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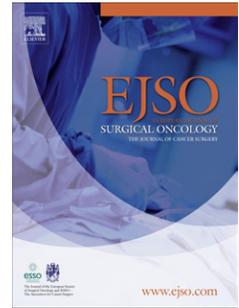
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Adjuvant radioimmunotherapy after radiofrequency ablation of colorectal liver metastases in an experimental model

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Conflict of interest statement

None of the authors of the above manuscript has declared any conflict of interest within the last three years which may arise from being named as an author on the manuscript.

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Key words

radioimmunotherapy; radiofrequency ablation; colorectal cancer; animal models

Introduction

Without any treatment, the 5-year survival of patients with colorectal liver metastases is negligible.[1] Surgical resection of these liver metastases has shown to improve 5-year survival of colorectal cancer (CRC) patients to about 30% (range 15-67%), but only 20% of patients are considered eligible for this surgical procedure.[2;3] If the metastases are not resectable, radiofrequency ablation (RFA) is a suitable alternative.[3] RFA induces coagulative necrosis by using high-frequency alternating current applied via electrodes placed within the tissue. RFA ablation can be applied percutaneously, laparoscopically or at open surgery. RFA has proven to be a safe procedure with a complication rate lower than <10%.[4] However, a high local recurrence rate is a major problem of RFA. Depending on the technique used, the number, the size and the location of the lesions, local recurrences are reported in 4 % to 55 % of cases.[5-7]

Radioimmunotherapy (RIT), using radiolabelled monoclonal antibodies (mAbs) directed against tumour-associated antigens, offers the opportunity to selectively irradiate tumour cells, while sparing normal tissues. Because tumour targeting with radiolabelled mAbs is more efficient in small tumour lesions, RIT is considered suited for minimal or residual disease. This concept has been demonstrated in preclinical as well as in clinical trials, showing an increased survival after resection of colorectal liver tumours when adjuvant treatment with RIT is applied.[8;9] Here, we hypothesized that RIT could also treat residual disease after RFA. Interestingly, *in vitro* studies with the human colon adenocarcinoma cell line LS174T have indicated that short hyperthermia results in a significant, temporarily, upregulation of carcinoembryonic antigen membrane expression[10], which potentially could result in a synergistic combination of RFA and RIT. The aim of the present study was to test the hypothesis that adjuvant RIT might be an effective way of preventing recurrent liver metastases after RFA in an experimental model using the mAb MG1, directed against a tumour-associated antigen expressed on the syngeneic colonic carcinoma cell line CC531.

Material and methods

Cell line and monoclonal antibody

The syngeneic rat colonic carcinoma cell line CC531 is derived from colonic tumours of Wag/Rij rats exposed to 1,2-dimethylhydrazine.[11] CC531 was cultured and suspended as described previously.[12] MG1 mAb was purchased from Antibodies for Research Applications BV (Gouda, The Netherlands). It is a murine IgG_{2a} antibody, raised by immunization of mice with CC531 colon carcinoma cells. It recognizes a cell surface antigen of about 80 kDa, localizes preferentially in the CC531 tumours when injected in rats and has minimal cross-reactivity with other cell types.[13;14] Moreover, it has a high affinity (1.0 nM) for the antigen expressed on CC531 tumour cells and shows high tumour uptake in experimental liver metastases after intravenous injection.[15;16]

Radiolabelling

¹⁷⁷Lu was purchased from IDB Holland BV (Baarle Nassau, The Netherlands). The MG1 antibody was labelled with ¹⁷⁷Lu and purified as described previously.[12] The specific activity of the ¹⁷⁷Lu-labelled diethylenetriamine penta-acetic acid (DTPA)-MG1 preparations (hereafter referred to as ¹⁷⁷Lu-MG1) used in these studies ranged from 169 to 627 kBq/μg. Radiochemical purity, determined by instant thin-layer chromatography on TEC Control chromatography strips (Biodex, Shirley, NY), using 0.1 M citrate buffer (pH 6.0) as the mobile phase, exceeded 92 % for all ¹⁷⁷Lu-MG1 preparations.

Animals and tumour induction

Male Wag/Rij rats, with a mean weight of 230 g, were used (Charles River Laboratories, Sulzfeld, Germany). They were accustomed to laboratory conditions for one week before use and housed under non-sterile, standard laboratory conditions (temperature 20–24 °C, relative humidity 50–60 %, 12-h light–dark cycle), with free access to animal chow and water. All experiments were conducted in accordance with the principles laid out by the revised Dutch Act on Animal Experimentation (1997) and approved by the institutional Animal Welfare Committee of the Radboud University Nijmegen.

All surgical procedures were carried out under clean conditions. Tumour induction was performed as described previously [16] with the alteration that, via a 2-cm midline laparotomy, tumour cells were injected subcapsularly in the left lateral lobe of the liver. Rat cages were kept on a warm mattress for

the first 24 h after operation. Before surgery and on the first and second day thereafter, analgesia was given in the form of subcutaneous carprofen injections (5 µg per 100 g bodyweight).

Radiofrequency ablation

Ten days after inoculation, animals were anaesthetized by isoflurane inhalation and the abdomen and back were properly shaved. After supine placement onto an electricity-conducting pad (grounding pad), the abdomen was opened via a 3-cm midline laparotomy. The left lateral lobe of the liver was lifted out of the abdomen and placed on a gauze to clearly visualize the tumour. A straight radiofrequency ablation needle with active tip of 8 mm (SMK-15, Cotop, Amsterdam, the Netherlands) was inserted and placed in the middle of the tumour. After placement of the radiofrequency ablation needle, impedance could be evaluated on the radiofrequency lesion generator system (Model RFG-3B, Radionics, Burlington, MA, USA). Next, treatment was started by delivering radiofrequency energy. During a treatment cycle of ~180 s, temperature could be monitored by means of a thermistor and thermocouple in the tip of the probe. Treatment was considered successful if a tip temperature of ~75–80 °C was reached. If the tumour had a size larger than 5 mm in diameter, the treatment was repeated, repositioning the needle in such a way that the whole tumour would receive one treatment cycle. After completing the identical RFA procedure in each rat, the abdomen was closed as described before.

Model validation

To determine the optimal model for recurrences and reproducibility of the model six Wag/Rij rats with a mean weight of 260 g had the tumour inoculation and treatment procedure as described above. Additionally, in the medial lobe of the liver a second tumour was inoculated, which was treated with 2 cycles of RFA per puncture. Ten days after the treatment, three randomly selected rats underwent a FDG-PET/CT scan acquired with an Inveon animal PET/CT scanner (Siemens Preclinical Solutions, Knoxville, TN).[17] One hour prior to scanning, the rats received an intravenous injection of 10 MBq ¹⁸F-2-deoxy-2-fluoro-D-glucose (FDG). To enhance liver contrast on the CT scan, two hours prior to scanning, 1.5 mL of glyceryl 2-oleoyl 1, 3-bis [7-(3-amino-2, 4, 6-triiodophenyl)] alkanoate (Fenestra LC; Advanced Research Technologies, Inc, Montreal, Quebec, Canada) was injected via the tail vein (RES-IV, reticuloendothelial system– specific contrast). Twenty minutes prior to scanning an

intraperitoneal injection of 1.5 mL ioversol (Optiray 320; Mallinckrodt Inc, 1:2 dilution) for intraperitoneal contrast was administered. After scanning, all the animals in the experiment were killed using oxygen/carbon dioxide asphyxiation and dissected. The liver was resected and sliced in 0.5-cm thick slices for routine histopathological haematoxylin & eosin (H&E) staining.

Adjuvant radioimmunotherapy

The therapeutic efficacy of RIT was determined after random assignment of 60 rats after the RFA procedure. One group of rats received an intravenous injection of ^{177}Lu -MG1 a couple of hours after the RFA procedure (RIT D10). Since timing of RIT might be crucial, a second group of rats received RIT 7 days after RFA (RIT D17). The administered activity dose in both groups was 300 MBq/kg in 0.25 - 0.50 ml PBS, which has been determined as the maximum tolerable dose (MTD) previously.[16] The third group did not receive any adjuvant treatment after the RFA procedure (control). The primary endpoint was survival calculated from the day of RFA. Toxicity was monitored by body weight measurement and assessment of general condition. Body weight was measured daily during the first week and twice weekly thereafter. The area under the curve (AUC) during the first month after RFA was calculated. When the humane endpoint (one of the following signs present: >20% body weight loss, abdominal distension and ascites, signs of physical discomfort like breathing problems) was reached, rats were killed using oxygen/carbon dioxide asphyxiation and dissected. Four months after the start of the experiment, the median survival was reached in all three groups and the remaining rats were euthanized and dissected. The abdominal cavity was carefully inspected, with special attention to the liver parenchyma. To objectify the humane endpoint, at dissection the peritoneal cancer index (PCI) was scored as described by Koppe et al.[12] Next, the left lateral lobe of the liver was sliced in 0.5-cm thick slices for routine histopathological haematoxylin & eosin (H&E) staining. Tumour growth was evaluated by an experienced pathologist at two levels in each slice and scored as either present or absent.

Statistical analysis

Statistical analysis was performed using the SPSS software (Chicago, IL) and GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego USA). Sample size (n=20) was determined by a power calculation based on observations from a previous animal experiment, with an estimated

75-days survival of 50% and 10% in the treated groups and in the control groups, respectively.[16] Alpha was set to 5% and the power was set to 90%. All analysis were performed on a per protocol basis. Kaplan-Meier survival curves for overall survival were generated and the log-rank test was used to identify the difference between the groups for overall survival. The level of statistical significance was set at a P-value of ≤ 0.05 . Means and standard deviations or 95%-confidence intervals (95%-CI) were used to describe continuous data and differences were calculated using one-way ANOVA and independent-samples two-sided T-tests. Median and range were used to describe non-binomially distributed data and differences were calculated using Kruskal-Wallis tests.

Results

Model validation

Ten days after tumour induction, all rats had a tumour in the medial and left lateral liver lobe with an average diameter of 5 mm. These tumours were all treated with RFA (one or two cycles). Ten days after the RFA procedure, central necrosis and a surrounding inflammatory zone could be identified microscopically in the RFA-treated areas (Fig 1). All tumours treated with one cycle showed, morphologically vital, tumour deposits, mainly present at the border of the treated area. Tumours treated with two cycles of RFA showed mainly morphologically disturbed tumour cells in the centre of the ablated area (not shown). In Figure 2, typical FDG-PET, CT and fused images after RFA are presented. FDG-PET/CT showed no uptake in the central parts of the two RFA treated areas of the liver. The FDG-PET scan also showed enhanced uptake at the edge of the tumour treated with one cycle only. Based on these results, recurrent tumour growth after RFA of liver tumours was expected to have the highest incidence after one RFA cycle and this procedure was selected for the subsequent experiment in which the efficacy of adjuvant RIT was investigated.

Efficacy of adjuvant radioimmunotherapy

All rats had developed a single metastasis (mean diameter 5 mm) in the left lateral lobe of the liver at the time of RFA. In 59 of the 60 rats RFA could be completed, whereas in one rat the location of the tumour prevented adequate RFA. This rat was excluded from the analysis. Four randomly selected rats that were assigned to treatment 7 days after RFA, were transferred to the control group because the available activity dose on the day of the administration was lower than expected. As a consequence, the control group consisted of 23 animals and the RIT D10 and RIT D17 groups of 20 and 16 rats, respectively. One rat (of the RIT D10 group) was censored in the survival analysis 77 days after RFA, since it was euthanized without fulfilling the criteria.

The relative body weight of the rats during the first month of the study, expressed as the percentage of the body weight on the day of tumour induction, is depicted in Figure 3. The mean AUC of the RIT-groups was significantly lower than the mean AUC of the control group ($P=0.007$), indicating that RIT significantly reduced the body weight of these rats. However, differences in the mean relative body weight per group were small (maximum difference between the RIT groups and the control group was

4% relative bodyweight), no other signs of clinical discomfort were observed during the initial post-treatment period and the body weight of the rats recovered quickly in the course of the study.

31 Rats died due to excessive tumour growth. Median (range) PCI found at dissection was 4 (0-13), 1.5 (0-13) and 0 (0-13) in the RIT D10, RIT D17 and control group, respectively, and did not differ between groups ($P=0.635$). The survival curves of the groups are shown in Figure 4. Kaplan-Meier survival analysis showed that the survival curves of the three groups did not differ ($P=0.902$). Median survival time was 119 days, 98 days and 116 days in the RIT D10, RIT D17 and control group, respectively. The 75th percentile survival was 74 days (95%-CI: 64 – 84 days), 67 days (95%-CI: 41 – 93 days) and 47 days (95%-CI: 15 – 79 days) in the RIT D10, RIT D17 and control group, respectively. Median (range) weight of the tumour in the liver was 4.8 (3.6-9.1), 4.7 (3.5-8.5) and 5.1 (1.7-18.2) in the RIT D10, RIT D17 and control group, respectively and differ not between the groups (0.999). 120 Days after tumour cell inoculation, the remaining 28 rats (9 in the RIT D10-group, 8 in the RIT D17-group and 11 in the control group) were euthanized. Histopathological examination of the livers of these remaining rats showed microscopic tumour nodules in 4 of these rats (one in each RIT group and two in the control group). Overall, local recurrence after RFA was detected in 45%, 47% en 50% of the animals of the RIT D10, RIT D17 and control group, respectively.

Discussion

RFA and recurrence rates

Currently, RFA offers a treatment option for patients with colorectal liver metastases, whom are not considered eligible for surgery. One of the major problems of RFA is the high local recurrence rate. Depending on the technique used and the number, the size and the location of the lesions, local recurrences rates of 4% to 55 % have been reported.[5] Tumour size is considered to be one of the most important prognostic indicators for local recurrence. In larger lesions, multiple probe insertions are necessary to obtain a treatment zone large enough to include all tumour tissue. Analysis of recurrence patterns has indicated that the efficacy of these overlapping treatment zones could be unreliable. Inadequate heating of the whole tumour can also be a result of the cooling effect of blood flow through adjacent large blood vessels.[18] Additionally, lower temperatures (42 to 46 °C), for example at the borders of the ablation area, result in reversible cell damage and could even induce synthesis of heat shock proteins, facilitating recovery of tumour cells from heat damage.[19] So, after RFA of larger tumours and of lesion that are located near large vessels, residual disease might cause recurrences. RFA is even thought to accelerate outgrowth of residual or microscopic metastases in the liver due to hypoxia related pathways.[20;21] Interestingly, studies on anti-tumour immune responses after tumour ablation by RFA have shown that tumour cell necrosis could generate a immunogenic source of tumour antigens.[22-24] These antigens can be up taken, processed and presented by dendritic cells and induce a potent immune response. However, initial cellular immune responses in animal models seem weak.

RFA and adjuvant therapy

Clinical trials on RFA are ongoing and US as well as European treatment guidelines do not contain any recommendations on adjuvant therapy after RFA yet. However, knowledge on beneficial effect of adjuvant systemic therapies after resection of colorectal liver metastases can be used as a rationale for a similar approach in RFA. In 2005 Elias et al. performed a study to determine the survival rate of patients with technically unresectable liver metastases when undergoing liver resection plus RFA,

along with optimal systemic chemotherapy. Unfortunately, perioperative chemotherapy regimens varied widely, thus the specific therapeutic impact on the median survival of 36 months could not be determined.[25] Two studies on the safety of hepatic arterial infusion of chemotherapy (six monthly cycles of 5-fluorouracil-based chemotherapy) after RFA report high complication and toxicity rates associated either to the pumps or to the chemotherapy.[26] Considering these results, there is an obvious need for alternative strategies and adjuvant RIT could be a good candidate. RIT with radiolabelled monoclonal antibodies directed against tumour-associated antigens, offers the opportunity to selectively irradiate tumour cells, while sparing normal tissues. The results of adjuvant RIT after surgical resection of liver metastases seem promising.[8;9] [de Jong et al. unpublished data]. However, the data in the present study show RIT to be ineffective in our model. Therefore, we conclude that adjuvant RIT does not influence survival in rats after RFA of colorectal liver metastases. Several biological factors could have contributed to these results. A key factor in the efficacy of RIT in solid tumours is the extent of accumulation of the antibody in the tumour.[27] Accumulation of radiolabelled antibodies has shown to be unfavourable in large tumours with limited vascularisation, increased interstitial pressure and a large transport distance in the interstitium.[28] In our study, these features could explain the absence of therapeutic efficiency of adjuvant RIT after RFA of the liver tumour. First of all, the tumour load after RFA could have been too high in this model to show any efficacy of RIT. Another explanation could be that changes in local tissue structure after RFA affected the antibody uptake. Vogt et al. studied the morphologic and functional changes in non-tumorous liver tissue at several time points after RFA in minipigs.[29] The study showed that three different zones could be identified on histopathologic evaluation of the ablative site immediately after RFA: a central zone demonstrating early signs of tissue necrosis, a transitional zone showing sinusoids engorged with blood (because the central ablative necrosis was obstructing outflow) and a peripheral zone demonstrating mild reactive changes. These findings, also present in our histological specimens ten days after RFA (Fig 1), indicate that in the transitional and peripheral zones of the ablated area vascularisation can be disturbed, interstitial pressure might be increased and the transport distances are increased due to the inflammatory response. As a result, localisation of systemically administered radiolabelled antibodies in the residual tumour cells present at the border of the ablated area might be hampered. Therefore, based on the present study, RIT cannot be recommended as an adjuvant therapy after RFA of colorectal liver metastases.

Conclusion

This study shows that adjuvant RIT using ^{177}Lu -labelled MG1 mAbs at MTD does not increase survival after RFA of colorectal liver metastases in rats.

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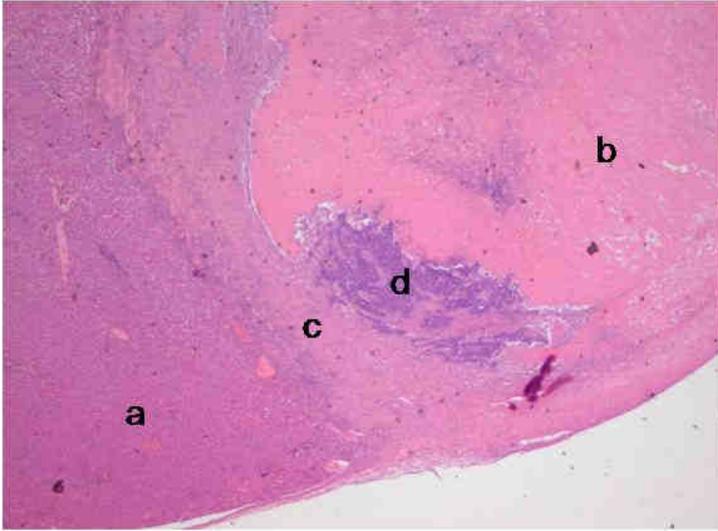
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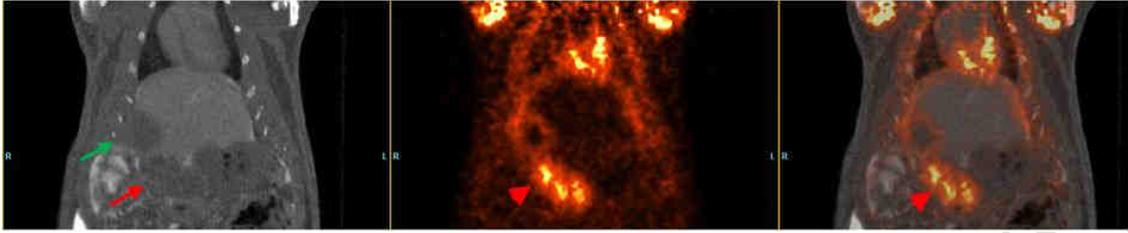
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