Local ablative therapies for colorectal liver metastases and the immune system

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Chapter 2

Systemic immune response and established metastases

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Introduction

Although the immune system is often activated in patients with malignancies, this mostly does not lead to an effective antitumour immune response[1]. Moreover, enhancement or induction of a tumour specific immune response by immunotherapy rarely results in tumour rejection or significant increases in survival rates [2-4]. Possible explanations are related to the immune system itself, such as insufficient levels of responsive T cells or insufficient avidity[5] of these immune cells for tumour cells[6]. In addition, the tumour may hinder rejection by expression of inhibitory cytokines like tumour growth factor β or lack of expression of necessary co-stimulatory molecules or human leukocyte antigens[7-9]. Another explanation could be that extensive extracellular matrix surrounding tumour cells from epithelial origin prevents sufficient contact between tumour cells and immune cells[10,11]. Circulating tumour cells, however, do not have this defence mechanism and can therefore be recognized by the immune system. As a result, lodging and proliferation of these cells is prevented. This is supported by studies concerning the occurrence of metastases in relation to a systemic immune response, showing that an immune response can prevent the formation of distant metastases[12,13]. In this study, we assessed the effect of a systemic immune response on both circulating tumour cells and established tumours. For our experiments, we used a rat liver and lung tumours model with the syngeneic colorectal cell line CC531 because of our extensive experience with this model and the immunogenicity of the CC531 cell line[14,15]. By first inducing CC531 liver tumours, that induces an anti-CC531 response, and subsequently exposing the rats to CC531 tumour cells intravenously, we could evaluate the effect of an anti-CC531 immune response on both circulating and established tumours.

By suppressing the immune system at time of inoculation of primary liver tumours, the induction of an antitumour immune response during the time the tumour needed to establish itself could be prevented and thus enabled differentiation between the effect of a systemic immune response on the primary tumour and on the subsequent intravenous injection of tumour cells.

Materials and methods

Animals

Male Wag/Rij rats weighing approximately 250 grams rats were used (Charles River, Zeist, The Netherlands). The animals had free access to food and water. The animals received care in accordance with established guidelines. 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

We used the colon adenocarcinoma cell line CC531 (1,2-dimethylhydrazine-induced) which is moderately differentiated and syngeneic to Wag/Rij rats [10]. Briefly, tumour cells were cultured in RPMI 1640 supplemented with 2mM L-glutamine (Gibco, Grand Island, NY, USA), 10% heat inactivated calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin sulphate (complete medium). 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 1×10^6 viable (trypan blue exclusion test) tumour cells per ml PBS. For liver tumour induction, 5×10^4 viable tumour cells (in 50 µl suspension) per site were injected subcapsulary into the liver at four sites.
To induce lung tumours, 4 million CC531 tumour cells were injected in the penile vein, in a 200 µl suspension containing 2×10^7 cells/ml.

Study design

Wag/Rij rats were randomly assigned to one of the following groups: (1) liver tumours + immune suppression + i.v. CC531 tumour cells, (2) immune suppression + i.v. CC531 tumour cells, (3) liver tumours + i.v. CC531 tumour cells, (4) i.v. CC531 tumour cells only, (5) liver tumours only or (6) neither liver tumours, immune suppression nor i.v. CC531 tumour cells. Each group contained a minimum of three evaluable rats. Tumours were inoculated in the liver at day 0. Immune suppression was achieved by intraperitoneal (i.p.) administration of 60 mg/kg cyclophosphamide for five consecutive days, starting the day before liver tumour inoculation. Rats received CC531 cells intravenously as described above at 13 days after liver tumour inoculation and were sacrificed at day 37. To confirm the obtained results, we repeated part of the experiment (without immune suppression, i.e. groups 3 to 6) with a minimum of 4 evaluable rats in each group. Blood samples were taken from all rats by orbital punction at time of inoculation and i.v. CC531 tumour cell administration or by aortal punction at time of sacrifice. If present, liver tumours were separately enucleated from the surrounding liver parenchyma and weighed. To macroscopically visualize lung tumours, 15 ml of a 15% black 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)[16]. After 24 hours, Fekete's solution was replaced by water.

Detection of anti-CC531 antibodies
Blood samples were centrifuged for 20 minutes at 5000 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 in 1:30 diluted sera from all rats by flowcytometry analysis. Briefly, CC531 tumour cells were harvested from culture and washed with PBS with 0.5% BSA w/v (PBS/BSA). Of each 1:30 diluted serum sample, 100 µl was added to the 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 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 flowcytometer (FACScalibur, Becton Dickinson Immunocytometry, San Jose, CA, USA). As a positive control serum from an intravenously CC531 boosted rat was used, that contained a high amount of antibodies. This sample was used in all flowcytometry experiments as an internal standard. 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.

Results

In this study, we investigated the presence of a systemic immune response after CC531 liver tumour inoculation and assessed its effect on both circulating tumour cells and solid tumours. Subcapsulary injection of CC531 tumour cells in the liver resulted in reproducible tumours, as no significant difference was seen in liver tumour weight between groups from two independently performed experiments (figure 1).

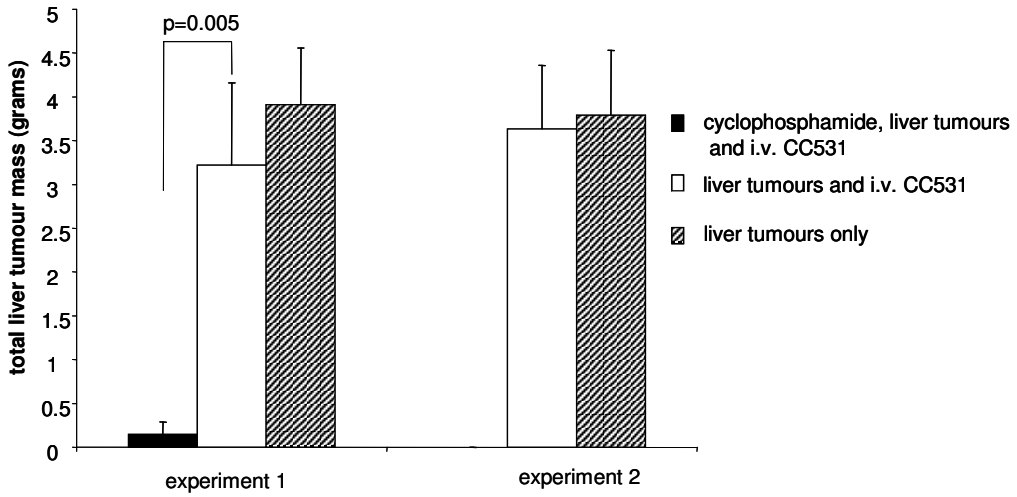

Figure 1. Total liver tumour mass (grams) in rats from experiment 1 and experiment 2. Liver tumours were inoculated by subcapsular injection of 5 x 10^5 viable tumour cells at 4 sites subcapsulary into the liver. At day 37 after inoculation tumours were removed from all rats and weighed. The added weight of all 4 liver tumours was used for statistical analysis. Total liver tumour mass in immune suppressed rats was significantly lower than rats that did not receive immune suppression (0.15 ± 0.14 grams in cyclophosphamide rats vs. 3.22 ± 0.94 grams in non-immune suppressed rats with liver tumours and rechallenge, p = 0.005), whereas there was no difference in liver tumour mass in rats with and without subsequent rechallenge

To detect the presence and effectiveness of a systemic anti-CC531 immune response, the ability to form lung tumours upon intravenous CC531 tumour cell injection was assessed as well as the level of anti-CC531 antibodies in the serum. I.v. administration of CC531 tumour cells in tumour free, non-immune suppressed rats led to formation of several lung tumours (figure 2a, table 1). All rats that did not receive i.v. CC531 tumour cells did not have lung tumours (table 1), indicating that i.v. injection of CC531 tumour cells was necessary for outgrowth of lung tumours. The presence of anti-CC531 antibodies, as detected by a flow cytometric assay, also showed a correlation with i.v. injection of CC531 tumour cells.

Anti-CC531 antibodies could only be detected if i.v. CC531 cells were administered, without this i.v. CC531 exposure no detectable antibodies were seen (figures 3 and 4).

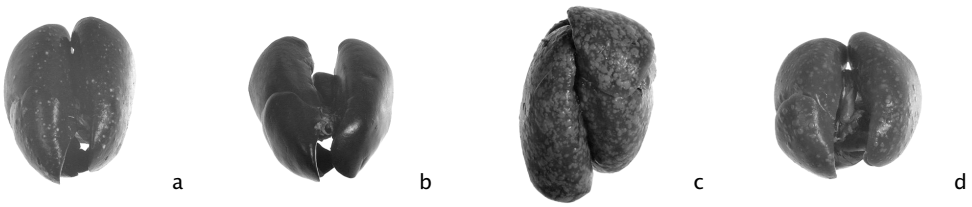

Figure 2. Lungs removed from rats at day 37 after tumour inoculation, i.e. 25 days after i.v. administration of 4.0 x 10^6 CC531 tumour cells. Fig. 2a shows lungs from a rat with i.v. CC531 cells only, with several metastases. Rats with liver tumours and rechallenge did not develop lung tumours (fig 2b), unless cyclophosphamide was administered at time of liver tumour inoculation (fig 2c). Rats with cyclophosphamide and i.v. CC531 cells had slightly less metastases (fig 2d)

Although i.v. CC531 administration alone was sufficient to induce detectable levels of anti-CC531 antibodies, significantly more antibodies were detected if liver tumours were present at time of i.v. CC531 cell administration (82% ± 17% of the level of the positive control vs. 49% ± 13% in exp 1, p=0.004 and 69% ± 11% vs. 38% ± 12% in exp 2, p=0.01; figure 3, table 1). These results indicated that CC531 tumour cells injected subcapsulary in the liver had interacted with the immune system resulting in CC531 specific B cells, enabling boosted antibody production upon i.v. administration. This increased specific anti-CC531 activity of the immune system was reflected by the absence of lung tumours in the rats that were i.v. injected with CC531 cells after liver tumour inoculation (figure 2b).

In contrast, the non-liver tumour bearing rats did develop lung tumours upon i.v. CC531 administration, showing that the boosted immune response upon liver tumour inoculation was able to prevent outgrowth of i.v. CC531 tumour cells into lung tumours. Despite this apparent anti-CC531 effect of the boosted immune system on i.v. injected CC531 cells, the weight of liver tumours did not significantly differ between liver tumour bearing rats with and without subsequent i.v. CC531 administration (figure 1), indicating that a systemic anti-tumour immune response could not affect liver tumour growth.

Group		*n* rats	Lung tumours	Anti CC531 antibodies
exp 1: cyclophosphamide	*liver tumours + i.v. CC531*	4	++	1.7 ± 0.4%
	i.v. CC531	4	++	2.1 ± 1.9%
exp 1: no cyclophosphamide	*liver tumours + i.v. CC531*	4	absent	82 ± 17%
	liver tumours	4	absent	3.5 ± 0.1%
	i.v. CC531	4	+	49 ± 13%
	none	3	absent	1.3 ± 0.2%
exp 2: no cyclophosphamide	*liver tumours + i.v. CC531*	5	absent	69 ± 11%
	liver tumours	5	absent	2.0 ± 2.9%
	i.v. CC531	5	+	38 ± 12%
	none	4	absent	0.1 ± 0.3%

Table 1. Presence of lung tumours and production of anti-CC531 antibodies in groups from experiment 1 (with immune suppression) and experiment 2 (without immune suppression). Presence of lung tumours is indicated by 'absent' (no lung tumours present), '+' (1-200 lung tumours present) or '++' (>200 lung tumours present). Level of antibodies is indicated as % mean fluorescence intensity of positive control, mean value of the number of rats indicated

Administration of cyclophosphamide during establishment of the liver tumours prevented the induction of an anti-CC531 immune response upon subcapsular injection of CC531 cells in the liver. This was shown by the appearance of numerous lung tumours and the absence of detectable anti-CC531 antibodies, regardless of the existence of primary liver tumours (figure 2c and 2d, table 1). Total liver tumour weight in these immune suppressed rats was significantly lower when compared to groups that did not receive cyclophosphamide ($p=0.005$, figure 1), presumably because cyclophosphamide did not only suppress the immune system but also acted as a cytostatic agent for the CC531 tumour cells.

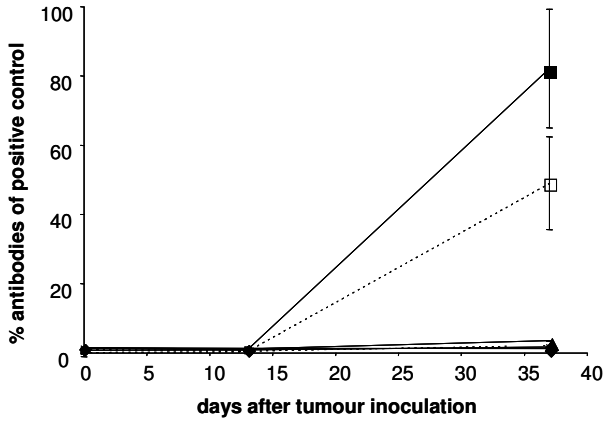

Figure 3. Amount of IgG antibodies directed against CC531 tumour cells in rats from experiment 1 (with cyclophosphamide). Quantity of antibody is represented as percentage of positive control and measured at day 0 (inoculation of liver tumours), day 13 (rechallenge) and day 37 (sacrifice). Antibody production was highest in rats without immune suppression with liver tumours and rechallenge (■; 82 ± 17 % of positive control). Antibody production was also detectable in non-immune suppressed rats with i.v. CC531 cells only (□; 49 ± 13%), with a significant difference between these positive groups (p = 0.023). Rats without immune suppression but with liver tumours only (▲) did not produce any antibody, nor did rats with cyclophosphamide and liver tumours with rechallenge (×), rats with cyclophosphamide and i.v. CC531 cells only (◊) or rats without any tumour or treatment (♦)

Discussion

Our study shows that a systemic antitumour immune response may not affect established solid tumours. The presence of antibodies to CC531 tumour cells indicated that a specific systemic immune response was present after tumour inoculation and rechallenge. While this immune response did not affect the growth of established liver tumours, it effectively prevented the formation of lung tumours upon rechallenge. The existence of this immune response is confirmed by the increased number of lung tumours when the immune

system was suppressed with cyclophosphamide during initial tumour inoculation. A ^{51}Cr release assay and apoptosis assay using T cells isolated from spleens of immune responsive rats in our experiments did not show anti-CC531 specific T cells (data not shown). This could indicate that only antibodies and no effector T cells were induced in CC531 injected rats, but it is also possible that the frequency of CC531 specific T cells was too low to detect in these assays.

It should be noted that the administration of cyclophosphamide did not only result in immune suppression, but also had a cytostatic effect on the inoculation of liver tumours. Cyclophosphamide is used both as an immune suppressant, leading to increased tumour growth and tolerance for xenografts[17] and as a cytostatic drug, resulting in tumour reduction[18]. The balance between these paradoxical effects is influenced by dosage, duration and time of administration[18,19]. In this study, we aimed to induce an immune suppressive effect, by administering high doses of cyclophosphamide before tumour inoculation. Apparently, the cytostatic effect exceeded this immune suppression, as development of liver tumours was significantly impaired. Two weeks later, at i.v. rechallenge, this balance was reversed in favour of the immune suppressive effect, as shown by the appearance of numerous lung tumours.

In our experiments, the systemic anti-CC531 immune response induced by induction of liver tumours was apparently strong enough to prevent circulating tumour cells from forming lung tumours but did not affect the already established liver tumours. These findings are in line with the disappointing reports on the application of tumour vaccination[2-4]. Apparently, activation of the immune system with either viable tumour cells or otherwise modulated tumour antigen can result in a systemic immune response, but this response mostly does not lead to a decrease in primary tumour mass or increase of survival. Possibly cells of the immune system cannot reach tumour cells as they are embedded in a solid tumour structure with a vast extracellular matrix surrounding the tumour nodules[10,11]. Also, the established tumour may express certain inhibitory cytokines or modulate expression of necessary co-stimulatory molecules while the single tumour cells did not develop these defence mechanisms yet[7-9]. There are several studies that do show an effect of the immune system on established tumours, but only when the immune response is enhanced by immune modulators such as interleukin-2[20-22], interleukin-12[12,23,23,24], tumour necrosis factor α[25,26], interferon α[27,28], interferon β[29] or granulocyte macrophage colony stimulating factor[20,30,31]. Apparently, the immune system is in fact capable of destroying established tumour cells, but only when the immune response is artificially enhanced. This strengthens the hypothesis that the capability of the immune system to destroy established tumour nodules is dependent on the quantitative presence of the immune response. This theory is also supported by the work of Perez-Diez *et al.*, who found that rejection of CT26.CL25 coloncarcinoma in mice was dependent on the quantity of the immune response, as measured by real-time quantitative reverse

transcriptase polymerase chain reaction[32]. These findings are congruent with the existence of a protective extracellular matrix surrounding established tumour cells, since a systemic immune response, like in our experiments, is often capable of killing circulating single tumour cells but not able to reject encapsulated tumour nodules.

Clinical studies usually evaluate the occurrence of a specific antitumour immune response by assessing the presence of cytotoxic T cells. Our results showed that the presence of a systemic antitumour immune response that effectively inhibits formation of lung tumours upon intravenous rechallenge was not sufficient to affect established tumours. Therefore, mere presence of specific T cells may not be sufficient to induce tumour rejection of established tumours. Further modulation of the immune system seems necessary to induce an immune response capable of rejecting established tumours.

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