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Local ablative therapies for colorectal liver metastases and the immune system

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Chapter 4

Locoregional therapies of liver metastases and immune response in vivo

F.H. van Duijnhoven, R.A.E.M. Tollenaar, O.T. Terpstra and P.J.K. Kuppen. Locoregional therapies of liver metastases in a rat CC531 coloncarcinoma model improve the anti-tumour immune response.

Introduction

Colorectal cancer is one of the most common malignancies in Europe and the USA with about 300 000 new cases and 200 000 deaths each year[1]. Metastatic disease, predominantly to the liver, eventually develops in 70% of the patients[2,3]. When confined to the liver, metastases may be curatively treated by hepatic resection. Unfortunately, only 10 to 25% of the patients with liver metastases are eligible for this treatment, as the number, localisation or size of the metastases or poor hepatic reserve often preclude radical hepatic resection[4]. When resection is not possible, locoregional therapies such as photodynamic therapy (PDT), radio frequency ablation (RFA) or hepatic artery infusion (HAI) can offer palliation and prolongation of disease-free and overall survival.

PDT, RFA and HAI have been applied clinically with varying success rates in the last decade. PDT involves the systemic administration of a tumour-localising photosensitising agent (photosensitiser) that is activated upon tumour illumination by light of an appropriate wavelength and then reacts with oxygen, producing reactive oxygen species. These reactive oxygen species lead to direct tumour cell damage and secondary effects like vascular damage, resulting in tumour necrosis. Currently, PDT is an established treatment option for various forms of cancer such as mesothelioma, bladder and oesophagus carcinoma[5-8]. For solid tumours such as colorectal liver metastases, the therapy has been successfully used in a rat model with CC531 liver metastases[9,10]. Apart from its advantages of non-invasiveness and tumour selectivity, PDT may also induce or increase a systemic immune response directed against the tumour cells[11]. Many studies have already shown the involvement of the immune system in the efficacy of PDT and indicated an increased specific immune response upon PDT[12-14]. For example, treatment of tumours with PDT led to an increased resistance to subsequent rechallenge, when compared to tumours treated with resection[12].

For RFA, a heat-producing probe is inserted into the tumour to deliver radiofrequency thermal energy. The high temperatures lead to local tissue necrosis. In colorectal cancer, RFA has been applied for colorectal liver metastases for several years now, with complete response rates of 52-95%[15,16]. No data are as yet published on its relation to or effect on the immune system. As RFA also involves the local destruction of tumour tissue which remains in situ after treatment, it may like PDT lead to the generation or increase of an anti tumour immune response.

In HAI, the chemotherapeutic drugs are administered directly into the hepatic artery. As established colorectal liver metastases derive most of their blood supply from the hepatic artery, in contrast with liver parenchyma[17,18], HAI theoretically leads to high drug concentrations within the tumour while the liver parenchyma is spared. Several randomised studies have shown good response rates of up to 41%, but sur-

vival benefit when compared to systemic chemotherapy is not significant[19,20]. This treatment differs from RFA and PDT as it is not local but regional, and destruction is not as acute as with the local ablative techniques. Furthermore, effects on the immune system are unknown.

If indeed a systemic immune response develops upon certain local or regional therapies, it can expand the effectiveness of these therapies to a systemic level. The activated immune system may be able to eliminate micro metastases or circulating tumour cells, thus reducing the development of extrahepatic metastases. In this study, we therefore aimed to elucidate the effect of an immune response induced by various locoregional treatments. We used a rat colon carcinoma liver metastases model, CC531. We have extensive experience with this model, which has proven to be adequately reproducible[9,21-23]. RFA and PDT were applied as local therapies and for regional therapy we used HAI with melphalan[24,25]. Various parameters were used to study the immunological effect of locoregional treatments on tumour growth: growth of untreated nearby livers tumours, outgrowth of locally de novo induced liver tumours and outgrowth of lung tumours after systemically de novo administered tumour cells. Furthermore, serum antibodies directed against CC531 tumour cells were detected.

Materials and methods

Animals

Male Wag/Rij rats weighing approximately 225 grams rats were used (Charles River, Zeist, The Netherlands). The animals had free access to food and water. The weight of the animals was followed throughout the experiment to monitor their general state. Principles of laboratory animal care were followed and, according to Dutch law, the Animal Welfare Committee of the Leiden University Medical Center approved the study.

Tumour model

For tumour inoculation, we used the colon adenocarcinoma cell line CC531 which is moderately differentiated and syngeneic to Wag/Rij rats[22]. Briefly, tumour cells were cultured in RPMI 1640 supplemented with 2mM L-glutamine, 10% heat inactivated fetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin sulphate (complete medium) (all from Gibco, Grand Island, NY, USA). Cells were maintained by serial passage. Tumour cells were harvested with a solution of 0.25% (w/v) EDTA and 0.25% (w/v) trypsine in HBSS (Sigma, St. Louis, MO, USA), washed three times in 0.9% (w/v) NaCl solution buffered with 1.4 mM phosphate buffered saline (PBS) and adjusted to a suspension containing 1 x 10^6 viable (trypan blue exclusion test)

tumour cells per ml PBS. For local liver tumour induction, 5 x 10^4 viable tumour cells (in 50 µl suspension) per site were injected subcapsulary into the upper lobe of the liver at two sites.
For local liver tumour rechallenge, 5 x 10^5 CC531 cells in 50 µl PBS were injected subcapsulary into the lower liver lobe. For systemic rechallenge, 2 x 10^6 CC531 cells were injected in the femoral vein, in 200 µl PBS.

Study design
Wag/Rij rats were randomly assigned to one of the following eight groups: (1) PDT and local rechallenge, (2) RFA and local rechallenge, (3) HAI and local rechallenge, (4) sham and local rechallenge, (5) PDT and systemic rechallenge, (6) RFA and systemic rechallenge, (7) HAI and systemic rechallenge (8) sham and systemic rechallenge. Two tumours were inoculated in the liver at day 0, number 1 on the left and number 2 on the right side of the upper lobe. At day 15, the rats were treated with the various treatment modalities. Tumour 2 was treated after laparotomy with RFA or PDT in rats from groups 1, 2, 5 and 6 and HAI with melphalan was performed in rats from groups 3 and 7. A laparotomy without treatment was performed in the sham groups 4 and 8. Rechallenge by subcapsular (groups 1 to 4) or intravenous (groups 5 to 8) injection of CC531 tumour cells in the lower liver lobe was performed on day 27, 12 days after treatment. Tumour developing at this site was indicated as tumour no. 3. Rats were sacrificed at day 42 and tumour presence in liver and lungs was determined.

Liver tumours were separately enucleated from the surrounding liver parenchyma and weighed. To macroscopically visualise the presence of lung tumours, 15 ml of a 15% black Indian ink solution in water was injected in the trachea of all rats. Lungs were then removed and put in 30 ml of Fekete's solution (86% alcohol 70% v/v, 8.6% formaldehyde 37% v/v and 4.4% acetic acid 99-100% v/v)[26]. After 24 hours, Fekete's solution was replaced by water. Blood samples were taken from all rats by orbital punction at time of inoculation and rechallenge and by aortal punction at time of sacrifice.

Treatment of tumours
Photodynamic therapy - At day 14, 1.0 mg/kg of the photosensitiser meta-tetra(hydroxyphenyl)bacteriochlorin (mTHPBC) was administered to the animals of groups 1 and 5 via the left femoral vein. The mTHPBC was dissolved in a mixture of 1,2-propanediol/ethanol (6:4, v:v), giving a solution of 2.5 mg/ml. The right tumour in the upper liver lobe of each rat was illuminated using a diode laser emitting light of 739 nm wavelength (Donald 0411-G, CeramOptec, Bonn, Germany). The power output was set at a fluence rate of 0.10 W. A bare tip fibre was used with a cross-section of 0.6 mm. After 100 seconds of irradiation, this resulted in a light dose of 10 J per tumour.

Radiofrequency ablation – Rats in groups 2 and 6 were treated with RFA using a RITA 1500X generator (RITA Medical Systems, Mountainview, CA, USA). A 2 cm expandable needle was inserted in the right tumour in the upper liver lobe and expanded to 1.5 cm, resulting in a lesion of 1.5 cm in diameter. Power output was set at 90 Watt, temperature was set at 90° Celsius and when this temperature was reached, an ablation of two minutes was performed.

Hepatic artery infusion with melphalan – Rats in groups 3 and 7 were treated by HAI with melphalan purchased from Glaxo Wellcome Pharmaceuticals (Zeist, The Netherlands). A melphalan solution (16.4 mM) was prepared by dissolving 1 mg melphalan in 200 µl 0.09% (w/v) hydrochloric acid, which was subsequently diluted with 0.9% NaCl. For hepatic artery infusion a cannula (PE-50, ∅ 0.61 mm) was inserted into the gastroduodenal artery with the tip in the common hepatic artery, leaving normal arterial blood flow intact. Rats received a dose of 4.5 mg/kg [25] melphalan infused over a period of 20 min followed by 5 minutes infusion with NaCl 0.9%, using an infusion pump (perfusor, B. Braun, Melsungen, Germany) at a flow rate of 25 µl/min. After infusion the gastroduodenal artery was tied off.

Detection of anti-CC531 antibodies

Blood samples were centrifuged for 10 minutes at 10 000 rpm (Beckman GS-6R centrifuge, Beckman Coulter, Fullerton, CA, USA), supernatants were collected and stored at -20°C until analysis. Anti-CC531 antibodies were detected by flow cytometry in sera from all rats diluted 1:30 in PBS with 0.5% BSA w/v (PBS/BSA). Briefly, CC531 tumour cells were harvested from culture and washed with PBS/BSA. Of each 1:30 diluted serum sample, 100 µl was added to 500.000 CC531 cells. After incubation for 30 minutes at 4°C, cells were washed twice with PBS/BSA. The second antibody, FITC labelled goat-anti-rat IgG (Southern Biotechnology Associates, Birmingham, AL, UK), was then added in 100 µl in a 1:100 dilution and incubated for 30 minutes at 4°C. Cells were washed once with PBS/BSA after which 300 µl of a 1:100 solution of propidium iodide in PBS/BSA was added. Cells were then analysed in a flow cytometer (FACScalibur, Becton Dickinson Immuno-cytometry, San Jose, CA, USA). As a positive control serum from an intravenously CC531 boosted rat that contained a high amount of antibodies was used. This sample was the internal standard in all flow cytometry experiments. Antibody levels were expressed as percentage mean fluorescence intensity of positive control.

Statistical analysis

Differences in tumour weight and anti-CC531 antibody levels were analysed with the Student's t-test, with $p < 0.05$ considered statistically significant. The absence or presence of lung metastases after i.v. rechallenge or of tumour in the lower liver lobe after local rechallenge was compared between groups with the Chi-squared test, with $p < 0.05$ considered statistically significant.

Results

Liver tumour growth

No complications occurred after laparotomy for tumour induction or treatment. The initial tumour induction was successful in all rats but one, in which tumour cells inadvertently leaked from subcapsular liver injection site to the abdominal cavity, resulting in a tumour in the omentum and lung metastases. This rat was excluded from further analysis. A minimum of 6 evaluable rats remained in all groups.

Treatment by RFA or PDT was effective in all rats, as tumours were largely necrotic and weighed significantly less than untreated tumours in the same rat ($p < 0.02$) or untreated tumours in rats from the sham group ($p < 0.05$) (figure 1a). Effective local treatment of tumour 2 did not affect tumour growth of untreated number 1, as average weight of tumours 1 in RFA and PDT groups did not significantly differ from sham group (figure 1a). Treatment with HAI resulted in total disappearance of both liver tumours in all but one rat, in which rat two very small tumours remained.

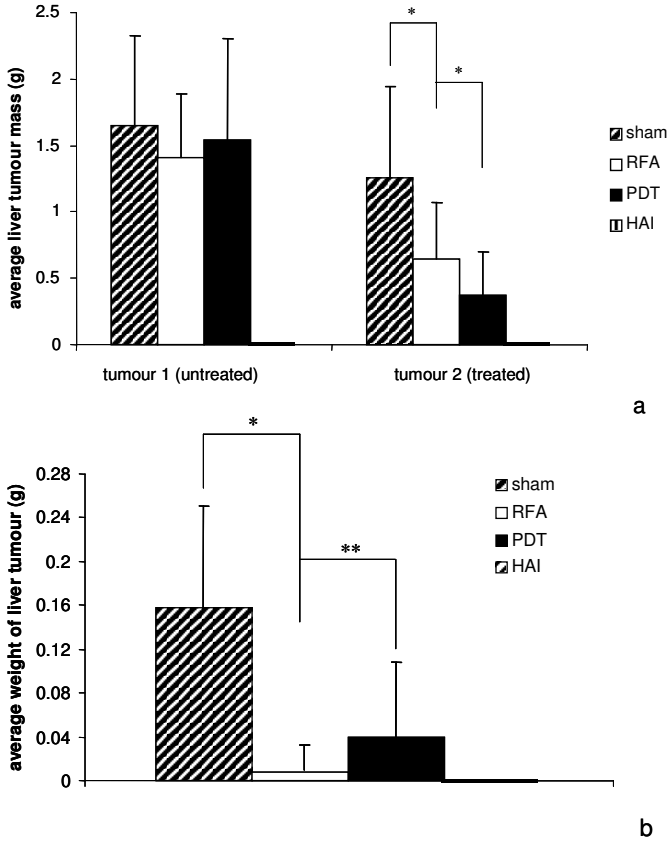

*Figure 1. Average weight of liver tumours in treatment and control groups. a. Tumour 1 (untreated) and tumour 2 (treated) in sham, RFA and PDT rats with * $p < 0.0001$ for sham vs. RFA and PDT. b. Tumour 3 after local rechallenge in sham, RFA and PDT rats with * $p < 0.0003$ for sham vs. RFA and ** $p < 0.02$ for sham vs. PDT*

Tumour rechallenge

Rats that were treated with locoregional therapy appeared to be more resistant to local rechallenge in the liver than rats whose liver tumours were not treated. This was shown by the development of a third liver

tumour at site of rechallenge in all but one rat from the sham group (table 1). In contrast, treatment with HAI effectively prevented any development of de novo induced liver tumour upon subsequent local rechallenge. RFA and PDT displayed similar protective effects, with 1 and 3 out of 8 rats respectively developing a liver tumour (table 1). For all treatment groups, differences with the untreated sham group were significant. Correspondingly, the average weight of this third liver tumour upon local rechallenge was significantly lower in RFA, PDT and HAI groups when compared to the sham group ($p < 0.02$)(figure 1b). There were no statistical differences regarding incidence or weight of de novo induced liver tumours between the three treatment groups.

Upon intravenous rechallenge, 4 rats in the sham group developed macroscopically visible lung tumours, whereas rats from treatment groups did not develop lung tumours at all. These findings were confirmed by immunohistochemical staining (data not shown). Difference between treated rats and sham rats was significant when comparing PDT rats with the sham group ($p < 0.03$). The number of rats in HAI and RFA groups was lower (n=6) and though these rats also did not develop any lung tumours, this difference was not statistically significant when compared to the sham group ($p = 0.06$).

type of rechallenge	treatment	n rats	liver tumour		p value vs. sham group (chi-squared test)
			present	absent	
local rechallenge	RFA	8	1	7	*0.004*
	PDT	8	3	5	*0.027*
	HAI	8	0	8	*0.000*
	sham	9	8	1	*NA*
			lung metastases		
			present	absent	
i.v. rechallenge	RFA	6	0	6	*0.06*
	PDT	8	0	8	*0.03*
	HAI	6	0	6	*0.06*
	sham	9	4	5	*NA*

Table 1. Presence of liver tumours after local rechallenge or lung tumours after systemic rechallenge in all treatment and control groups as described in material and methods

Production of anti-CC531 antibodies

Upon local treatment of liver tumours and subsequent rechallenge by subcapsular administration of CC531 cells in the liver, there was no increased level of anti-CC531 serum antibodies, regardless of treatment modality of liver tumours (figure 2). However, when CC531 cells were administered intravenously, production of CC531 antibodies was boosted to approximately twice the pre-inoculation level ($p < 0.05$, figure 3). This is seen in all treatment groups as well as in sham-operated rats. There was no statistical difference between treatment and sham groups.

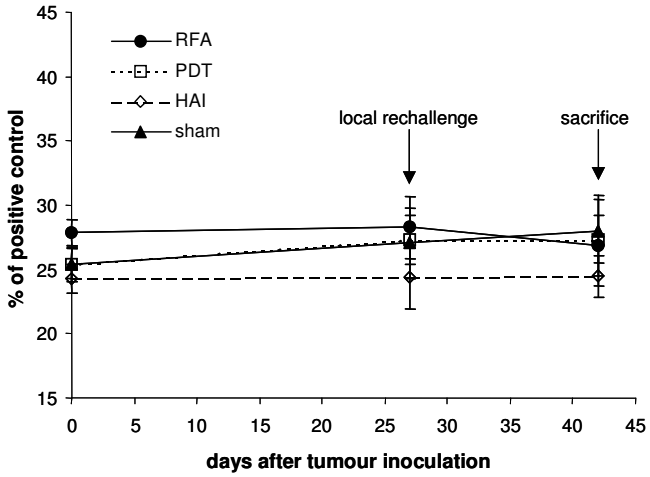

Figure 2. Amount of IgG antibodies directed against CC531 tumour cells. Quantity of antibody is represented as percentage of positive control and measured at day 0 (inoculation of liver tumours), day 27 (rechallenge) and day 42 (sacrifice). Antibody production does not increase in rats after local rechallenge, independent of treatment

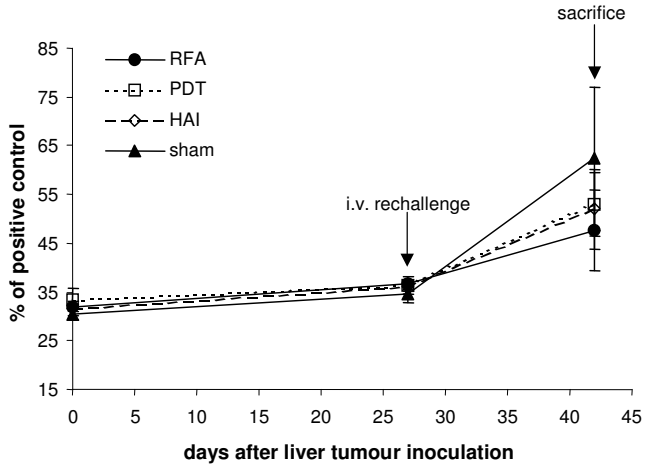

Figure 3. Amount of IgG antibodies directed against CC531 tumour cells. Quantity of antibody is represented as percentage of positive control and measured at day 0 (inoculation of liver tumours), day 27 (rechallenge) and day 42 (sacrifice). Antibody production increases in all rats after systemic rechallenge, with no differences between treatment and control groups

Discussion

The results of this study indicate that a systemic anti-tumour immune response is generated or increased by local treatment with either RFA or PDT of experimental colorectal liver metastases, since rats that were not treated by local therapy developed either a de novo liver tumour upon local rechallenge or lung tumours upon systemic rechallenge.

At this moment, only one study on the effect of RFA on the immune system has been published, showing the presence of circulating tumour specific T cells after RFA treatment of VX2 hepatoma in 11 rabbits as well as increased T cell infiltration in tumour margins after RFA[27]. A relation between heat-based local ablative therapy and the immune system is also shown in studies concerning laser induced thermotherapy (LITT)[28,29]. In interstitial LITT a laser generates local energy, resulting in 46°C heat in the tumour and, consequently, tumour destruction. As RFA also destructs tissue by heat generation, albeit by higher temp-

eratures, treatments are to some extent comparable. Both LITT studies showed that LITT of liver tumours in rats decreased the occurrence of intraperitoneal tumour spread[28,29]. In our study we found that effective treatment with RFA or PDT of one out of two liver tumours did not affect growth of the untreated tumour. The LITT study by Isbert *et al.* is similar in design and rat tumour model to our study but showed results that are only partly in accordance with our findings[28]. In this study, treatment of one out of two CC531 liver tumours by LITT did inhibit growth of the nearby, untreated tumour as well as reduce macroscopic peritoneal tumour spread at 21 days after treatment. As we sacrificed rats not at 21 but at 27 days after treatment, this could explain the different outcome of our studies since the growth inhibiting effect of LITT is possibly partly due to systemically active growth factors and may therefore be only temporary. For PDT, we also found that PDT of one out of two liver tumours did not affect growth of the established, untreated tumour, whereas development of a de novo liver tumour or lung tumours after administration of tumour cells was effectively inhibited. An earlier study by our group confirmed the absent effect of PDT on the growth of established tumours. In this study, we also treated one out of two CC531 liver tumours with PDT and found that however effective, this treatment did not affect growth of nearby, untreated tumours[23], nor was there influx of macrophages or lymphocytes in these tumours.

Both RFA and PDT of liver tumours increased resistance to local or systemic administration of tumour cells in suspension, but did not affect established tumours. A previous study by us already showed that a systemic anti-CC531 response did prevent outgrowth of lung metastases upon intravenous CC531 administration but did not affect growth of established liver tumours[30]. A possible explanation for this lack of response against established tumours in these studies is the existence of a protective tumour structure. When CC531 tumour cells settle in tissue, they develop a tumour structure in which tumour nodules with epithelial cells grow separately from tumour stroma, and both compartments are divided by a basal membrane like structure. This structure may prevent immune cells from reaching the epithelial compartment, thus prohibiting the development of an effective anti-tumour response. This hypothesis is supported by findings that cells of the immune system scarcely infiltrate the tumour epithelial cell compartment but remain enclosed in the tumour stroma[31]. Local treatment of liver tumours may disrupt this structure and enable contact between tumour cells and cells of the immune system, inducing or increasing a systemic tumour specific immune response by "in situ" vaccination. This may involve a cellular rather than a humoral immune response, as increased production of anti-CC531 antibodies was correlated in this study not with local treatment but with systemic exposure to a large quantity of tumour cells.

Treatment of liver metastases by HAI with melphalan was very effective, as no liver tumours remained at all. After tumour cell administration, no de novo liver tumours could be induced, nor lung tumours

developed. Taking into account the (transient) immunosuppressive effect of an invasive procedure like HAI, rats showed impressive resistance to both systemic and local rechallenge in these rats, indicating a significant immunostimulatory effect of this treatment. Possibly, this is due to the very effective destruction of tumour tissue, with no tumour present at all after treatment. Tumour antigens may circulate systemically in large quantities over a longer period of time, as destruction is not as acute as in RFA and PDT. Thus, an adequate immune response may develop upon this antigen exposition.

Local therapies are being improved continually, as their broad applicability can provide a considerable contribution to current treatment strategies. Induction or enhancement of a tumour specific systemic immune response by local therapies may further improve results with these therapies. With the use of immunoadjuvants, an immune response could be enhanced, possibly resulting in a beneficial effect on recurrence, metastases and survival. Further research into both the effect of local therapies for colorectal liver metastases in a clinical setting and into the possibilities of using immunoadjuvants in in vivo experiments seems therefore indispensable.

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