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Radioimmunotherapy in an Immunocompetent Tumor Model utilizing a 177Lu-mAb

Elgström, Erika

2015

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Elgström, E. (2015). *Radioimmunotherapy in an Immunocompetent Tumor Model utilizing a 177Lu-mAb*. [Doctoral Thesis (compilation), Tumor microenvironment]. Oncology, Kamprad Lab.

Total number of authors:
1

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Radioimmunotherapy in an Immunocompetent Tumor Model utilizing a ^{177}Lu-mAb

Studies of Cell Death, Immune Response, and Metastases

Erika Elgström

LUND UNIVERSITY

DOCTORAL DISSERTATION
which, by due permission of the Faculty of Medicine, Lund University, Sweden, will be defended at Strålbehandlingshuset, Klinikgatan 5, Lund, on Thursday November 19th 2015, at 9.00 am.

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Document name
DOCTORAL DISSERTATION

Date of issue
2015-11-19

Sponsoring organization

Title and subtitle
Radioimmunotherapy in an Immunocompetent Tumor Model utilizing a ^{177}Lu-mAb
Studies of Cell Death, Immune Response, and Metastases

Abstract
Radioimmunotherapy (RIT) is a therapeutic strategy in which radionuclides are conjugated to monoclonal antibodies that bind to tumor-associated antigens in the tumor, so that the decay of the radionuclide takes place inside the tumor. The aim of the research presented in this thesis was to investigate the effects of RIT (utilizing the radioconjugated antibody ^{177}Lu-BR96) on an inoculated tumor and the development of metastases. The main emphasis was on the immune response after RIT, since it has been shown that radiation can lead to reprogramming of tolerogenic immune cells to become immunogenic (promote rejection of the tumor cells). Several studies have shown that tumors can reprogram the immune cells of the host to be tolerogenic instead of rejecting tumor cells. The studies described here were carried out in an immunocompetent syngeneic rat colon carcinoma model with the potential to develop distant metastases. Thus, the model simulates the clinical situation of metastatic disease.

Following RIT, the tumor cells of the inoculated tumor succumbed due to both caspase-3-dependent and caspase-3-independent cell death. Treatment with the unlabeled BR96 resulted only in caspase-3-independent cell death, indicating that unlabeled BR96 and ^{177}Lu-BR96 induce different cell death mechanisms. Other studies have shown that cell death in which activated caspase-3 is expressed might result in an immunogenic type of cell death, although apoptosis has previously been considered to be immunogenic silent, in contrast to necrosis. The evaluation of immune cell markers showed that markers related to immune rejection were expressed to a higher degree than immune cell markers related to immune tolerance, in both untreated tumors and tumors treated with RIT. Both T cells and macrophages were present in untreated tumors of this model and decreased during RIT. The depletion of CD8-positive cells did not result in any delay in the rejection of the inoculated tumor after administration of RIT.

The depletion of CD8-positive cells during RIT appeared to result in a higher frequency of animals developing metastases. This might indicate that RIT induces long-term immunity to the tumor cells. Metastases developing after RIT showed a reduced expression of the targeted antigen compared to untreated inoculated tumors in 17 of 23 metastases. However, none of the metastases or remaining primary tumors completely lacked expression of the targeted antigen, indicating the possibility of repeating RIT with BR96.

It was concluded that the mechanisms of cell death in tumors were different when using RIT than with the unlabeled antibody. Immune cells are present in this syngeneic tumor model, although CD8-positive cells are not mainly responsible for the rejection of the primary tumor. However, CD8-positive cells seemed to prevent the development of metastases. Some of the metastases that developed after RIT exhibited reduced targeted antigen expression, but none of the metastases or remaining tumors lacked targeted antigen expression completely.

Key words: Radioimmunotherapy, Immunology, Tumor microenvironment, Immune cells, Cell death, CD8, Antigen expression, Metastases, Syngeneic, Immunocompetent, Rat, Colon carcinoma

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language: English

ISSN and key title
1652-8220

ISBN
978-91-7619-163-7

Recipient's notes

Number of pages

Price

Security classification

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Studies of Cell Death, Immune Response, and Metastases

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LUND UNIVERSITY

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Faculty of Medicine, Department of Clinical Sciences, Lund
ISBN 978-91-7619-163-7
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2015

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List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I. Change in cell death markers during (177)Lu-mAb radioimmunotherapy-induced rejection of syngeneic rat colon carcinoma
Erika Elgström, Otto Ljungberg, Sophie E. Eriksson, Anders Örbom, Sven-Erik Strand, Tomas G. Ohlsson, Rune Nilsson, Jan Tennvall
Cancer Biotherapy and Radiopharmaceuticals, 2014, 29(4):143-52

II. Evaluation of immune cell markers in tumor tissue treated with radioimmunotherapy in an immunocompetent rat colon carcinoma model
Erika Elgström, Sophie E. Eriksson, Otto Ljungberg, Pär-Ola Bendahl, Tomas G. Ohlsson, Rune Nilsson and Jan Tennvall
EJNMMI Research, 2015, 5:47

III. Role of CD8-positive cells in radioimmunotherapy utilizing (177)Lu-mAbs in an immunocompetent rat colon carcinoma model
Erika Elgström, Sophie E. Eriksson, Tomas G. Ohlsson, Rune Nilsson, Jan Tennvall
EJNMMI Research, 2015, 5:3

IV. Pattern of antigen expression in metastases after radioimmunotherapy of a syngeneic rat colon carcinoma utilizing the BR96 antibody
Erika Elgström, Sophie E. Eriksson, Tomas G. Ohlsson, Jan Tennvall, Rune Nilsson
Experimental Hematology & Oncology, 2012, 13;1(1):34

Related publications

- Successful radioimmunotherapy of established syngeneic rat colon carcinoma with 211At-mAb
  Sophie E. Eriksson, Tom Bäck, Erika Elgström, Holger Jensen, Rune Nilsson, Sture Lindegren, Jan Tennvall
  EJNMMI Res. 2013, 4;3(1):23

- The intratumoral distribution of radiolabeled 177Lu-BR96 monoclonal antibodies changes in relation to tumor histology over time in a syngeneic rat colon carcinoma model
  Anders Örbom, Sophie E. Eriksson, Erika Elgström, Tomas G. Ohlsson, Rune Nilsson, Jan Tennvall, Sven-Erik Strand
  J Nucl Med. 2013, 54(8):1404-10

- Sequential radioimmunotherapy with 177Lu- and 211At-labeled monoclonal antibody BR96 in a syngeneic rat colon carcinoma model
  Sophie E. Eriksson, Erika Elgström, Tom Bäck, Tomas G. Ohlsson, Holger Jensen, Rune Nilsson, Sture Lindegren, Jan Tennvall
  Cancer Biother Radiopharm. 2014, 29(6):238-46

Thesis at a glance

Paper I

Aim: To investigate intratumoral cell death during radioimmunotherapy by evaluating fragmented DNA and the activation of caspase-3.
Findings: The analysis of cell death markers after treatment with ^{177}Lu-BR96 revealed that tumor cells succumbed due to both caspase-3-dependent and caspase-3-independent cell death. Treatment with unlabeled BR96 resulted in caspase-3-independent cell death only, indicating that unlabeled BR96 and ^{177}Lu-BR96 induce different cell death mechanisms.

Paper II

Aim: To investigate the intratumoral changes in the infiltration of immune cells during radioimmunotherapy by immunohistochemical staining of immune cell markers of T cells and macrophages.
Findings: Immune cell markers related to immune rejection were expressed to a higher degree than immune cell markers related to immune tolerance for both T cell and macrophage markers in both untreated tumors and tumors treated with ^{177}Lu-BR96. The immune cell markers demonstrated that T cells and macrophages were present in untreated tumors of this model and decreased during radio-immunotherapy.

Paper III

Aim: To study the effects of CD8-positive cells on the rejection of tumors and the establishment of metastases after treatment with ^{177}Lu-BR96.
Findings: The depletion of CD8-positive cells did not result in any delay in the rejection of tumors treated with ^{177}Lu-BR96. However, the initial depletion of CD8-positive cells seemed to result in a higher frequency of animals developing metastases. Thus, a long-term immunity to the tumor cells might have been induced in the rats not developing metastases.

Paper IV

Aim: To evaluate the antigen expression in metastases after treatment of the local tumor with ^{177}Lu-BR96 compared to untreated tumors.
Findings: The expression of the targeted antigen in metastases after radioimmunotherapy was reduced in 17 of 23 metastases. However, none of the metastases or remaining primary tumors completely lacked the targeted antigen expression, indicating the possibility of repeating radioimmunotherapy with BR96.

Abbreviations

BN	Brown Norway
CR	complete response
DAMP	damage-associated molecular patterns
DC	dendritic cell
DOTA	S-2-(4-isothiocyanatobenzyl)-1, 4, 7, 10-tetraazacyclododecane tetraacetic acid
FDA	the U.S. Food and Drug Administration
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IHC	immunohistochemical
IgG	immunoglobulin G
LeY	Lewis Y
LR	local recurrence
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
NK	natural killer
p.i.	post injection
RIT	radioimmunotherapy
ROS	reactive oxygen species
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

Abstract

Radioimmunotherapy (RIT) is a therapeutic strategy in which radionuclides are conjugated to monoclonal antibodies that bind to tumor-associated antigens in the tumor, so that the decay of the radionuclide takes place inside the tumor. The aim of the research presented in this thesis was to investigate the effects of RIT (utilizing the radioconjugated antibody ^{177}Lu-BR96) on an inoculated tumor and the development of metastases. The main emphasis was on the immune response after RIT, since it has been shown that radiation can lead to reprogramming of tolerogenic immune cells to become immunogenic (promote rejection of the tumor cells). Several studies have shown that tumors can reprogram the immune cells of the host to be tolerogenic instead of rejecting tumor cells. The studies described here were carried out in an immunocompetent syngeneic rat colon carcinoma model with the potential to develop distant metastases. Thus, the model simulates the clinical situation of metastatic disease.

Following RIT, the tumor cells of the inoculated tumor succumbed due to both caspase-3-dependent and caspase-3-independent cell death. Treatment with the unlabeled BR96 resulted only in caspase-3-independent cell death, indicating that unlabeled BR96 and ^{177}Lu-BR96 induce different cell death mechanisms. Other studies have shown that cell death in which activated caspase-3 is expressed might result in an immunogenic type of cell death, although apoptosis has previously been considered to be immunogenic silent, in contrast to necrosis. The evaluation of immune cell markers showed that markers related to immune rejection were expressed to a higher degree than immune cell markers related to immune tolerance, in both untreated tumors and tumors treated with RIT. Both T cells and macrophages were present in untreated tumors of this model and decreased during RIT. The depletion of CD8-positive cells did not result in any delay in the rejection of the inoculated tumor after administration of RIT.

The depletion of CD8-positive cells during RIT appeared to result in a higher frequency of animals developing metastases. This might indicate that RIT induces long-term immunity to the tumor cells. Metastases developing after RIT showed a reduced expression of the targeted antigen compared to untreated inoculated tumors in 17 of 23 metastases. However, none of the metastases or remaining primary tumors completely lacked expression of the targeted antigen, indicating the possibility of repeating RIT with BR96.

It was concluded that the mechanisms of cell death in tumors were different when using RIT than with the unlabeled antibody. Immune cells are present in this

syngeneic tumor model, although CD8-positive cells are not mainly responsible for the rejection of the primary tumor. However, CD8-positive cells seemed to prevent the development of metastases. Some of the metastases that developed after RIT exhibited reduced targeted antigen expression, but none of the metastases or remaining tumors lacked targeted antigen expression completely.

Introduction

Cancer is a common cause of death, and the incidence is generally increasing. The incidence of different types of cancer differs throughout the world, probably due to lifestyle and pre-existing risk factors (*e.g.* cancer-related infections, inherited mutations, and environmental factors) [1]. The main reason for cancer-related death is the development of distant metastases (disseminated disease) [2, 3].

The tumor is a complex structure consisting of both tumor cells and normal cells. Tumor cells interact with normal cells (*e.g.* endothelial cells, immune cells) to promote tumor growth [4-8]. Tumor cells are normal cells that evolve progressively to a neoplastic state. Hanahan and Weinberg [9] have defined ten hallmarks of tumors, including resistance to cell death, induction of angiogenesis, activation of invasion and metastasis, avoidance of immune destruction, and tumor-promoting inflammation.

The effects of radiation

Radiation is commonly used for the treatment of cancer. The effects of radiation depend on both the hallmarks of cancer and the radiobiology, often described as the 5Rs [10, 11], which are:

1. *Repair* of DNA damage in the cell after irradiation,
2. *Repopulation* of the cells remaining in the tumor,
3. *Redistribution* of cell cycle phases in the remaining cells (cells have different radiation sensitivity in the different phases of the cell cycle),
4. *Reoxygenation* radiation damages normoxic cells to a higher extent than hypoxic cells leading to reoxygenation of hypoxic cells, and
5. *Radiosensitivity* the sensitivity of different cells (both normal and tumor cells) to radiation differs, thus the dose must be adapted to the radiosensitivity of the target cells.

Radiation causes damage to cell DNA by direct effects such as formation of double or single strand breaks, as well as indirect effects *e.g.* by formation of reactive oxygen species (ROS). The damage can result in cell death by several mechanisms, *e.g.* apoptosis, necrosis, and mitotic catastrophe, depending on the absorbed dose and the properties of the irradiated cell [12, 13].

Radiation can induce damage in non-irradiated cells in the proximity of irradiated cells (bystander effect). It has been suggested that the bystander effect is induced by cytokines, nitric oxide, and/or ROS [14, 15]. The bystander effect may thus enhance the therapeutic response to radiation.

Effects of radiation on the immune system

In rare cases, therapeutic effects of external radiation have been observed on distant non-radiated tumor cells (abscopal effect). The explanation of this is, at least partly, the induction of the immune response [16-23]. However, immune cells are radiosensitive, and are affected to different degrees by radiation, for example, lymphocytes are more radiosensitive than macrophages [16, 24]. In the ideal case, radiation should be used to induce a systemic response to tumor cells, but more research is needed to determine how the abscopal effect can be elicited and exploited.

Radiation has the ability to activate an immune response by the induction of immunogenic cell death [25-28]. Immunogenic cell death is defined by three damage-associated molecular patterns [27, 29]. One of these is the cell surface translocation of calreticulin, normally expressed in the endoplasmic reticulum. The expression of calreticulin on cell surface is a potent “eat me” signal to the immune cells. The other two damage-associated molecular patterns is the extracellular release of high-mobility group protein box 1 and adenosine triphosphate which activate immune cells and act as a “find me” signal that attracts immune cells.

Cancer and the immune system

One of the hallmarks of cancer is tumor-promoting inflammation [9]. Chronic inflammation has been associated with the development of colon cancer, hepato-cellular carcinoma, and cervical cancer associated with human papilloma virus [30, 31]. Tumor cells can reprogram stromal cell functions for their own benefit, creating a microenvironment that supports tumor progression [31].

Immune cells can be either tumor tolerogenic or immunogenic, *e.g.* NK (natural killer) cells act to promote tumor rejection while regulatory T cells (T_{reg} cells) promote the growth of the tumor. Tumors also have the ability to reprogram immune cells, from immunogenic to tolerogeneic [32, 33]. This can be illustrated by the dual actions of T helper cells which can be divided into type 1 (T_{H1} cells) and type 2 (T_{H2} cells), where T_{H1} cells promote tumor rejection and T_{H2} cells promote tumor growth. Macrophages can similarly be divided into type 1 (M1) or 2 (M2), promoting tumor rejection or growth, respectively.

The concept of cancer immunoediting describes the balance between the immune system and tumor cells (during development and in established primary tumors and metastases), as is illustrated in Figure 1 [34, 35]. During tumor development the phases can change several times, the change can depend on *e.g.* tumor development or therapy. The goal of immunotherapy is to boost the immune response, or to remove whatever is limiting the immune response, in order to change the balance from tolerance (escape) to rejection (elimination).

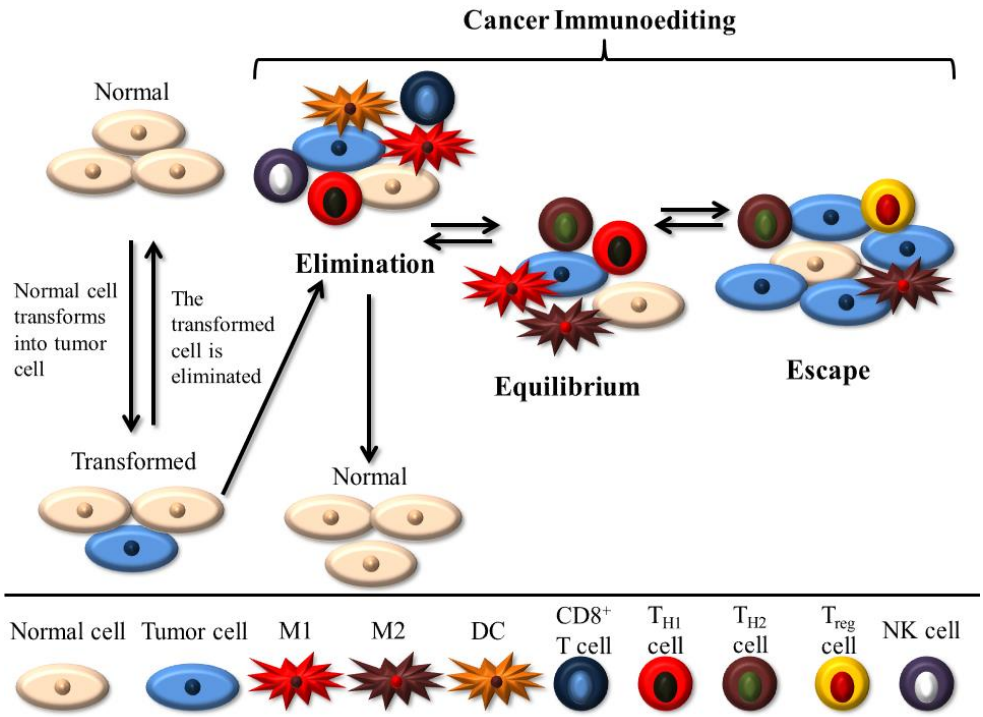

Figure 1. The concept of cancer immunoediting
Cancer immunoediting describes the balance between the immune system and tumor cells (during development and in established tumors), and consists of three phases. The first phase is elimination, in which the immune response is activated and the number of tumor cells decreases. Elimination can stop tumor development before it becomes clinically apparent. The second phase is equilibrium, in which the immune system prevents tumor growth, and the tumor volume is constant: a type of dormancy or stable disease. The third phase is escape, in which the tumor has acquired the ability to circumvent immune recognition and/or destruction and the tumor will grow and develop. The figure is adapted from [34-36].

M1: macrophage type 1, M2: macrophage type 2, DC: dendritic cell, $CD8^+$ T cell: cytotoxic T cell, T_{H1} cell: type I T helper cell, T_{H2} cell: type 2 T helper cell, T_{reg} cell: regulatory T cell, NK cell: natural killer cell.

The U.S. Food and Drug Administration (FDA) has recently approved immunotherapies for cancer treatment, such as immune checkpoint inhibitors *e.g.* CTLA-4 (Ipilimumab) and PD-1 (Nivolumab). These agents inhibit the inactivation of T cells which can induce an immune response to tumor cells and also have their own cytotoxic activity [37-40]. Another type of immunotherapy for cancer recently approved is a type of vaccination with autologous peripheral-blood mononuclear cells that have been activated *ex vivo* by a recombinant fusion protein associated with prostate cancer (Sipuleucel-T) [37, 40]. The optimal use of these new immunotherapies is still under investigation.

Radioimmunotherapy

Radioimmunotherapy (RIT) is a therapeutic strategy in which radionuclides are conjugated to monoclonal antibodies. The antibody binds to an antigen expressed in the tumor, and deposits the radionuclide in the tumor, were it decays and causing cell damage which might result in cell death. Two radioimmunoconjugates have been approved by the FDA, both targeting the antigen CD20 in the treatment of B-cell non-Hodgkin's lymphoma. The radioimmunoconjugates are Bexxar® (^{131}I-tositumomab) and Zevalin® (^{90}Y-ibritumomab), which are both based on mouse antibodies. The therapeutic response to Bexxar has been shown to be dependent on both the radiation (delivery of the radioisotope ^{131}I) and the antibody effects (antibody dependent cell-mediated cytotoxicity and the direct induction of apoptosis) [41, 42]. The effects of RIT on solid tumors are unfortunately limited. This may be because solid tumors are less radiosensitive than lymphomas, and the vasculature of solid tumors can result in limited uptake of the radioimmunoconjugate [43, 44].

The targeting effects of RIT are dependent on the antibody and its associated antigen. The specificity of the antibody should be high and the antigen should be as specific as possible to the tumor, *i.e.*, the antibody should bind mainly to the tumor but not to normal tissue, in order to achieve high efficacy and avoid toxicity. The stability of the antibody is also important to avoid early excretion due to degradation, resulting in low amounts of antibody reaching the tumor. The physical range (varying from µm to mm) and the half-life of the radionuclide (from hours to days) should be matched with the biodistribution of the radioimmmunoconjugate. In general, beta-emitting radionuclides have a longer range (than alpha-particle-emitting radionulides) resulting in the irradiation of not only the target cells, but also surrounding cells; this effect is called crossfire. The crossfire effect can compensate for heterogeneous distribution of the radionuclides in the tumor, but may also cause irradiation of normal tissue. Radionuclides emitting alpha particles have a shorter range and a higher linear energy transfer, which means that

they cause more ionization along their path through tissue. One of the side effects of RIT is damage to normal tissue. This may be a result of antigen expression in normal tissue, decay of the radionuclide outside the tumor, or due to crossfire. Critical organs, *i.e.*, the bone marrow, liver, lungs and kidneys are often exposed to radiation by the circulation or excretion.

RIT differs from external beam radiation in that the latter is administered at a high dose rate (*i.e.* a high radiation dose is given over a short period) and the tumor is often irradiated homogenously in repeated fractions. In RIT, the dose is administered at a low dose rate (the dose given during a long period, often days), and the dose is often heterogeneously distributed within the solid tumor. External beam radiation is generally directed towards the tumor and regional lymph nodes, while RIT is given systemically with the intention of targeting the tumor-associated antigens. Using RIT, it is possible to treat microscopic or diffuse tumors, providing the radioimmunoconjugate reaches the tumor, and the tumor cells (or adjacent cells) express the targeted antigen.

Aims of this work

The general aim of the work presented in this thesis was to investigate the effects of RIT (using ^{177}Lu-BR96) on an inoculated tumor and the development of metastases in an immunocompetent rat colon carcinoma model. The specific aims were:

- to study the intratumoral changes during RIT, by evaluating cell death (Paper I) and markers of T cells and macrophages (Paper II),
- to investigate the effects of CD8-positive cells on the rejection of the tumor and the establishment of metastases during RIT by the depletion of CD8-positive cells prior to RIT (Paper III), and
- to evaluate antigen expression in metastases after treatment of the local tumor with ^{177}Lu-BR96 (Paper IV).

The tumor model

The tumor model used in this work was a syngeneic immunocompetent rat colon carcinoma model, meaning that the cell line used was established from an animal of the same strain as used in the studies. The animals have an intact immune system and develop tumors after inoculation, without the tumor cells being recognized as foreign. The tumor model enables the tumor cells to interact with the cells of the host, *e.g.* the immune cells and stromal cells. Xenograft models, in contrast, are usually based on human tumor cells injected into animals that must be immunocompromized in order for tumors to develop after inoculation of tumor cells.

The therapeutic antibody, BR96

The chimeric (mouse/human) monoclonal IgG1 antibody BR96 (Seattle Genetics Inc., Seattle, WA, USA) was first developed in a murine form by the immunization of mice with a human cell line from metastatic breast adenocarcinoma [45]. The chimeric mouse/human antibody used in this work was produced by conversion of the hybridoma cell line [46].

BR96 binds to the Lewis Y (LeY) antigen (also known as CD174), which is a blood-group-related antigen expressed on the cell membrane [47]. The LeY antigen is expressed on several human carcinomas, *e.g.* breast, colon, lung, and ovary [45], and it has been suggested that increased expression of LeY is associated with increased cell motility [47].

As with the majority of cancer-associated antigens, LeY is also expressed in normal tissue, mainly epithelial cells from the gastrointestinal tract [45] and in some hematopoietic progenitor cells [48]. The binding affinity between BR96 and the cell line used is high; the dissociation constant has been determined to be 4 nM [49].

Radioimmunoconjugation

The radionuclide used in these studies was ^{177}Lu, which is considered a suitable candidate for RIT. ^{177}Lu is a beta emitter and has a physical half-life of 6.7 days and a maximal range in tissue of 1.8 mm. The range can compensate to some degree for heterogeneous tumor uptake of the radioimmunoconjugate by the crossfire effect (cell diameter approximately 10 µm).

The radiochemical method used to prepare radioimmunoconjugates is chosen based on the physical properties of the radionuclide. Lutetium is a radiometal and requires a chelate molecule (in this case, DOTA, S-2-(4-isothiocyanatobenzyl)-1, 4, 7, 10-tetraazacyclododecane tetraacetic acid) to be conjugated to the antibody. Conjugation of BR96 and DOTA was performed as described by Forrer *et al.* [50], and human serum albumin was added to prevent radiolysis. The conjugation method is described in more detail in the papers.

Three different conjugates were used in this work. The batches contained on average 2.4 (Papers I and II), 2.6 (Paper III), and 2.3 (Paper IV) DOTA moieties per BR96, and had an immunoreactivity (given by the ratio: K_d[BR96]/K_d[DOTA-BR96]) of 0.9 (Papers I, II, and IV) and 0.8 (Paper III). The specific activities (MBq/mg antibody) and radiochemical purity (% radioactivity bound to the antibody) are reported in the papers.

The cell line

The BN7005-H1D2 cell line originates from a colon carcinoma induced by 1,2-dimethyl-hydrazine in a Brown Norway (BN) rat [51]. The H1D2 clone was established after limiting dilution of BN7005-H1D2 in the absence of selection pressure [52]. The cell line has a short doubling time during exponential growth *in vitro*, of approximately 10 h. The radiosensitivity of BN7005-H1D2 expressed as the fraction of survival after exposure to 2 Gy has been determined to be 0.5 (^{137}Cs radiation source) [53]. This is similar to the radiosensitivity of human colorectal carcinoma cell lines [54].

The cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 10 mM HEPES buffer, and 14 mg/L gentamicin at 37°C in a humidified environment containing 5% CO_2. The cells were washed with phosphate-buffered saline and detached by treatment with trypsin. Cells in the exponential growth phase were used in all experiments.

The expression of BR96-binding antigen was evaluated *in vitro* by limiting dilution. The BN7005-H1D2 cells were diluted to a concentration of 0.5 cells/well and added to six 96-well plates. After culturing for 6-7 days the cell colonies were detected by microscopic examination. The plates were labelled with BR96 (0.5

µg/well) and the detection antibody (Anti-human IgG H+L donkey $F(ab)_2$ HRP, Jackson ImmunoResearch Laboratories), and the colonies expressing BR96-binding antigen were stained with diaminobenzidine (Dako). All 105 colonies expressed BR96-binding antigen.

The rats

Male BN rats were used in all studies (Harlan Laboratories Inc.). BR96-binding antigen is expressed in some normal tissues in these rats, mainly in the epithelium of the gastrointestinal tract [55]. Thus, the BR96 antibodies are tumor selective rather than tumor specific in this tumor model, illustrating the clinical situation.

The animals were housed under standard conditions, with fresh water and standard pellets *ad libitum*. Animals were sacrificed with an overdose of isoflurane and heart puncture when tumor growth reached the maximal permitted size (20 x 20 mm), or if their general health was affected (signs of metastatic disease or severe weight loss, >15% of normal body weight), or at the end of the study. All experiments were conducted in compliance with Swedish legislation on animal protection, and were approved by the Regional Ethics Committee on Animal Experiments.

The tumor

Inoculation with the tumor cell suspension was performed 12-14 days before injection of the radioimmunoconjugate. At the time of treatment the tumors were established and were approximately 1 cm in diameter.

Inoculation

Tumor cells were injected between the peritoneum and the abdominal muscle (sub-peritoneal). This was done by cutting the skin and abdominal muscle, along linea alba (maximum 2 cm). Homeostatic forceps were used to lift the abdominal muscle and the needle was placed beneath the peritoneum, and the cell suspension was injected (3×10^5 cells in 0.05 mL). As the syringe was removed, the cells were prevented from leaking out by applying pressure to the needle track with tweezers. The muscle (continuous locking) and skin (interrupted stitches) were then closed with sutures. This invasive procedure was performed under aseptic conditions, requiring general anesthesia with isoflurane and analgesia with burprenorphine.

Characterization

The tumors became established and grew rapidly, leading to a solid tumor of approximately 1 cm in diameter within two weeks after inoculation (*i.e.* the time for treatment). The tumors could be easily palpated and measured with a digital caliper. The tumor volumes were calculated as: length x width^2 x 0.4.

The histology of the tumors is poorly differentiated and consists of dense tumor cell growth with infiltrating granulation tissue, often with necrotic regions of various sizes. This differs from the tumors arising from subcutaneous inoculation of the same cell line, which generally have a large necrotic core and limited granulation infiltration. The structures of subcutaneous and sub-peritoneal tumors, and areas of tumor cells are illustrated in Figure 2. The presence of granulation tissue and vascular interaction, and the ability to form metastases makes this syngeneic sub-peritoneal tumor model more relevant than many subcutaneous xenograft models, which often lack these properties [56, 57].

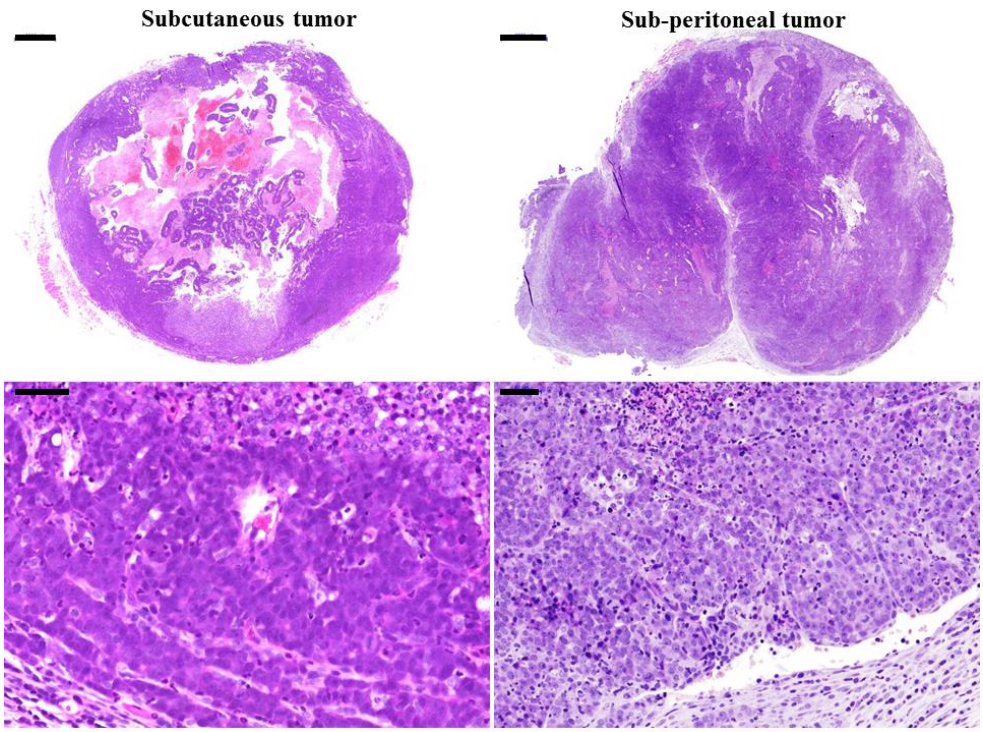

Figure 2. Histological structures of subcutaneous (*left*) and sub-peritoneal (*right*) tumors
Above: Section of the entire tumor, scale bars: 1 mm *Below*: Areas of tumor cells at the capsule, scale bars: 50 µm.

Antigen expression is of major importance in treatment with antibodies. The expression of BR96-binding antigen in tumors was visualized using immunohistochemical (IHC) staining. Both snap-frozen and paraffin-embedded untreated tumors showed complete membranous staining of apparently all tumor cells.

Radioimmunotherapy with ^{177}Lu-BR96

In all the *in vivo* studies described in this thesis the injected activity was 100 MBq/animal, corresponding to approximately 400 MBq/kg body weight. This activity normally results in the rejection of the majority of tumors within two weeks. The volume of the injected radioimmunoconjugate was 0.4 mL, and the amount of BR96 was adjusted to 150 µg per animal, resulting in approximately 0.6 mg BR96/kg body weight. The radioimmunoconjugates were administered in the tail vein using a cannula. The activity in the syringes was measured before and after injection to calculate the injected activity.

Biodistribution and intratumoral distribution

The biodistribution and distribution of the radioimmuneconjugate within the tumor are important as they influence the therapeutic effect and the risk of toxicity. The maximal tumor uptake was approximately 8% of the injected activity per g tumor tissue and was reached 24-48 h after injection of the radioimmunoconjugate. The determination of maximal tumor uptake is, however, uncertain due to the decrease in tumor volume [58]. The maximal activity in normal tissue (the blood-rich organs such as the liver and kidneys) reached approximately 2% of injected activity per g tissue 2 h post-injection (p.i.) and then decreased. The activity in normal organs does not seem to be related to antigen expression [53].

The uptake of the radioimmunoconjugate by the tumor depends on the properties of the tumor (*e.g.* tumor volume, vessel permeability, and extracellular matrix composition) and the drug (*e.g.* size, affinity, and dose) [59-61]. When injecting 400 MBq/kg body weight we found a heterogeneous distribution within the tumor, where areas of high activity were correlated to low tumor cell density 24 h p.i. (Paper I). The uptake was monitored for 8 days p.i. and the activity became more homogeneous as the number of viable tumor cells decreased, but the high-activity areas were still correlated to tumor cells with low viability. In a previous study using 25 and 50 MBq/kg, we showed that during the first 24 h p.i. the activity was correlated to viable antigen-expressing tumor tissue [58]. Later in that study, the activity was correlated less with viable antigen-expressing tumor tissue and more with granulation tissue, probably due to the therapeutic effects on the originally targeted tumor cells [58]. The difference in the findings 24 h p.i. in these

two studies probably due to the greater therapeutic response resulting from the administration of 8-16 times higher activity (Paper I), although lower activities also resulted in some therapeutic response.

Therapeutic effects

The maximal tolerable activity of ^{177}Lu-BR96 in this model has been determined to be 600 MBq/kg body weight [62]. Maximal tolerable activity is defined as the highest injected activity allowing 100% survival without any signs of infection, bleeding, or diarrhea, and with <20% body weight loss. In order to reduce the toxicity, the minimal effective activity of ^{177}Lu-BR96 was determined, and found to be 400 MBq/kg body weight in this model [49]. The minimal effective dose was defined as the activity resulting in complete response (CR) of the inoculated tumor in 5 out of 6 rats. Repeated studies have shown that this activity results in metastatic development in about half of the animals, between 50 and 100 days p.i. [49, 63]. When administering the maximal tolerable activity half of the animals develop metastatic disease [62], thus the administered activity does not have a major effect on the fraction of animals developing metastatic disease.

Observed immunological effects in the tumor model

The therapeutic response to RIT observed in this tumor model is greater than that observed in other models. One reason for this could be that our model is syngeneic rather than xenogeneic. Previous studies by our group using this model have indicated that the immune response could be activated.

Untreated tumors show dense tumor cell growth, while tumors from animals treated with 1 or 10 mg unlabeled BR96/kg body weight showed an increased fraction of granulation tissue and necrosis. The administration of unlabeled BR96 thus results in histological changes [58]. Furthermore, the administration of 15 mg/kg body weight BR96 alone has been found to induce transient CR in four of six rats using this model [64]. However, all the animals had to be sacrificed within 80 days p.i. due to local recurrence (three before day 45 p.i.), or metastatic disease (one, day 77 p.i.) [64]. A dose of 0.6 mg unlabeled BR96/kg body weight has also been found to induce CR in one animal, which showed no local recurrence or detectable metastases 100 days after injection [65]. It has also previously been indicated that murine BR96 has direct cytotoxic effects, and can induce antibody-dependent cell-mediated cytotoxicity, and complement-dependent cytotoxicity [45, 66].

Cyclosporine A has been given to prevent the development of rat anti-human antibodies after RIT by inhibition of T-cell activation, in order to enable repeated

administration of BR96 [67-72]. Treatment with cyclosporine A and RIT in the present model prolonged the time to CR compared with animals treated with RIT only, but the fraction of animals exhibiting CR was similar [63]. Neither the time to metastatic disease nor the fraction of animals developing metastatic disease was affected by cyclosporine A [63]. This finding shows that cyclosporine A affects the rejection of the tumors during treatment with ^{177}Lu-BR96, indicating that T cells might be involved in the rejection process.

A previous study indicated that intrahepatic tumors of BN7005-H1D2 resulted in systemic suppression of the anti-tumor immune response in rats carrying established tumors [52]. The systemic suppression of the anti-tumor immune response was detected by a second injection of tumor cells expressing IL-18 (pro-inflammatory cytokine).

Intratumoral effects in tumors during radioimmunotherapy

Tumors are complex structures consisting of cellular and non-cellular components such as tumor cells (different cell clones), the extracellular matrix, and stromal cells such as endothelial cells, immune cells, pericytes, adipocytes, and fibroblasts [4-8]. The microenvironment of the tumor promotes tumor growth by the secretion of paracrine stimulatory factors, angiogenesis, and immune mediated interactions [5]. However, tumors are often hypoxic and have a high intratumoral pressure, at least partially, due to leaky and dysfunctional vasculature, and a lack of lymphatic drainage, which may limit tumor growth [6].

One of the difficulties in treating tumors is to reach all the tumor cells, as tumors consist of different cell clones with different gene mutations. Two theories have been proposed to describe the establishment of different tumor cell clones. The first is clonal evolution, in which genetic instability within the tumor cell population leads to the accumulation of additional mutations within single cells. The other is the stem cell hypothesis, in which only cancer stem cells can participate in clonal evolution and drive tumor progression, while other cells are evolutionary dead ends [5].

It is possible to study the effects of various agents on one cell type or the interactions between few cell types using *in vitro* studies. However, *in vivo* studies make it possible to evaluate the complex interactions within the tumor.

An important aspect that should be borne in mind during the evaluation of the effects on the tumor when using the present model is that the activity is not homogeneously distributed within the tumor during RIT, Paper I.

Histopathological evaluation – Paper I

The tumors evaluated in this study were excised 1-8 days after injection of 400 MBq/kg body weight ^{177}Lu-BR96. Tumors from untreated animals served as control. All tumors were individually evaluated regarding the proportion of viable tumor cells, necrotic cells, granulation tissue, fibrous tissue, and the infiltration of granulocytes and lymphocytes.

The fraction of viable tumor cells decreased continuously from 1 day p.i., resulting in the first CR 4 days p.i. The fraction of necrotic tissue initially increased, showing a maximum 2 days p.i., and then decreased and returned to about the same level as in untreated tumors. The fraction of granulation tissue initially increased, showing a maximum 4 days p.i., before declining to values similar to those in untreated tumors. The fraction of fibrous tissue started to increase a few days after treatment and increased throughout the entire study. The results regarding the infiltration of granulocytes and lymphocytes were not as clear as for the other characteristics, and these should therefore be interpreted with caution. The histological appearance of untreated tumors and tumors from animals treated with RIT is shown in Figure 3.

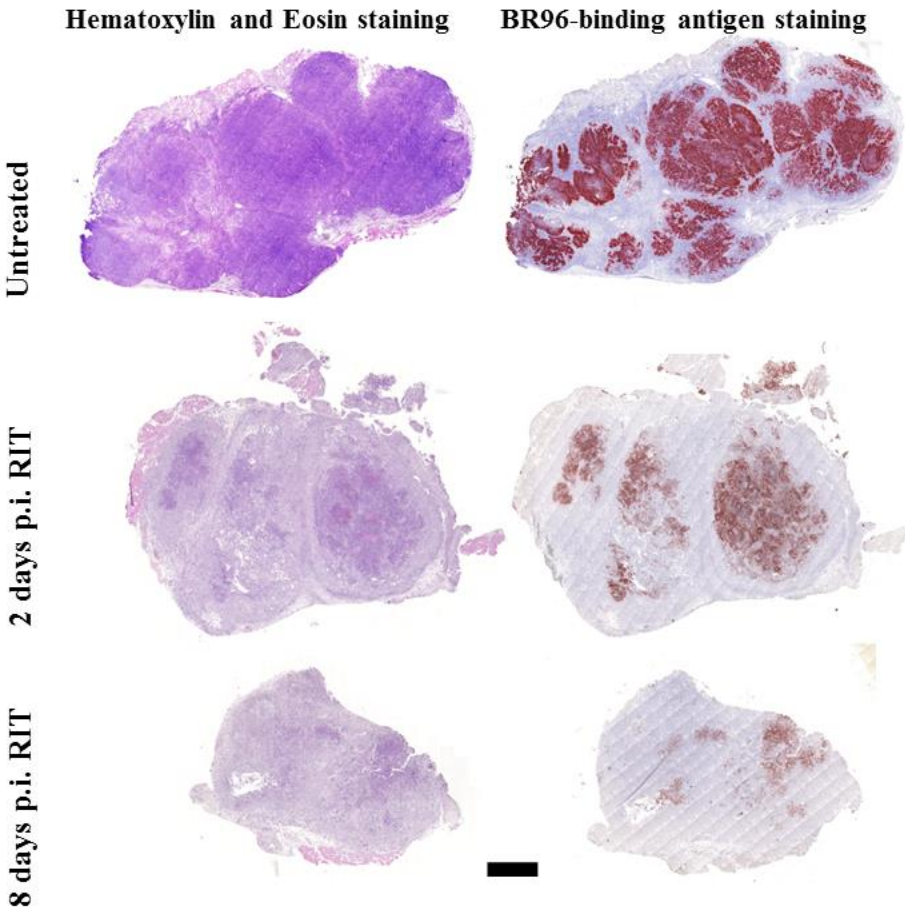

Figure 3. Histological evaluation of untreated tumors and tumors from animals treated with RIT

Top: Untreated tumor. *Middle*: Tumor from animals 2 days after injection of the radioimmuno-conjugate. *Bottom*: Tumor from animals 8 days after injection of the radioimmunoconjugate. *Right*: Hematoxylin and eosin staining. *Left*: BR96-binding antigen (red), stained with BR96. Scale bar: 1 mm.

Histological changes consisting mainly of granulation infiltration within the tumor cell areas could be seen 48 h after injection with the same amount of unlabeled BR96 (0.6 mg/kg body weight). The degree of histological changes was correlated to the amount of antibody administered (0.1 vs. 1 mg/kg body weight). The histological changes seen after the administration of unlabeled antibody were less than those after RIT.

Cell death – Paper I

Cell death mechanisms can influence the response to therapy. Radiation has been shown to have the ability to induce immunogenic cell death, thus radiation can awaken the immune response to tumor cells, *i.e*. immune response can enhance the effects of radiation on the tumor [26-28, 73]. Cell death by activated caspase-3 has been found to result in immunogenic cell death, resulting in protection against re-challenge by viable tumor cells [74-76]. This contradicts that apoptosis is immunogenically silent, in contrast to necrosis [74-76].

Most studies on cell death have been performed *in vitro*. However, it is also important to evaluate cell death *in vivo* due to the complex, interactive, and dynamic nature of tumors which consist of more than simply tumor cells. The cell death markers evaluated in the present work were fragmented DNA which occurs late in all cell death mechanisms (by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay), unpacking of DNA/double strand breaks (γH2AX by IHC staining), and executer caspase during apoptosis (activated caspase-3 by IHC staining) [77, 78].

In untreated tumors, cells staining positive for activated caspase-3, TUNEL, and γH2AX were mostly seen within and in the vicinity of necrotic areas, as can be seen in Figure 4. One to two days after injection of the radioimmunoconjugate, activated caspase-3- and TUNEL-positive cells were more prevalent and had infiltrated areas of viable tumor cells, while γH2AX-positive cells (more than foci staining, indicating staining of more than double strand breaks) were seen over most of the tumor section. Three to eight days p.i., the expression of activated-caspase-3-positive cells was the same as in the untreated tumors. Three to four days p.i., TUNEL-positive cells were observed in large parts of the remaining tumor cell areas, but later, 6-8 days p.i., TUNEL-positive cells were expressed as in the untreated tumors. After 3-8 days, the extent of γH2AX-positive cells was lower than after 1-2 days p.i., but they were still expressed over large areas of the tumor, showing no correlation to tumor cells. In animals given unlabeled BR96 the tumors showed activated-caspase-3- and γH2AX-positive cell expression as in the case of untreated tumors, while the higher dose of BR96 led to TUNEL-positive cell expression within tumor cell areas, as can be seen in Figure 4.

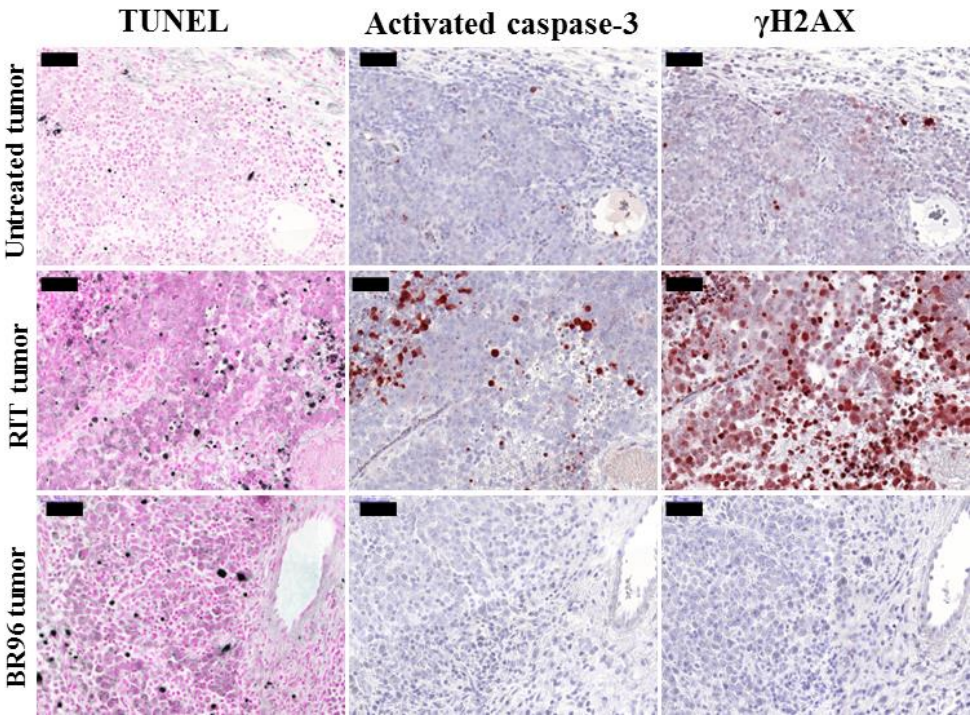

Figure 4. Tumor sections from untreated animals and animals treated with RIT and BR96, stained for various cell death markers
Top: Untreated tumor. *Middle*: Tumor from an animal 2 days after injection of the radio-immunoconjugate. *Bottom*: Tumor from an animal 2 days after injection of 1 mg/kg BR96. Tumors were stained with *Left*: TUNEL assay. *Middle*: Anti-activated caspase-3. *Right*: Anti-γH2AX. Scale bars: 50 µm.

In this study, tumors treated with RIT displayed intense nuclear staining of γH2AX in almost the entire tumor 1-2 days p.i., and elevated levels of positive cells 3-8 days p.i. Although a difference was seen in the γH2AX staining pattern between treated and untreated tumors, the staining pattern was not in foci formation. This indicates that not only double strand breaks are detected in our *in vivo* model, which is also confirmed by other studies [79, 80]. Thus, the results of γH2AX staining should probably be interpreted with caution.

Cells exposed to external beam radiation have generally been reported to succumb to apoptosis or mitotic catastrophe [13]. Mitotic catastrophe has been detected 2-6 days after external beam radiation *in vitro* [13], compared with apoptosis which was observed 4-6 hours after exposure [77]. The therapeutic response in this study was fast, with the first CR observed only 4 days after injection of the radioimmunoconjugate, indicating rapid cell death.

In this study, using the immunocompetent model, tumor cells died through both caspase-3-dependent and -independent cell death, *e.g.* caspase-3-independent apoptosis, necrosis, and/or necroptosis, after treatment with ^{177}Lu-BR96. In

contrast, BR96 induced only caspase-3-independent cell death, which is in accordance with a previous *in vitro* study using another cell line and treatment with BR96, resulting in morphological changes that appeared to be necrosis rather than apoptosis [66].

Immune cell markers – Paper II

Immune cells can promote the tolerance of the immune system or its response to tumor cells (rejection) [32, 81, 82]. For example NK cells promote tumor rejection, while T_{reg} cells promote the growth of the tumor, as illustrated in Figure 5. Also, the tumor has the ability to reprogram immune cells making them tolerogenic by secreting cytokines, growth factors, and proteases [32, 33]. This can be illustrated by the dual actions of T helper cells which can be divided into T_{H1} cells and T_{H2} cells the former promoting tumor rejection and the latter promoting tumor growth. Macrophages can similarly be divided into type 1 (M1) and 2 (M2). The possibility of evaluating the infiltration of immune cells in the primary tumor in order to identify patients with the poorest prognosis has been proposed [83-87]. Tumors are then classified based on the number of infiltrating immune cells, giving an immunoscore [88, 89]. The immunoscore could be used to predict which patients need additional therapy, and could also contribute to the choice of suitable therapies.

The tumors evaluated in this study (Paper II) were the same (untreated and RIT treated) as those used in the histopathological and cell death evaluation (Paper I). The paraffin-embedded tumors were IHC stained for T cell markers (CD2, CD3, CD8α), and macrophage markers (CD68, and CD163) (see Table I). Antibodies against additional immune cell markers were tested, but did not result in reliable IHC staining. The positive cells were counted in areas of viable tumor cells, necrotic cells, and in granulation tissue between and surrounding areas of tumor cells. The positive cells were counted in two hot spots per tumor in each area. The change in the number of positive cells resulting from RIT, the difference between the immune cell markers at the same location, and the difference in location of the same immune cell markers were evaluated.

CD2, CD3, and CD8α decreased during RIT, while CD68 and CD163 showed only a tendency towards a decrease during RIT. This could be the result of the higher radiosensitivity of lymphocytes than macrophages, NK cells, and dendritic cells (DCs) [16, 24]. Also, CD8α decreased less than the other T cell markers (CD2 and CD3). CD8α is not only expressed by cytotoxic T lymphocytes, but also by the less radiosensitive NK cells and DC subset, which could explain why CD8α decreased less than the other T cell markers. Since immune cell markers are rarely expressed by one cell type only (as can be seen in Table I), this type of evaluation is quite complex.

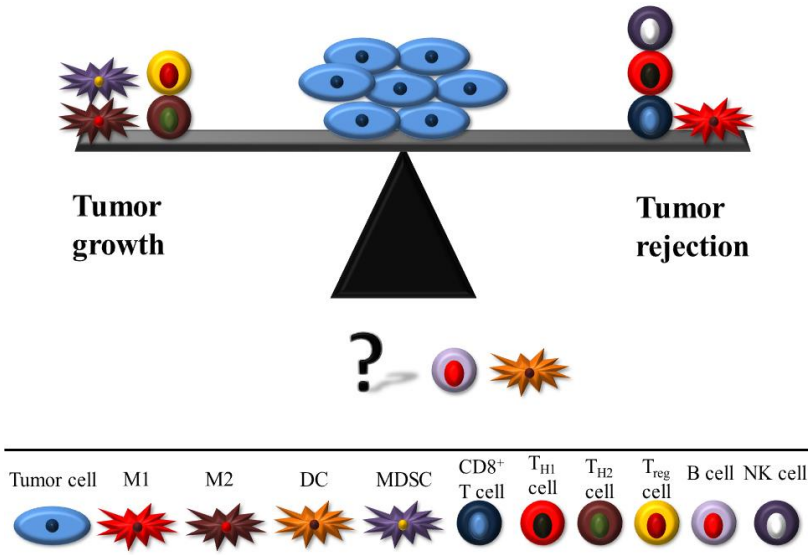

Figure 5. Balance between immune cells that promote either tumor growth or tumor rejection
Immune cells can promote tolerance (leading to tumor growth) or immune response (leading to tumor rejection), for example, by phagocytic activity and the production of cytokines. The immune cells infiltrating the tumor can be primed by secreted cytokines, growth factors, and proteases, thus the balance can shift due to the plasticity of immune cells. However, conflicting results have been reported for some immune cells while others have not been sufficiently well evaluated to determine their effects. The figure is adapted from [33, 90].

M1: macrophage type 1, M2: macrophage type 2, DC: dendritic cell, MDSC: myeloid-derived suppressor cell, $CD8^+$ T cell: cytotoxic T cell, T_{H1} cell: T helper cell type 1, T_{H2} cell: T helper cell type 2, T_{reg} cell: regulatory T cell, NK cell: natural killer cell.

Table I. The cellular expression of the markers evaluated

Antigen	Cellular expression
CD2	T cells, B cells, NK cells, thymocytes
CD3	T cells, thymocytes
CD8α	Cytotoxic T cells, NK cells, DC subset, thymocytes
CD68	Macrophages, monocytes, DC, myeloid progenitors (including MDSC), neutrophils, basophils
CD163	Monocytes, protumor macrophages (M2) [91, 92]

MDSC, myeloid-derived suppressor cells

There were more positive cells in the granulation tissue (both between and surrounding tumor cell areas) than within the tumor cell areas, for all the immune cell markers evaluated. This demonstrates the difficulty of immune cells in infiltrating tumor cell areas due, for example, to the high interstitial pressure within tumors. Others have shown that the infiltration of CD3- and CD8α-positive cells in cancer cell nests is correlated to improved overall survival in colorectal cancer [84].

In this study more cells stained positive for CD8α than for CD2 and CD3, and for the macrophage markers there was a trend towards more positive cells for CD68 than CD163, in both untreated tumors and in tumors treated with RIT. Thus, it was shown in this syngeneic immunocompetent rat tumor model that there was a higher expression of immune cell markers related to immune rejection than immune tolerance of tumor cells during RIT. The number of positive cells for all the evaluated immune cell markers decreased during RIT compared with untreated tumors. The positive cells for all immune cell markers were mostly located within granulation tissue, which could indicate difficulties for the immune cells to infiltrate tumor cell areas.

Effects of CD8-positive cells on tumor rejection – Paper III

Several studies have demonstrated that the administration of antibodies against CD8 reverses the effects of external beam radiation [93-96], which indicates that CD8-positive cells play a crucial role in the therapeutic response to radiation in these models. In a previous study by our group it was observed that the administration of cyclosporin A (a substance known to mainly affect T cells [67-72]) resulted in a prolonged time to CR after treatment with RIT, compared to animals treated with RIT only. However, the fraction of animals that exhibited CR or developed metastases did not differ between the groups [63]. Based on these findings, the study described in Paper III was carried out to determine whether CD8-positive cells are involved in the therapeutic response to RIT in this rat tumor model. This was investigated by administering ^{177}Lu-BR96 and antibodies against CD8 in 15 tumor-bearing rats, while 15 other tumor-bearing rats received ^{177}Lu-BR96 only. The depletion and the recovery of CD8-positive lymphocytes was analyzed by flow cytometry.

All animals in the group given ^{177}Lu-BR96 only exhibited CR, while in the group given ^{177}Lu-BR96 and antibodies against CD8 all but one exhibited CR. No difference was found between the groups regarding the time to CR. The mean and individual tumor volumes are presented in Figure 6. These findings indicate that CD8-positive lymphocytes are not a major player in the rejection of the inoculated tumor after treatment with ^{177}Lu-BR96. Our previous study on the effects of

cyclosporine A [63] resulted in a difference in time to CR, indicating that T cells are involved in tumor rejection, either by other T cells, *e.g.* CD4+ T_{helper} cells, or a complex T cell response involving more than CD8-positive lymphocytes.

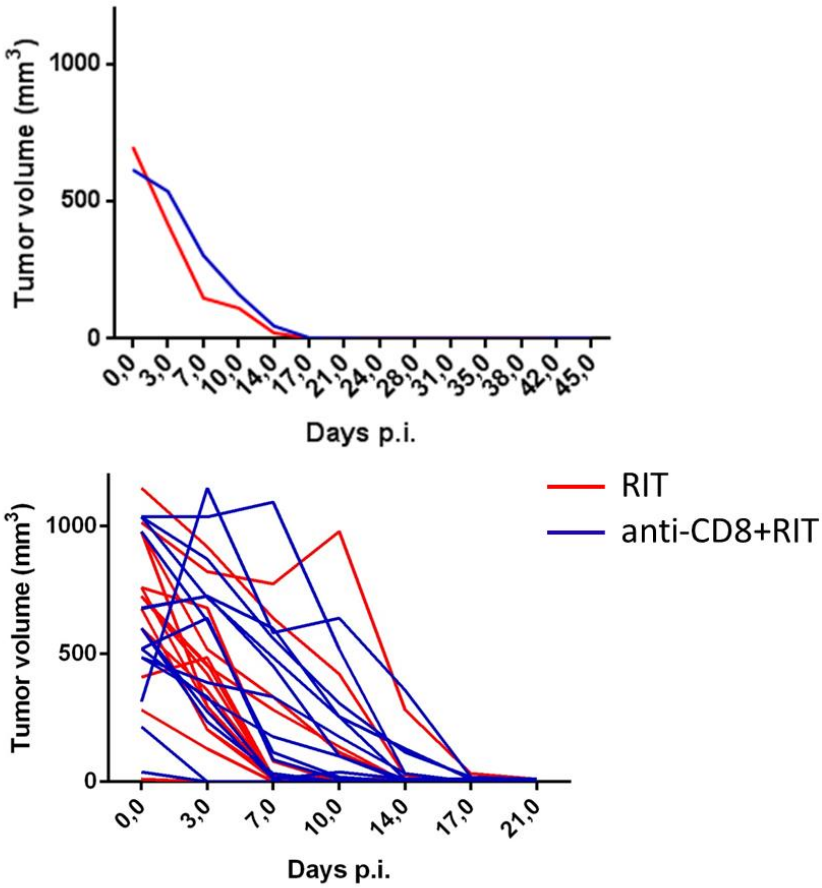

Figure 6. Tumor volumes from animals treated with antibodies against CD8 and radioimmunotherapy (anti-CD8+RIT) (*blue lines*) and with radioimmunotherapy only (RIT) (*red lines*)
Left: The average tumor volume. *Right*: The individual tumor volume. Note the difference between the x-axes.

Effects on metastases after radio-immunotherapy

Metastases can appear years after of undetectable tumors, due to the dormancy of tumor cells [97, 98]. Dormancy can be divided into three categories [97, 98]:

1. *Cellular dormancy* where intrinsic and/or extrinsic mechanisms drive single or small groups of tumor cells to enter quiescence,
2. *Angiogenic dormancy* where the tumor mass is constant due to the balance between dividing cells and dying cells due to poor vascularization, and
3. *Immune-mediated dormancy* where the immune system prevents the proliferation of tumor cells by persistent cytotoxic activities.

Dormancy may thus provide a window of opportunity, after the treatment of the primary tumor, to prevent the development of metastases.

It has been shown that far less than 1% of disseminated tumor cells succeed in forming a macrometastatic growth [99-101]. Most tumor models do not have the ability to metastasize. In our colon carcinoma model in immunocompetent rats, metastases develop in about half of the animals within 100 days after injection of the radioimmunoconjugate. It is thus possible to evaluate the establishment of metastases and possible differences between the inoculated tumor and metastases in this model.

Effects of CD8-positive cells – Paper III

Others have shown that the depletion of cytotoxic T cells by the administration of anti-CD8 antibodies reverses the anti-tumor effects of external beam radiation therapy [93-96]. This shows that CD8-positive cells are involved in the therapeutic response to radiation in those studies. The setup used in the present study was described in the section above, entitled “Effects of CD8-positive cells on tumor rejection – Paper III”. The follow-up after administration of ^{177}Lu-BR96 was 99 days.

All animals treated with antibodies against CD8 exhibited depletion of CD8 lymphocytes on the day of treatment with RIT. The CD8 lymphocytes started to recover about one week after the antibodies were administered. At the end of the study, the number of CD8 lymphocytes in both groups was similar, as can be seen in Figure 7.

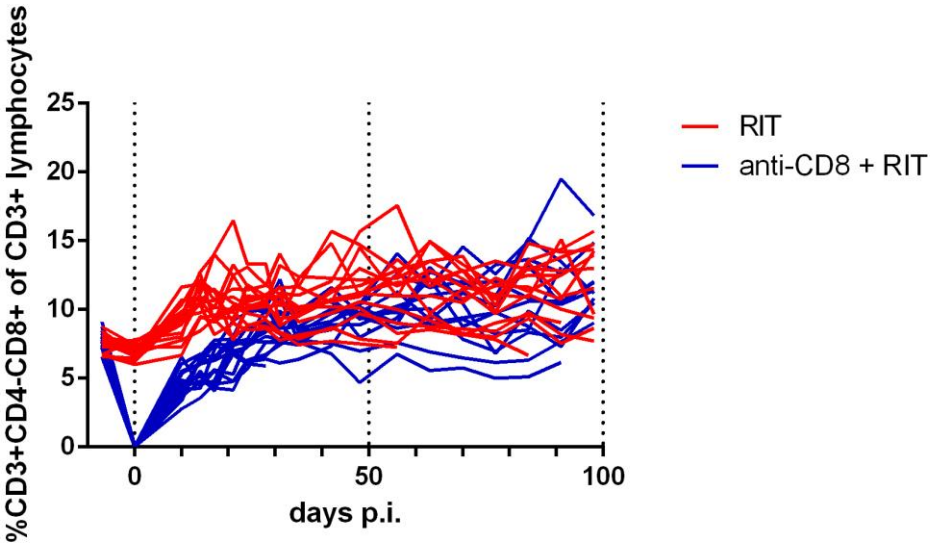

Figure 7. The depletion of CD8-positive lymphocytes
The individual percent of CD8-positive lymphocytes (defined as within the lymphocyte gate and positive for CD3 and CD8, but negative for CD4) of the total lymphocytes (defined as within the lymphocyte gate and positive for CD3) in animals treated with antibodies against CD8 and radioimmunotherapy (anti-CD8+RIT) (*blue lines*) and with radioimmunotherapy only (RIT) (*red lines*).

Five animals in the group treated with both antibodies against CD8 and RIT were sacrificed due to metastatic disease (28, 42, 45, 91, and 96 days p.i.). At the final autopsy (99 days p.i. of radioimmunoconjugate), 4 additional animals had detectable metastases. In the group given RIT only, 4 animals were sacrificed due to metastatic disease (56, 84, 91, and 91 days p.i.) and none of the animals had detectable metastases at the end of the study. Thus, in the group given RIT only, 11 animals were free from detectable metastases 99 days p.i., while only 6 were free from detectable metastases in the group given antibodies against CD8 and RIT. These findings indicate that an initial depletion of CD8 lymphocytes prior to RIT resulted in an increased risk of developing metastases.

Targeted antigen expression – Paper IV

One risk associated with targeting therapies is the down-regulation of the target, thus limiting the possibility of repeated administration. An advantage of RIT compared with other targeted therapies is that not all the cells need to be directly targeted, as irradiation from targeted cells results in crossfire, leading to damage of adjacent untargeted cells.

Early studies *in vivo* have shown that RIT with ^{131}I-labeled anti-breast mucin antibodies resulted in minimal loss of antigen in the primary tumor [102]. In a subsequent study, the administration of ^{131}I-labeled monoclonal antibodies in up to four fractions did not result in a reduction in the target antigen in the remaining tumors [103]. In contrast, repeated administration of the same unlabeled antibody resulted in a decrease in antigen expression [104]. A study in another model indicated that RIT can induce a reduction in carcinoembryonic antigen when ^{90}Y-labeled anti-carcinoembryonic antigen was administered [105]. An explanation of this difference could be that carcinoembryonic antigen may not be essential for survival of the carcinoma cells and can be down-regulated, whereas mucin may be essential for tumor cell survival and could consequently not be down-regulated.

The intention of the study described in Paper IV was to compare the expression of the BR96-binding antigen in primary tumors to that in metastases detected after the administration of ^{177}Lu-BR96. Thus, determine whether the BR96-binding antigen expression is down-regulated in metastases after treatment of the inoculated tumor with RIT in our model. Thirty-five tumor bearing rats were treated with ^{177}Lu-BR96 while 11 were untreated. Primary tumors, local recurrence (LR) of tumors, and metastases were stained with BR96 by IHC to identify the BR96-binding antigen and scored on a scale from 0 to 3. A score of 0 corresponded to less than 10% of the tumor cells showing strong and complete membranous staining, Score 1 corresponded to 10-50%, Score 2 to 50-90%, and Score 3 to over 90%.

Thirty-two of the RIT-treated animals exhibited CR of the primary tumor, two of which later showed LR, *i.e.* three animals did not exhibit CR. In the group exhibiting consistent CR of the inoculated tumor (30 animals), 11 animals developed metastases and two additional animals were found to have metastases at the final autopsy. All animals with non-CR (3 animals) and LR (2 animals) had metastatic findings. In the untreated group, none of the animals had developed detectable metastases at the day for treatment (13-14 days after inoculation).

The results are presented in Table II. All untreated and LR tumors had a score of 3, while the non-CR tumors had scores of 2 and 3. The majority of metastases had scores of 2 or 3. The metastases with reduced expression of the BR96-binding antigen showed a reduction in antigen expression in either specific areas or over the entire tissue section; none of the metastases completely lacked expression (see

Figure 8). There was no correlation between the expression of BR96-binding antigen between different metastases from the same animal.

Table II. The relationship between treatment, outcome, and the expression of the BR96-binding antigen

Treatment and outcome	Tissue analysed, n		Score 0 (< 10 %[a])	Score 1 (10 – 50 %[a])	Score 2 (50 – 90 %[a])	Score 3 (> 90 %[a])
RIT with CR	Primary tumor	0	N.A.	N.A.	N.A.	N.A.
	Metastases	23	1	7	7	8
RIT with CR and LR	Primary tumor	2	0	0	0	2
	Metastases	3	0	1	1	1
RIT without CR	Primary tumor	3	0	0	2	1
	Metastases	2	0	0	2	1
Untreated without CR	Primary tumor	11	0	0	0	11
	Metastases	0	N.A.	N.A.	N.A.	N.A.

[a] - % of tumor cells with strong complete membranous staining

n - the number of tissue analyzed

CR - complete response

LR - local recurrence

N.A. – not applicable

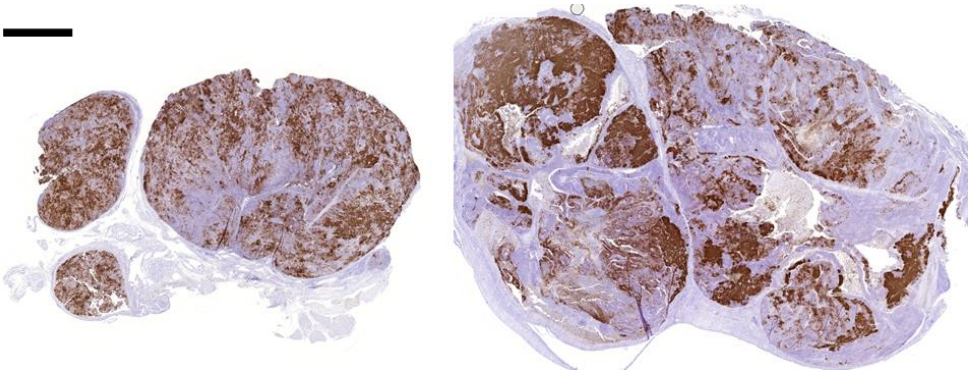

Figure 8. Expression of BR96-binding antigen in metastases from animal treated with radio-immunotherapy
Down-regulation of the targeted antigen, IHC stained with BR96, in specific areas and over large areas of the section. The paraffin-embedded metastases are from different lymph nodes in the same animal. Scale bar: 1 mm.

It is not known whether the reduced expression of the BR96-binding antigen is the result of treatment with ^{177}Lu-BR96, or a result of the metastatic process, or a combination of both. Furthermore, it is not known whether the tumor cells disseminate before or during treatment. Thus, it is not known whether the potential selection pressure arising from RIT acts on single tumor cells or on small metastases already established before RIT, or both. In the untreated animals the tumor reached the maximal permitted volume on days 21-28, *i.e.* prior to the earliest detectable metastatic disease. It would be interesting to evaluate metastases in animals not treated with RIT, but this would require the removal of the inoculated tumor to prolong the survival of these animals to allow the development of metastases.

Another possible mechanism causing antigen reduction in our syngeneic model is immune modulation. Others have shown that immunoediting can modulate antigen expression, thus a strong immune response can lead to antigen loss, although the mechanism is not clear [106, 107]. The therapeutic effect of RIT may be a combination of the direct cytotoxic effect of radiation and an immunological effect. It is, however, difficult to differentiate between these effects, which might act synergistically.

In an experimental study of treatment with the immunotoxin BR96-doxorubicin in a rat brain tumor model, Muldoon *et al.* [108] observed changes in the antigen staining pattern in residual tumors. The immunotoxin resulted in the outgrowth of tumors with areas of low or no antigen staining, as well as areas with moderate to intense staining, while the untreated tumors showed uniform intense staining for BR96. These results demonstrate that the BR96-binding antigen has the ability to be down-regulated in tumors.

In the present study, the untreated tumors had a very high BR96 antigen expression (above 90% of that of viable tumors with complete membranous

staining) compared with other antigens used as a target for therapy, *e.g.* HER2, which is scored as ‘strongly positive’ when over 10% of breast cancer cells are stained with complete membranous staining (according to the scoring guidelines of Herceptest™, Dako). In the present study, none of the metastases or remaining primary tumors completely lacked BR96-binding antigen expression, thus it may be possible to repeat RIT with BR96 utilizing a radionuclide with a relatively long range, resulting in crossfire from targeted to adjacent untargeted cells.

Conclusions

The studies described in this thesis were carried out to evaluate the effects of RIT on the inoculated tumor and metastases in an immunocompetent rat colon carcinoma model. The main conclusions that can be drawn are presented below.

- The analysis of cell death markers after administering ^{177}Lu-BR96 revealed that tumor cells succumbed due to both caspase-3-dependent and caspase-3-independent cell death. Treatment with unlabeled BR96 resulted in caspase-3-independent cell death, indicating a difference in cell death mechanisms for unlabeled BR96 and ^{177}Lu-BR96.
- The immune cell markers demonstrated that T cells and macrophages were present in untreated tumors in this model, and decreased during RIT. T cells decreased more than macrophages. The immune cell markers related to immune rejection were expressed to a higher degree than immune cell markers related to immune tolerance, for both T cells and macrophage markers, in both untreated tumors and tumors treated with RIT.
- The depletion of CD8-positive cells prior to RIT did not result in any delay in the rejection of the tumor after RIT. However, the initial depletion of CD8-positive cells seemed to result in a higher frequency of animals developing metastases in this immunocompetent rat colon carcinoma model. These results may indicate that long-term immunity to the tumor cells might have arisen in animals treated with RIT who showed no metastases.
- The expression of BR96-binding antigen in metastases after RIT was reduced, compared with untreated tumors, in 17 of 23 metastases. However, none of the metastases or remaining inoculated tumors completely lacked BR96-binding antigen expression. Thus, it may be possible to repeat RIT with BR96 using a radionuclide with a relatively long range, utilizing the crossfire from targeted to adjacent untargeted cells.

Future perspectives

The studies included in this thesis were carried out to evaluate the effects of ^{177}Lu-BR96 on a tumor in an immunocompetent animal model. The results obtained must be confirmed using another antibody, and another tumor model. Another radionuclide,for example, another beta-emitting radionuclide or an alpha-particle-emitting radionuclide, should also be evaluated.

Several studies have indicated that the activation of caspase-3 results in immunogenic cell death, which offers a protection against re-challenge by viable tumor cells [74-76]. The studies described in this thesis showed that ^{177}Lu-BR96 resulted in caspase-3-mediated and caspase-3-independent cell death (Paper I), and that CD8-positive lymphocytes do not affect the rejection of the tumor (Paper III). Fewer positive cells for all the evaluated immune cell marker were found in tumors treated with RIT than in untreated tumors, and the immune cell markers were found mainly in granulation tissue, not in tumor cell areas (Paper II). Thus, these results indicate that the immune response is not mainly responsible for the response of the inoculated tumor after injection of ^{177}Lu-BR96 (possibly due to the rapid CR). However, the initial depletion of CD8-positive lymphocytes does prevent the development of metastases (Paper III) in this tumor model. Based on the results of this work, it cannot be determined whether RIT induces immunity to the tumors in animals that did not develop metastases. This could be investigated by re-challenge, by inoculating the animals with a small number of tumor cells subcutaneous after CR of the inoculated tumor. If the control animals then develop palpable tumors, and animals treated with ^{177}Lu-BR96 develop tumors later or not at all, this would indicate that the treatment has evoked an immune response to the tumor cells.

Markers of cell death and immune cells were evaluated, but this is quite complicated. The cell death markers currently available are not linked to the immunological response, or to the classical definitions of cell death. The markers of immune cells are expressed by a variety of cell types, and are not specifically linked to cells favoring tumor rejection or growth. Hopefully, better markers will be available in the future for both cell death and immune cells.

One way of inducing or strengthening immunity to tumor cells is to combine RIT with immunotherapy. The combination of radiation and immunotherapy has shown considerable potential in inducing *in situ* vaccination by immunogenic cell death, implying that the dead tumor cells act as a vaccine in the treated animals, inducing an immune response to many different tumor antigens [109-112]. It

would be interesting to evaluate RIT in our tumor model in combination with immunotherapy, such as checkpoint inhibitors like anti-CTLA-4, or anti-PDL1/anti-PD1 [109-111, 113-115].

RIT has not yet been approved for the treatment of solid tumors. Hopefully, the studies described in the thesis will help to understand why RIT is effective in this model and lead to the optimization of RIT of solid tumors in the clinic.

Populärvetenskaplig sammanfattning

Cancer har tills nyligen främst behandlats med kirurgi, strålning och/eller cellgifter. Under de senaste åren har man utvecklat olika målsökande behandlingar som söker upp tumörceller genom att känna igen olika specifika antigen (måltavlor). I denna avhandling använder vi oss av radioimmunoterapi (RIT). RIT består av radioaktiva isotoper (avger strålning) som är kopplade till en antikropp (målsökande del). RIT går ut på att man strålar tumören genom att rikta den strålande antikroppen mot ett antigen på tumörceller, som kan vara spridda i hela kroppen. Vi studerar effekterna av RIT genom att använda en modell av tjocktarmscancer i råttor. Denna tjocktarmscancermodell illustrerar problematiken med cancer i människor väl, dels för att råttorna har ett fullt fungerande immunförsvar och dels för att den har kapaciteten att bilda metastaser (spridd cancer).

När vi studerar den behandlade tumören kan vi se att tumörcellerna dör på ett annat sätt när de behandlas med RIT än om de behandlas med samma antikropp utan påkopplad isotop. Detta visar att antikroppsbehandling och RIT har olika celldöds mekanismer vilket kan leda till olika effekter mot de döda tumörcellerna, t.ex. genom eventuell aktivering av immunförsvaret mot tumörcellerna.

Immunförsvaret kan antingen angripa tumören eller underlätta tumörens tillväxt. Det är därför viktigt att utvärdera både antalet och typen av immunceller som finns i tumören. Vi har valt att studera både obehandlade tumörer och tumörer som behandlats med RIT. Vi såg att det fanns fler immunceller i obehandlade än i behandlade tumörer. Detta kan förklaras med att immunceller är känsliga för strålning. Vi fann också att det var fler immunceller som var associerade till att angripa tumören än främjande av tumör tillväxten, både i obehandlade och behandlade tumörer.

Då man tar bort en av de immunceller som ansvarar för att angripa tumören ($CD8^+$ T-celler) fann vi att metastaser utvecklas i fler djur efter behandling med RIT. Dock påverkades inte antalet djur där tumören försvann eller tiden till tumörens försvinnande. Detta tyder på att denna typ av celler inte påverkar den lokala tumörens svar på behandlingen även om den verkar förebygga uppkomsten av metastaser.

Då man använder målriktad behandling finns det en risk att tumörcellerna förlorar uttrycket av antigenet som behandlingen är riktad mot. Vi har därför även studerat förekomsten av detta antigen i metastaser och kvarvarande tumörer. Vi fann att hos 17 av 23 metastaser minskade uttrycket av antigenet jämfört med

obehandlade tumörer. Det var dock inga metastaser eller tumörer, som helt saknade antigenet. Om man jämför med andra antigen som kan användas för målriktad behandling har vår modell en hög förekomst av antigenet i tumören. Då inga metastaser helt saknade antigenet, finns möjligheten att upprepa den målriktade behandlingen med samma antikropp i denna modell.

Denna avhandling visar att i denna modell har immunförsvaret en inblandning i behandlingssvaret med RIT. Dessa resultat är baserade på en modell där vi har använt en och samma tumör, antikropp och isotop i alla delarbete. För att se om dessa resultat är allmängiltiga, behöver dessa studier bekräftas med andra tumörer, antikroppar, isotoper innan det tas till kliniska studier.

Acknowledgements

Many people have contributed to my work at the Division of Oncology and Pathology, at Lund University. I would like to express my gratitude to everyone who has helped me and encouraged me during my work. Here I would like to take the opportunity to thank some of them especially.

First, I would like to thank my supervisors for their never ending engagement and support: my main supervisor, *Jan Tennvall,* for his great patience with my grammatical imperfections and my stubbornness to do things my way; my co-supervisor, *Rune Nilsson*, for his support in both good times and bad in the laboratory, and for believing in my abilities even when I doubted them myself; and my other co-supervisor, *Sophie Eriksson*, for being the mentor I needed, for answering my endless questions, and for her wonderful sense of humor. I am truly grateful to all of you, and I cannot imagine what it would been like to undertake this journey without you.

Anna Ebbesson for introducing me to the art of immunohistochemical staining, and for her great patience during the countless antibody experiments.

My co-authors: *Otto Ljungberg*, for sharing his vast expertise in histopathological evaluation, *Pär-Ola Bendahl*, for giving me an insight into statistics, *Tomas Ohlsson*, for performing the radiochemistry, and *Anders Örbom* and *Sven-Erik Strand*, for introducing me to the world of radionuclides and activities.

Tina Gustavsson, for her help at the animal facility.

Lotta Welinder, for performing the MALDI-MS analyses, and for always being prepared to lend a helping hand.

Hans-Olov Sjögren, for sharing his many years of experience regarding the animal model, and for inspiring discussions.

Lars Ekblad, for running the division and creating a stimulating and safe environment, and *Susanne André,* for solving all my administrative problems so I could concentrate on all the other problems.

Bo Baldetorp, for his engagement in all areas from technical issues (*e.g.* improving the FACSCalibur equipment) to running the department, and for making the mornings more pleasant with his jokes and smiles.

All the other PhD students and colleagues at the Division of Oncology and Pathology and the Department of Medical Radiation Physics in Lund, for creating such a pleasant working atmosphere. Thanks especially to *Julien Menard* for the optimization of the FACSCalibur run, and everyone in *the Targeted Alpha*

Therapy Group in Göteborg for creative discussions and exciting collaboration with the alpha emitter ^{211}At.

Last, but not least, *my friends and family (ingen nämnd ingen glömd)*. All the laughs and memories have reminded me of the world outside academia. Thanks for letting me be devoted to both horses and dogs, and for giving me free rein to take on larger projects, and for always supporting my ideas. This made me a happier person!

A special thanks to *my better half, Martin,* who has shown the patience of an angel! I cannot count the times I came home late from the stable and dinner was cold, or when you cleaned the house because our friends were coming over. You balance my life, as I am the notorious time optimist and always try to do more than I can alone. It is thanks to you that my life works.

This research was supported by grants from the Swedish Cancer Society, Mrs. Berta Kamprad's Foundation, Gunnar Nilsson's Foundation, Governmental Funding of Clinical Research within the National Health Service, King Gustaf V's Jubilee Foundation, The Lund University Medical Faculty Foundation, The Lund University Hospital Fund, and travel grants from the John and Augusta Persson Foundation.

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