and anti-IRS-1 Abs and then immunoblotted with anti-pTyr mAb. Membranes were stripped and reprobed with anti-IGF-IR, anti-β1 integrin, and anti-p85 PI3-K Abs. A total of 60 μg of each lysate was also resolved by 8% SDS-PAGE, and immunoblotted with anti-pAKT and anti-pERK Abs. α-tubulin serves as loading controls. B, serum-starved cells were incubated with IGF-I (100 ng/ml) or PMA (100 nM) for 10 min or pretreated with αIR3 (5 μg/ml) for 30 min before incubation with IGF-I (100 ng/ml) for 10 min. One mg of cell lysates was immunoprecipitated with 4 μg of anti-β1 integrin Abs. Immunoprecipitates were analyzed by immunoblotting with anti-IGF-IR and anti-β1 integrin Abs.
In the article by G. Yousef et al., titled “Human Kallikrein 5: A Potential Novel Serum Biomarker for Breast and Ovarian Cancer,” which appeared in the July 15, 2003 issue of Cancer Research (pp. 3958–3965), figure 4 was printed incorrectly. Below is the correct figure.

Fig. 4. Fractionation of three biological fluids (serum, ascites fluid from an ovarian cancer patient, and breast milk) by size-exclusion liquid chromatography. The elution profile of molecular mass standards is denoted by arrows. In serum, hK5 elutes as two immunoreactive peaks, one with an apparent molecular mass of 50 kDa (fractions 37–39) and one with an apparent molecular mass of approximately 150–180 kDa (fractions 31–33). The elution profile of another kallikrein with a similar theoretical molecular mass, hK6, is also shown by dashed lines. This kallikrein elutes at a molecular mass of ~35 kDa, corresponding to free hK6. In ascites fluid, the same comments apply as for serum. In breast milk, hK5 elutes mainly as a single immunoreactive peak. hK6 elutes as two distinct peaks, one at a molecular mass of ~35 kDa and another one at a molecular mass of ~100 kDa.
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