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EXPERIMENTAL

RADIOIMMUNOTHERAPY

AND

EFFECTOR MECHANISMS

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ABSTRACT

Experimental Radioimmunotherapy and Effector Mechanisms
Radioimmunotherapy is becoming important as a new therapeutic strategy for treatment of tumour diseases. Lately monoclonal antibodies tagged with radionuclides have demonstrated encouraging results in treatment of hematological malignancies. The progress in treatment of solid tumours using radioimmunotherapy, however, has been slow. New strategies to improve the treatment response need to be evaluated. Such new strategies include the combination of radioimmunotherapy with other treatment modalities but also elucidation and exploration of the death effector mechanisms involved in tumour eradication.
As the combination of radioimmunotherapy and radiotherapy provides several potential synergistic effects, we started out by optimising a treatment schedule to detect benefits combining these treatment modalities. An anti-cytokeratin antibody labelled with ^{125}I administered before, after, or simultaneously with radiotherapy, indicated that the highest dose to the tumour was delivered when radiotherapy was given prior to the antibody administration. The optimised treatment schedule was then applied therapeutically in an experimental study on HeLa Hep2 tumour bearing nude mice given radiotherapy prior to administration of ^{131}I-labelled monoclonal antibodies. Combining these treatment regimes enhanced the effect of either of the treatment modalities given alone, and a significant reduction in tumour volumes could be demonstrated. This treatment caused a dramatic change in tumour morphology, with increased amounts of connective tissue, giant cells and cysts. Furthermore cellular alterations like heterogeneity of nuclear and cytoplasmic size and shape were observed, and at least a fraction of the tumour cells presented some characteristics of apoptosis.

The induced sequential events in Hela Hep2 cells exposed to 2.5-10 Gy of ionizing radiation were studied further, with special emphasis on cell cycle arrest, mitotic aberrations and finally cell death. Following radiation HeLa Hep2 cells initiated a transient G2/M arrest trying to repair cellular damage. This arrest was followed by a sequence of disturbed mitoses with anaphase bridges, lagging chromosomal material, hyperamplification of centrosomes and multipolar mitotic spindles. These mitotic disturbances produced multinuclear polyploid cells and cells with multiple micronuclei, cells that were destined to die via mitotic catastrophes and delayed apoptosis.
Induction of apoptosis in HeLa Hep2 cells following radiation doses and dose-rates equivalent to those delivered at radioimmunotherapy was concurrently studied *in vitro*. Significant induction of apoptosis was obtained and found to be induced relatively slowly, peaking 72-168 hours post irradiation. Caspases from the intrinsic pathway as well as the extrinsic pathway were found to be activated in response to ionizing radiation. Furthermore caspase-2, which has recently been acknowledged for its role as an initiator caspase was found to be activated following radiation and seems to play an important role in this delayed apoptosis.

ORIGINAL PAPERS

This thesis is based on the following papers:

1. Johansson A, **Eriksson D**, Ullen A, Löfroth PO, Johansson L, Riklund-Åhlström K, Stigbrand T. The combination of external beam radiotherapy and experimental radioimmunotargeting with a monoclonal anticytokeratin antibody. Cancer. 2002 Feb 15;94(4 Suppl):1314-9.

2. **Eriksson D**, Joniani HM, Sheikholvaezin A, Löfroth PO, Johansson L, Riklund Åhlström K, Stigbrand T. Combined low dose radio- and radioimmunotherapy of experimental HeLa Hep 2 tumours. Eur J Nucl Med Mol Imaging. 2003 Jun;30(6):895-906.

3. **David Eriksson**, Per-Olov Löfroth, Lennart Johansson, Katrine Åhlström Riklund, Torgny Stigbrand. Cell cycle disturbances and mitotic catastrophes following 2.5-10 Gy of ionizing radiation. Manuscript.

4. Mirzaie-Joniani H, **Eriksson D**, Johansson A, Löfroth PO, Johansson L, Åhlström KR, Stigbrand T. Apoptosis in HeLa Hep2 cells is induced by low-dose, low-dose-rate radiation. Radiat Res. 2002 Nov;158(5):634-40.

5. **David Eriksson**, Homa Mirzaie-Joniani, Per-Olov Löfroth, Lennart Johansson, Katrine Åhlström Riklund, Torgny Stigbrand. Apoptotic signalling pathways induced in HeLa Hep2 cells following 5 Gy of ionizing radiation. Manuscript.

ABBREVIATIONS

^{60}Co	cobalt-60
^{67}Cu	copper-67
^{90}Y	yttrium-90
^{125}I	iodine-125
^{131}I	iodine-131
^{137}Cs	cesium-137
^{177}Lu	lutetium-177
^{186}Re	rhenium-186
^{188}Re	rhenium-188
^{211}At	astatine-211
^{212}Bi	bismuth-212
^{213}Bi	bismuth-213
ADCC	antibody-dependent cell-mediated cytotoxicity
Ag	antigen
Apaf-1	apoptotic protease-activating factor-1
ATM	ataxia-telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related protein
CAM	cell adhesion molecule
CDC	complement dependent cytotoxcicity
CDK	cyclin dependent kinase
CDR	complementarity determining region
CEA	carcinoembryonic antigen
CH	constant heavy chain
Ck8	cytokeratin-8
CL	constant light chain
CLL	chronic lymphocytic leukaemia
DISC	death induced signalling complex
EGFR	epidermal growth factor receptor
FADD	Fas-associated death domain
FAP	fibroblast activation protein
Gy	Gray
HAMA	human anti-mouse antibodies
ICAD	inhibitor of caspase-activated deoxyribonuclease
LET	linear energy transfer
NF-κB	Nuclear Factor kappaB
NHL	non-Hodgkin's lymphoma
PDGF	platelet-derived growth factor
PARP	poly(ADP-ribose) polymerase
PEM	polymorphic epithelial mucin
PI3	phosphatidylinositol 3

PLAP	placental alkaline phosphatase
pRB	retinoblastoma protein
PSMA	prostate-specific membrane antigen
RBE	relative biological effectiveness
scdsFv	single chain disulfide stabilized variable fragment
scFv	single chain variable fragment
$(scFv)_2$	divalent tandem single-chain variable fragment
TAG-72	tumour-associated glycoprotein-72
TGF-α	transforming growth factor-alpha
TNF-α	tumor necrosis factor-alpha
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TSP-1	thrombospondin-1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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INTRODUCTION

Carcinogenesis

The expected length of a human life has steadily been increasing but is also positively correlated to the increased prevalence of cancer. With age the incidence of several types of cancer is increasing, reflecting that the transformation of a normal cell into a cancer cell is a multistep process comprising various combinations of sequentially acquired cancer-related mutations. Initiation of this process may be due to exposure of cells to chemical, physical or viral carcinogens. Acquired mutations most often affect genes not related to the induction of the carcinogenic process or genes indispensable for survival and might be lethal for the cell. Occasionally, however, mutations affect genes giving the cell growth advantages because of accelerated proliferation or reduced cell death. Such genes comprise the oncogenes, which when mutated, may be activated and stimulate proliferation or protection against cell death, and the tumour suppressor genes which will inactivate genes that normally inhibit proliferation. Each successive genetic alteration may confer to the cell one or another type of growth advantage, which eventually converts the normal cell into a cancer cell.
The alterations in cell physiology that gears the transformation from a normal cell to a malignant can be divided into distinct acquired properties summarised in Figure 1.

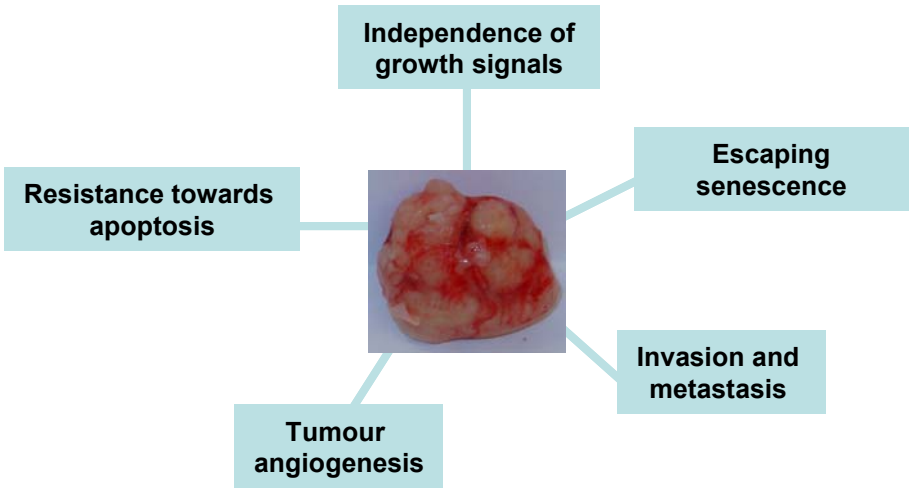

Figure 1. Acquired properties important for carcinogenesis.

Independence of growth signals
A normal cell halted in a quiescent state requires exogenous growth signals to be able to leave this state and start to proliferate. Growth-factors bind to specific receptors and transmit signals into the cell to initiate proliferation. Tumour cells are independent of external growth stimuli as they may acquire the capability to produce their own growth factors to which they are responsive (PDGF (*1-4*), TGF-α (*5-7*)). Furthermore tumour cells often overexpress the receptors involved in the signalling cascade (EGFR (*8, 9*), HER2/neu (*8, 10*)) making them hyperresponsive, or the cell presents alterations in the downstream intracellular signalling pathways, which make them constitutively activated, thus eliminating requirements for external growth factors (*8, 9, 11*).
An additional difference between a normal and a tumour cell concerning growth signals is that a normal cell responds to anti-proliferative signals, while a tumour cell is insensitive. Anti-growth signalling molecules bind their receptors and reduce the proliferation by shifting the cell status from active proliferation into a quiescent state or alternatively drive the cell into a postmitotic state. Insensitivity to anti-growth signals can be achieved in tumour cells by interference of the retinoblastoma protein (pRB) as many anti-proliferative signals normally are transferred via this pathway. Disruption of this pathway can be achieved in several ways including elimination of the pRB function via sequestration by viral oncogenes like the E7 protein from human papillomavirus (*12*).

Resistance towards apoptosis
An apoptotic response to unscheduled proliferation is a general response in normal cells to prevent transformation. Normal cells display a continuous surveillance of the extracellular surroundings and of the intracellular conditions to ensure that these fulfil standard requirements. If any abnormalities are detected, an apoptotic signalling pathway should be activated and force the cell into a programmed cell death. Resistance towards this apoptotic response is an important attribute that tumours need to acquire in order to grow and expand. An important alteration that induces resistance to apoptosis can be obtained by mutations in the p53 tumour suppressor gene. The P53 protein normally passes signals on from sensors detecting DNA damage, hypoxia or oncogene hyperexpression, to downstream effectors and thereby induces the apoptotic signalling cascade. Mutations of this gene is seen in more than 50 % of all human cancers (*13-15*), which is an indication of the importance the inactivation of p53 exerts for the transformation of a normal cell into a malignant one. Other alterations include activation of the PI3-kinase-AKT/PKB pathway, which

transmits survival signals, and interruption of the Fas-signalling pathway (*11*).

Escaping senescence

A normal cell can exert a finite number of cell divisions before its replicative potential ceases and the cell enters a senescent state. The number of replications is correlated to the length of the telomeres, situated at the ends of the chromosomes, which protect the DNA. Telomerase is an enzyme responsible for maintaining the length of the telomeres and is a key component for unlimited replication (*16, 17*). Telomerase is activated in the vast majority of human cancers but not sufficiently active in normal cells to maintain telomere length (*18*).

Tumour angiogenesis

Any cell in a tissue is dependant on the supply of nutrients and oxygen from surrounding blood vessels. The formation of blood vessels is normally strictly regulated by inducers and inhibitors, but this balance is altered in tumours in order to induce growth of new blood vessels (angiogenesis) (*19, 20*). Angiogenesis is an important prerequisite for the rapid expansion associated with macroscopic tumours. Several known inducers of new blood vessels are known, and among these angiogenic factors, the vascular endothelial growth factor (VEGF) is probably the best recognized. VEGF binds to its specific receptors on endothelial cells inducing neovascularisation, and VEGF expression is frequently upregulated in tumours (*21*). The most recognised angiogenic inhibitor is thrombospondin-1 (TSP-1)(*22*). TSP-1 binds its receptor on endothelial cells and hampers the formation of new blood vessels via inhibition or apoptosis. The p53 tumour suppressor protein positively regulates TSP-1 (*23, 24*) and as the p53 gene is frequently mutated or downregulated in most tumours, TSP-1 levels will decrease, releasing the endothelial cells from the inhibitory effect of TSP-1.

Invasion and metastasis

Traits important for the late stages of cancer progression include the ability to invade surrounding tissues and the capacity of tumour cells to disseminate and form new colonies at distant sites in the body. To acquire such traits, a reduction of the cell-to-cell contact inhibition has to take place, as well as a decrease of the cell and microenvironment interactions, which normally tightly regulate and restrain such cellular behaviour (*11*). Several types of molecules are involved in the cell-cell contacts and may be altered in cells which have acquired metastatic and invasive capabilities. These molecules include the cell-cell adhesion molecules (CAMs) and the integrins (*25*). E-cadherin is a CAM and is expressed on all epithelial cells.

Interactions between E-cadherin molecules on neighbouring cells cause induction of antigrowth signals (*26*). In normal cells this causes an inhibition of both invasion and metastasis. The functional inactivation of E-cadherins seen in most epithelial tumours leads to the opposite, i.e. the capacity to invade and establish metastases (*26*).
Another property important for the potential to invade neighbouring tissues and metastasise in distant organs is the upregulation of extracellular protease activity (*27, 28*). This upregulation can be induced within the tumour cell or by recruited stromal and inflammatory cells. The coupling of these extracellular proteases to the cell surface of the tumour cell increases the efficiency by which the cells invade and metastasise to new areas within the body.

Treatment of malignant diseases

When designing treatment strategies of malignant diseases the projected outcome might sometimes, due to non-controlable circumstances, diverge from the initial wish to completely eradicate the primary tumour, i.e. it could be to reduce the tumour growth, to find and eradicate distant metastases or to offer relief but not cure from the disease.
Malignant diseases can be treated in several ways and treatment is geared by factors such as origin of the tumour, stage, location and the general health status of the patient. Radiotherapy, chemotherapy and surgery are three classical regimes widely used for treating tumour diseases, and biotherapy, including radioimmunotherapy, is a more recent group of therapies under rapid progression. All of these treatment modalities can also be combined, given either simultaneously or sequentially in order to optimise the treatment effect.

Surgery
Surgery is the oldest and still the most widely used treatment modality available for cancer patients, when possible to perform. If the tumour is detected at an early stage before spreading, surgery alone might be sufficient to reach complete remission. When there has been spreading with distant metastases, surgery is frequently used in combination with radiotherapy or chemotherapy.

Chemotherapy
At chemotherapy a wide range of therapeutics, generally eradicating rapidly dividing cells are employed. As tumour cells often lose the capacity to regulate proliferation, they will continue to divide even when exposed to these drugs and consequently be killed. Unfortunately rapidly dividing cells are not only malignant cells, but also normal cells from bone marrow and gastrointestinal tract. Compared to surgery and radiation therapy, chemotherapy has one advantage - it is able to eliminate cancer cells throughout the entire body, not only the primary tumour. There are several major categories of chemotherapeutic agents which include:

Antimetabolites
Drugs that interfere with the formation of key biomolecules within the cell including nucleotides, the building blocks of DNA. These drugs utltimately interfere with DNA replication and therefore cell division.

Genotoxic drugs
Drugs that damage DNA. By causing DNA damage, these agents interfere with DNA replication, and cell division.

Spindle inhibitors
These agents prevent proper cell division by interfering with the cytoskeletal components, which enable one cell to divide.

Other chemotherapeutic agents
These agents inhibit cell division by mechanisms that are not covered in the three categories listed above.

Radiotherapy
Radiotherapy, also known as radiation therapy, provides high-energy ionizing radiation administered as X- or γ-rays. Ionizing radiation deposits energy in cells, which causes damage to crucial molecules, disrupts cellular processes and prevents proper cell division, making it impossible for these cells to grow. As radiation is not specific to malignant cells, it can also damage normal cells, but more efficiently tumour cells, because of their rapid proliferation. Normal cells also recover from the effects of radiation more easily and may actually accelerate the cell division after radiation. Radiation therapy is applied locally, affecting only cells in the treated area, and can not be used to treat non identified metastases. To achieve killing of secondary tumours and stop growth of any remaining tumour cell, radiation

therapy is often used in conjunction with other treatment modalities like chemotherapy and surgery.
Radiation therapy can be delivered via **external radiation** from a source outside the body directing the radiation to the tumour or by an **internal radiation** source (brachytherapy), which is positioned inside the body, adjacent to or inside the tumour.

Radioimmunotherapy

Historical aspects
Antibody therapy has moved significantly forward since the discovery of the immune system and its capability to recognise bacteria and foreign cells. Paul Ehrlich, who received the Nobel Prize in 1908, is one of the founders of immunology, and he was the first to recognise antibodies for their ability to differentiate between normal cells and transformed malignant cells. He is generally recognised as the inventor of the term "magic bullets", describing the potential of an antibody to specifically target tumour cells. He specifically introduced immunotherapy as a potential treatment modality for targeting and treating tumours (*29*). Pressman and Keighley were the first to be able to inject antibodies specific for rat kidney labelled with radioactive isotopes, and document their localisation at the target site (*30*). This technique evolved into a trial in which Pressman and Korngold were able to demonstrate an accumulation of anti-tumour antibody that was larger than in normal tissues (*31*). In 1965, Gold and Freedman discovered carcinoembryonic antigen (CEA) (*32, 33*), the first well defined tumour-associated antigen and as a result of this finding purified polyclonal anti-CEA antibodies were shown to localise to CEA expressing tumours *in vivo* (*34, 35*). In 1975 Köhler and Milstein reformed the field of radioimmunotargeting as they introduced the hybridoma technology, a method that made it possible to produce large quantities of monoclonal antibodies with high purity and reproducibility (*36*). Since then numerous antigen-antibody systems have been established and several of the antibodies have been taken to clinical trials. Today radioimmunotherapy is mainly used for therapy of hematopoietic malignancies, such as non-Hodgkin´s lymphoma (*37-40*). The progress in this area is due to the sensitivity of these cells to low doses of radiation and they are easily accessible by systemic therapy. The therapeutic advancement of solid tumours has been much slower, but several investigations using different radionuclides, engineered antibodies, and methods to increase antibody accumulation and penetration are currently being evaluated and have so far shown promising results (*41-44*).

Tumour antigens

In radioimmunotherapy tumour antigens are used as targets for radiolabelled monoclonal antibodies. In order to distinguish the tumour cell from a normal cell, an antigen should selectively be expressed by the tumour cell, and it should be expressed stably and homogeneously in high amounts, without shedding into the circulation, and furthermore it should be easily accessible by the monoclonal antibody. Up to date, however, no tumour antigen is known to fulfil all these criteria.

Most tumour antigens instead are expressed not only by tumour cells but also at least by a subgroup of healthy normal cells, but expressed in higher quantities or in an atypical mode on the tumour cells. These antigens are not tumour-specific but are referred to as tumour-associated antigens.

Tumour antigens are a diverse group of molecules, which have been identified in a variety of malignancies (Table 1)

Table 1. Categories of tumour antigens used as targets in radioimmunotherapy*

Antigen category	Antigen name	Tumour type	Clinical studies
Hematopoietic differentiation antigens	CD20	NHL	FDA approved antibodies (Bexxar and Zevalin)
	CD22	NHL	Phase III
	HLA DR	NHL, CLL	Phase II/III
	CD33	Myelocytic leukemia	(*45-47*)
Cell-surface differentiation antigens			
Glycoproteins	CEA	Colorectal, breast, lung, pancreatic, stomach carcinoma	Phase III
	TAG-72	Ovarian, colorectal, breast, prostate carcinoma	(*48*), (*49, 50*), (*51, 52*), (*53*)
	PEM (MUC1)	Ovarian, breast, bladder carcinoma	Phase III
	A33	Colorectal carcinoma	(*54*)
	PSMA	Prostate carcinoma	(*55*)
Carbohydrates	Lewis Y antigen	Breast, lung, colon, prostate and ovary carcinoma	(*56*)
Growth-factor receptors	EGFR	Glial tumours	(*57*)
	HER2/neu	Breast carcinoma	(*58*)
Angiogenesis and stromal antigens	FAP	Epithelial tumours (metastatic colon cancer)	(*59, 60*)
	VEGFR		(*61*)

Abbrevations: CEA, Carcinoembryonic antigen; TAG-72, Tumour-associated glycoprotein; PEM, Polymorphic epithelial mucin; PSMA, Prostate-specific membrane antigen; EGFR, Epidermal growth factor receptor; FAP,Fibroblast activation protein; VEGFR, Vascular endothelial growth factor receptor; NHL, non-Hodgkin's lymphoma; CLL, chronic lymphocytic leukaemia

* Modified from ref. (*62-64*).

Placental Alkaline Phosphatase (PLAP)

PLAP is one of four members of the alkaline phophatase family. Alkaline phosphatases are present in many cell-types, but their specific functions are still largely unknown. Alkaline phosphatases are often located on absorptive surfaces on tissues indicating a role in the transport of nutrients or ions across the plasma membrane (*65*). PLAP is expressed in the syncytiotrophoblasts of the placenta (*66, 67*) and trace amounts of PLAP can also be detected in tissues like testis, lung, liver and intestinal mucosa, together with tissue-non-specific or specific alkaline phosphatases (*68*). PLAP is a dimeric enzyme anchored in the plasma membrane and has also been suggested to be related to cell division both in normal and transformed cells (*69, 70*). Furthermore, PLAP has been claimed to be involved in the transfer of maternal IgG to the fetus, but this is still controversial (*71, 72*). Oncofetal antigens are tumour associated antigens normally expressed in fetal tissues and present only in trace amounts in normal tissues. High expression of the antigen in adult tissues is usually due to malignant transformation.
In 1968 PLAP levels were found to be elevated in the blood of a patient with broncogenic carcinoma thereby establishing PLAP as one of the first oncofetal antigens (*73*). Later PLAP was also detected in serum from patients with other malignancies such as ovarian and gastrointestinal carcinomas, as well as seminomas (*74*). Since then, several reports have been published on PLAP and its role as a tumour marker and PLAP is currently used clinically as a tumour marker for seminomas (*68, 75*). PLAP has also been used as a target at experimental radioimmunotargeting (*76, 77*) as well as for imaging of PLAP-positive tumours in patients (*78*).

Cytokeratins

The cytoskeleton of eukaryotic cells is composed of three filament systems in the cell, i.e. microtubules, microfilaments and intermediate filaments. The cytoskeleton, together with its associated proteins, cooperate in several essential functions such as maintenance of cell shape, cell movement, cell replication, apoptosis, cell differentiation and cell signalling (*79*). The intermediate filaments are a family of proteins in which the two largest groups are the keratins (acidic and basic keratins) (*80*). Fortynine functional keratin genes have been identified from the human genome sequence database and 34 of these are cytokeratins and the rest hair keratins. Cytokeratins are the most abundant proteins in many types of epithelial cells and their complex expression pattern is both tissue-specific and

differentiation-specific (*81*). In addition, the expression pattern of the cytokeratins is usually maintained in transformed malignant cells (*82, 83*). This provides opportunities to identify the origin of malignant cell types by characterisation of their cytokeratin composition. Cytokeratin 8, 18, and 19 are the most abundant in carcinomas, confirming that most of these tumours are of simple epithelial origin (*82*). Furthermore the abundance of these cytokeratins also makes them appropriate antigens for radioimmunotargeting (*84-90*).

Antibodies

To achieve the most favourable outcome at radioimmunotherapy, the antibody has to be functionally efficient. Properties like optimal affinity, penetration, antibody kinetics and clearence from circulation as well as minimal immunogenicity have to be taken into consideration. Today several different categories of antibodies are available, all with different characteristics making it possible to choose the most favourable antibody for each new treatment setup.

Polyclonal and Monoclonal antibodies

When the immune system encounter a foreign antigen an immune response is elicited and many different B-lymphocytes and plasma cells are activated to generate a polyclonal antibody response. In the beginning of the radioimmunotherapy era, polyclonal antibodies were used, but did not result in significant improvements in treating cancer because of problems with cross-reactivity. In 1975 the hybridoma technology was established (*36*), making it possible to produce monoclonal antibodies, which were very important for the continuation of immunotherapy. The use of monoclonal antibodies for radioimmunotherapy increases the targeting specificity and the tumour to non-tumour ratios of accumulated radionuclide. Monoclonal antibodies are also easily produced and compared to polyclonal antibodies more homogenous. Monoclonal antibodies have specificity for a single antigen epitope and are produced by hybridoma cells that originate from a single B-lymphocyte, which has been fused to a myeloma cell making it immortal. When intact monoclonal antibodies are used in radioimmunotherapy, they have the potential to kill targeted tumour cells by several mechanisms (*62, 91, 92*). Firstly these antibodies may act via their inherent immune effector mechanisms by recruiting and activating effector cells via the Fc portion of the antibody molecule leading to antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement

dependent cytotoxcicity (CDC). Cell killing then proceeds via a cell-dependent phagocytosis or a cell-independent lysis of the targeted cell. Secondly, antibodies may be used as carriers, more or less selectively delivering radionuclides to the tumour, where they may exert radiation-induced tumour cell death. Finally, antibodies may neutralise ligands or block membrane receptors thereby interfering with receptor-ligand interactions and signal transduction. This might reduce the proliferative potential or induce apoptosis in the targeted tumour cells.

Recombinant antibodies

If mouse monoclonal antibodies are used for repetitive injections in humans, the immune system recognises them as foreign and responds by producing human anti-mouse antibodies (HAMA). This problem limits the use of monoclonal antibodies and has stimulated the development of recombinant antibodies with reduced immunogenicity, including chimeric antibodies, humanised antibodies and fully human antibodies (Figure 2) (*93*). Furthermore, although murine monoclonal antibodies may induce ADCC and CDC with human effector cells, chimeric, humanised and fully human antibodies with human IgG1 constant regions are preferred, as they elicit larger cytotoxicity in the presence of human complement and human effector cells. Chimeric antibodies consist of the mouse variable regions linked the to human constant regions and in humanised antibodies the complementarity determining regions (CDR) are grafted onto an equivalent human frame (*94, 95*). Fully human antibodies may be obtained from single chain variable fragment- (scFv) or Fab-phage display libraries (*96*). Human antibodies have also been obtained from transgenic mice which contain human immunoglobulin genes and genetically disrupted endogenous immunoglobulin loci. Immunisation elicits the production of human antibodies, which may be recovered using hybridoma technology (*97*).

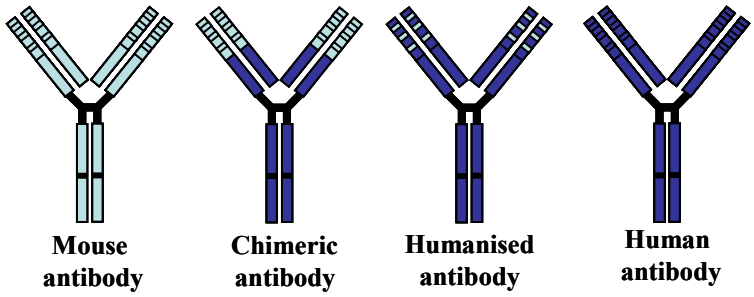

Figure 2. Antibodies with reduced immunogenicity can be created for repetitive injections of antibodies in humans.

In attempts to diminish the problem of low overall tumour uptake of radiolabelled antibodies, compared to levels in blood and normal tissues, a variety of different recombinant antibodies have been generated. These antibodies can be constructed in an effort to optimise properties, such as tumour penetration, clearence and binding affinity. To this category belong scFv:s, nonstabilised or stabilised with a disulfide bond, diabodies (mono- and bi-specific), $(scFv)_2$ (mono- and bi-specific) to mention a few (Figure 3)(*63, 94, 98*).
Different types of antibodies also have different targeting properties such as tumour binding (uptake, duration, optimal accretion time), biological (immune effector function, $t_{1/2}$ blood, target organ) and physical (molecular weigth) (*63*).

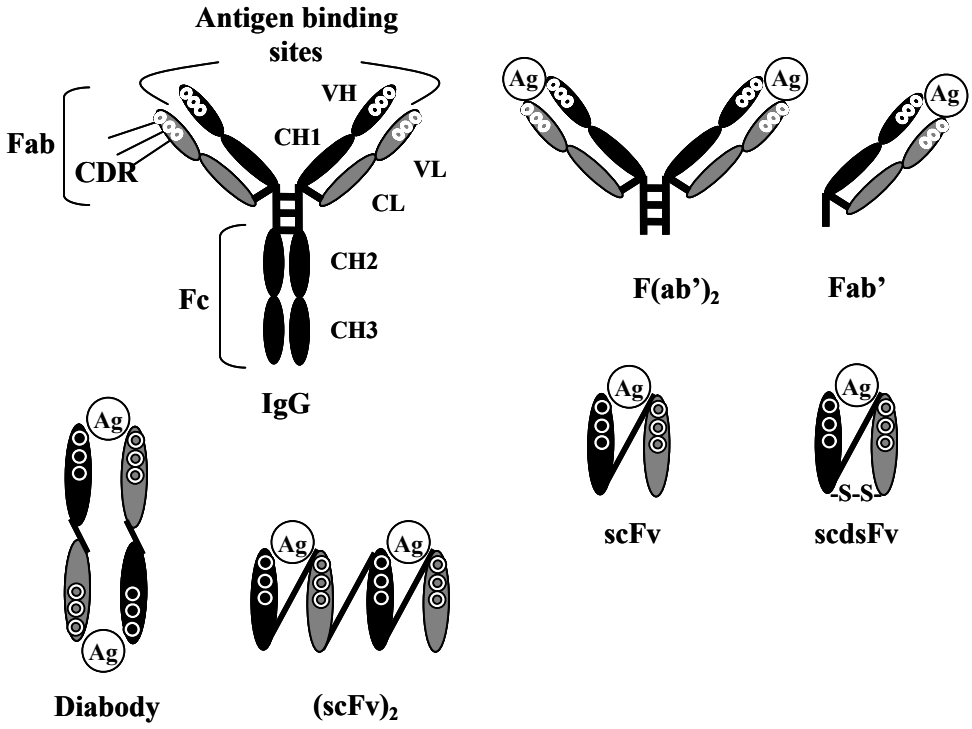

Figure 3. Schematic pictures of some antibody derivates with potential for use in radioimmunotherapy.

Antibodies in this thesis

Two monoclonal antibodies have been employed in this work. The first one, TS1, is a monoclonal antibody specific for cytokeratin 8, an intracellular antigen deposited extracellularly in necrotic regions of the tumour.
TS1 has been shown to localise efficiently to HeLa Hep2 tumours in several studies (*84-90*) and targets mainly necrotic parts of the tumour (Figure 4).
The second antibody H7 is a monoclonal antibody against placental alkaline phosphatase (PLAP), a plasma membrane oncofetal antigen. H7 efficiently targets experimental HeLa Hep2 tumours (*76, 77*) and will bind mainly to viable parts of the tumour (Figure 4).

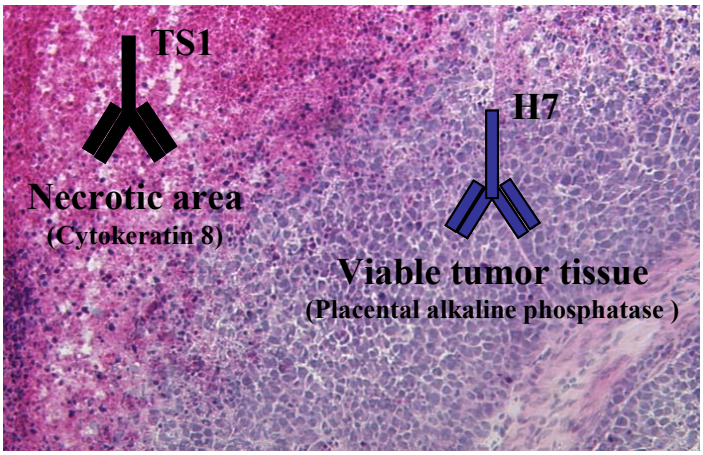

Figure 4. Two monoclonal antibodies targeting different tumour antigens.
TS1 recognises cytokeratin 8 and binds to necrotic areas of tumours and H7 is specific for PLAP and binds viable tumour tissue. HeLa Hep2 tumour section stained with haematoxylin-eosin.

Clinical applications with antibodies

Radioimmunotherapy of hematopoietic malignancies has lately advanced significantly and it seems likely that radioimmunotherapy in the future will play a major role in treatment of these diseases. Treatment responses in solid tumours are improving, but have not been as encouraging as expected, compared to hematopoietic malignancies, mainly due to a higher degree of inherent radioresistence in these tumours and heterogeneity in expression of tumour antigens and variability in vasculature. Numerous improvements

remain to be made before radioimmunotherapy can be included as a standard treatment modality of solid tumours.

Hematological malignancies

Radioimmunotherapy has been most successful in the treatment of hematological malignancies. Currently, several radioimmunotherapeutics are being evaluated in Phase III clinical trials and two have been approved for clinical use (Table 1). These two radioimmunotherapeutics are murine monoclonal antibodies, specific for CD20, used for treatment of non-Hodgkin's lymphomas (NHL). CD20 is an antigen expressed on B cells from the pre-B cell stage to the B cell lymphoblast stage and further expressed on most malignant B cells. Bexxar is one of the approved radioimmunotherapeutics and is a mixture of unconjugated tositumomab and ^{131}I-labelled tositumomab. The second approved radioimmunotherapeutics is ^{90}Y-Zevalin, an immunoconjugate with a stable bond between the monoclonal antibody ibritumomab and the linker-chelator tiuxetan, which provides a high affinity, conformationally restricted chelation site for ^{90}Y. Other examples of antibodies used for treatment of NHL, currently in clinical trials, recognising B-cell target antigens include oncolym (^{131}I Lym-1), a monoclonal antibody labelled with ^{131}I specific for the HLA-Dr10 protein and epratuzumab (Lymphocide), a humanised antibody labelled with ^{90}Y that targets CD22 receptors on B-cell lymphomas. Furthermore some potential T-cell epitopes that may be targeted for treatment of NHL include CD33 and CD25.

Solid tumours

Treatment responses to radioimmunotherapy have been evaluated across the full spectrum of malignancies including breast, ovarian, colorectal, medullary thyroid and brain tumours. A significant number of antibodies specific for target antigens like carcinoembryonic antigen (CEA), tumour-associated glycoprotein (TAG-72), polymorphic epithelial mucin (PEM, MUC1), Lewis Y, prostate-specific membrane antigen (PSMA), and A33 have been used in clinical studies, predominantly labelled with ^{131}I or ^{90}Y (Table 1). Pemtumomab is a radioimmunotherapeutics currently in Phase III clinical development for ovarian cancer. It is an ^{90}Y-labelled mouse monoclonal antibody directed against MUC-1, a form of mucin found on several tumour cells. Furthermore, labetuzumab, a humanised monoclonal antibody labelled with ^{131}I and targeting carcinoembryonic antigen is in a pending Phase III status for treatment of liver metastases of colorectal cancer.

Although there are some promising radioimmunotherapeutics for treatment of solid tumours, the progress has been much slower when compared to studies on hematopoietic neoplasms. Consequently, novel strategies to improve the treatment response need to be developed, if radioimmunotherapy is to become a standard treatment in the future, and several strategies are currently being investigated.

Radionuclides

Labelling antibodies with suitable radionuclides is an important aspect of radioimmunotherapy. The selection of a radionuclide for radioimmunotherapy depends on the situation and no single radionuclide is likely to tackle every therapeutic aspect. Several properties, including physical data on the radionuclide, its availability, cost, labelling-chemistry and available biological characteristics have to be considered (*63, 99, 100*). The type of radiation emitted by the nuclide, required energy necessary for imaging or therapy and half-life of the radionuclide, which should correspond to the pharmacokinetics of the antibody *in vivo* are important physical parameters. Chemical parameters governing the choice of radionuclide include achievable specific activity, stability of the radionuclide/antibody complex after labelling, and that the labelling procedure does not interfere with the immunological activity of the antibody. Biological parameters to consider include tumour type, size, location, antibody kinetics, antigen density and heterogeneity and antigenicity. All these parameters determine the tumouricidal effect, but also the way the patient responds toxicologically.

Table 2 lists radionuclides of current interest in radioimmunotherapy and their physical characteristics.

Table 2. Radionuclides presently used for radioimmunotherapy*

Radionuclide	Half-life	Emission	Imageable	Mean tissue range (mm)	Maximum tissue range (mm)	E_{max} (MeV)
Iodine-131 (^{131}I)	8.0 d	β, γ	Yes	0.4	2	0.81
Yttrium-90 (^{90}Y)	2.7 d	β	No	2.76	12	2.3
Lutetium-177 (^{177}Lu)	6.7 d	β, γ	Yes	0.28	1.5	0.5
Copper-67 (^{67}Cu)	2.6 d	β, γ	Yes	0.6	1.8	0.6
Rhenium-186 (^{186}Re)	3.8 d	β, γ	Yes	0.92	5	1.1
Rhenium-188 (^{188}Re)	17 h	β, γ	Yes	2.43	11	2.1
Bismuth-212 (^{212}Bi)	1 h	α	Yes	0.04-0.1	0.09	6.09
Bismuth-213 (^{213}Bi)	0.77 h	α	Yes	0.04-0.1	<0.1	5.87
Astatine-211 (^{211}At)	7.2 h	α	Yes	0.04-0.1	0.08	5.87
Iodine-125 (^{125}I)	60.1 d	Auger, γ	Yes	0.001-0.02		0.035

* Modified from references (*63, 92, 100*)

Three main categories of radionuclides have been investigated for therapeutic potential in radioimmunotherapy including β-particle emitters, α-particle emitters, and Auger electron-emitters following electron capture. Furthermore γ-ray emission may accompany the above mentioned radiation types during the decay of some radionuclides.

So far the vast majority of preclinical and clinical studies have made use of β-emitting radionuclides such as ^{131}I or ^{90}Y. β-emitters have the advantage of being sufficiently long ranged to treat large solid tumours, often heterogenous in target antigen expression and local hemodynamics (*101, 102*). Therapeutic benefits using β-emitters can be obtained by crossfire, implying that the cell which is targeted by the radiolabelled antibody is not necessarily the target of the decay event. This phenomenon reduces the requirement to target every single tumour cell, thereby bypassing tumour antigen heterogeneity and inadequate vascularisation of the tumour.

α-particle emitting radionuclides are short ranged, high-energy helium nuclei with a high linear energy transfer (LET). As a consequence α-emitters have a high relative biological effectiveness (RBE), which means

that a very low number of nuclear traversals from α-particles are needed to affect the targeted cell. This radiation reduces the ability of cells to repair their damaged DNA and also efficiently kill hypoxic cells. This makes tumour cells targeted by the α-particle emitting antibody and the immediate neighbouring cells sensitive targets (*103*). Consequently, α-emitters like ^{211}At, ^{212}Bi and ^{213}Bi are particularly attractive for treatment of easily accessible tumour cells in the circulation such as leukaemic cells and also micrometastases derived from solid tumours (*104, 105*).

Like α-particle emitters, Auger-electron emitters like ^{125}I have a high LET. Auger-electron emitters deposit a concentrated amount of energy in even shorter distances than α-emitters. This means that these radionuclides need to be located in the vicinity of the tumour cell nucleus to be effective. For this reason, antibodies labelled with Auger-emitting radionuclides need to target the entire tumour cell population for efficient therapy.

Radiobiology

Radiobiology is the branch of biology with focus on the effects of radiation on living organisms. Ionizing radiation is highly energetic and consists of α-, β-, γ and X-rays. These groups can be divided into non-particulate radiation, such as X-rays and γ-rays, and radiation transmitted by energetic charged particles, such as α- and β-rays. Some atoms are radioactive and referred to as radionuclides. These atoms can disintegrate randomly and will loose energy by emitting radiation as α-rays, β-rays and/or γ-rays in a process called radioactive decay. This radiation, if absorbed by a tissue, removes an orbital electron from an atom or molecule, referred to as ionization. X-rays have the same properties as γ-rays when they interact with matter, the only difference being that X-rays are emitted by electrons and not by the nuclei. Ionizing radiations can also be subdivided on basis of intensity of the ionization. This is measured in terms of linear energy transfer (LET), which is the energy transferred per unit length of track. The importance of LET is that if the intensity of ionization can be increased, there will be an increased probability that the radiation energy will be deposited in a biological target, thus increasing the biological effect (*106*). High-LET radiation includes the α-rays which have an immediate impact and directly transfers its energy to vital target molecules. Low-LET radiation includes β-rays, γ-rays and X-rays, which ionize sparsely with more indirect effects, since they interact and transfer their energy to molecules like water. These molecules then generate very reactive free

radicals, which in turn damage DNA. Several types of DNA lesions are induced by ionizing radiation, including changes in the bases of the nucleic acid, breaks in the continuity of the strands of the double helix. Abnormal cross-links formed within the DNA or between DNA and cellular proteins may occur (*106*). The most severe lesions are the DNA double strand breaks which will be lethal for the cell if not repaired.

The cell cycle and radiation effects

DNA damage checkpoints

DNA damage checkpoints are pathways activated in response to DNA damage and when in operation they will delay or arrest cell cycle progression. When exposed to radiation, it is important for the cell to control the structural integrity of genomic DNA for any damage. DNA double strand breaks have for long been implied as the most important DNA lesions for activation of cell death and 1 Gy of radiation has been shown to induce approximately 40 DNA double-strand breaks in an ordinary cell (*107*). Several other DNA lesions including intra-strand and inter-strand cross-links and single-strand breaks can also be induced by radiation (*106*) and the DNA damage checkpoints have virtually to respond to all these types of lesions. If DNA damage is detected, reparation processes are started immediately and signals are initiated and mediated to effector proteins, which activate DNA damage checkpoints, thereby preventing progression through the cell cycle. Activation of the DNA damage checkpoints adds extra time to the reparation machinery to fix the DNA lesion. This delay stimulates and may contribute to organise these reparation processes and may furthermore stimulate induction of apoptosis, since proteins involved in the cell cycle arrest also participate in activation of apoptosis. This is important since DNA damages have the potential to cause genetic mutations and chromosomal rearrangements, which will be inherited by the daughter cells.

DNA damage checkpoints can delay cell-cycle progression in the G1, S or G2 phases. The most important checkpoints for DNA damages acquired by radiation are those in G1, arresting the cell prior to entry into the S-phase, and in G2, arresting the cell prior to mitosis.

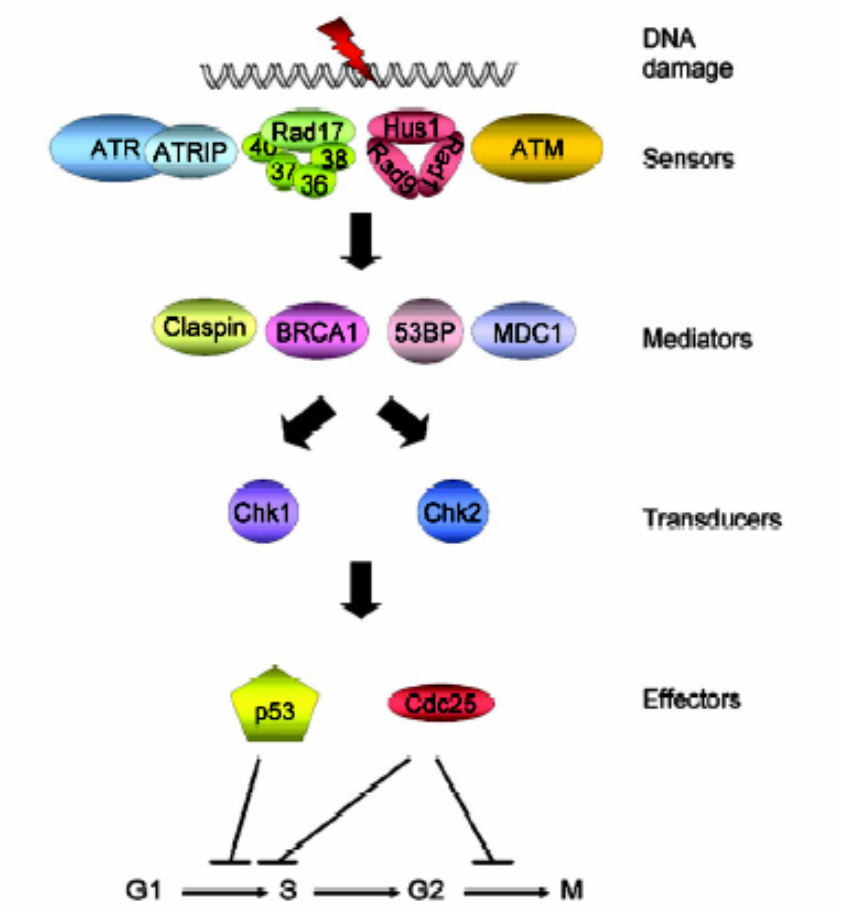

Figure 5. Components of the DNA damage checkpoints in human cells. The damage is detected by sensors that, with the aid of mediators, transduce the signal to transducers. The transducers, in turn, activate or inactivate other proteins (effectors) that directly participate in inhibiting the G1/S transition, S-phase progression, or the G2/M transition.

G1/S DNA damage checkpoint

In cells irradiated in the G1 phase of the cell cycle, ataxia-telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases work as sensors detecting DNA damage. Following activation, ATM and ATR phosphorylate and activate downstream proteins including p53, which in turn transcriptionally upregulates the cyclin-dependent kinase inhibitor p21. P21 then binds to the CDK-cyclin complex inhibiting the G1 to S transition or inducing apoptosis.

G2/M DNA damage checkpoint

The G2/M DNA damage checkpoint is responsible for the detection of DNA damage in cells that are about to enter mitosis and when activated should block this entry. Also at this checkpoint ATM and ATR kinases work as sensor molecules, detecting the DNA damage and transfer regulatory signals to downstream targets, thereby controlling proteins like Cdc2/CyclinB, Wee1, and Cdc25a, proteins crucial for the G2 to M transition. Furthermore p53 has been shown to sustain a G2 arrest following DNA damage by activation of the downstream target molecule p21 and cells either p53-deficient or p21-deficient may initiate a G2 arrest, but may also rapidly escape from this arrest (*108*).

Cell death

Cell death can be executed by different mechanisms, and was earlier looked upon as either necrosis or apoptosis. Apoptosis has for long been considered as a controlled programmed type of cell death, whereas necrosis has been an accidental form of cellular dying. Furthermore apoptosis characteristically induces cellular shrinkage, condensation and margination of the chromatin, and irregularities of the plasma membrane finally will break up the cell into apoptotic bodies, which will be recognised and engulfed by macrophages. Necrosis on the other hand is characterised by cellular swelling, often accompanied by chromatin condensation, which might lead to cellular lysis and a subsequent inflammatory reaction. Nowadays the distinction between apoptosis and necrosis is less clear and several reports indicate that biochemical and morphological characteristics of both modes of cell death can be found in the same cell. This indicates that apoptosis and necrosis are the extremes of a continous spectrum of cell deaths. Classification of cell death has furthermore gone from being regarded as either apoptotic or necrotic to literally explode into new definitions describing different cell death types (apoptosis, autophagy, necrosis and oncosis, mitotic catastrophe, programmed cell death, interphase cell death, senescence) making this area complex and challenging as one cell death type often can be described at least partly by more than one of these definitions (*109-111*).

Apoptotic signalling pathways

Execution of apoptosis is closely linked to the activation of a family of proteases called caspases. At normal conditions these caspases exist in the cell as inactive procaspases and will be activated when the cell encounter

external or internal inducers of the apoptotic machinery. Initiator caspases like caspase-8 and caspase-9 are activated via oligomerization and they then cleave and activate effector caspases like caspase-3, which subsequently activate/inactivate “cellular death substrates”, involved in the regulation and execution of apoptosis. These death substrates include poly(ADP-ribose) polymerase (PARP), inhibitor of caspase-activated deoxyribonuclease (ICAD) and actin (Figure 6).

There are two major pathways which can be involved in the activation of the caspase cascade. The extrinsic pathway (the death receptor pathway) is activated when for instance Fas-ligand binds to its specific cell death receptor Fas, a member of the TNF receptor superfamily. The receptors are localised on the cell membrane and binding of the ligand reorganises the receptors, thereby inducing their oligomerisation. This causes the formation of the death induced signalling complex (DISC), which then recruits and activates caspase-8, which subsequently activates effector caspases (Figure 6).

The intrinsic pathway (mitochondrial pathway) is activated by various extracellular and intracellular stress signals such as DNA damage, hypoxia, growth factor withdrawal, or transcription induction of oncogenes. Generally, radiation induced apoptosis occurs via activation of this pathway, which involves permeabilisation of the outer mitochondrial membrane. This permeabilisation releases cytochrome C that facilitates the formation of the apoptosome-containing adaptor Apaf-1 and caspase-9. Caspase-9 is thereby activated and subsequently activates the rest of the caspase cascade (Figure 6).

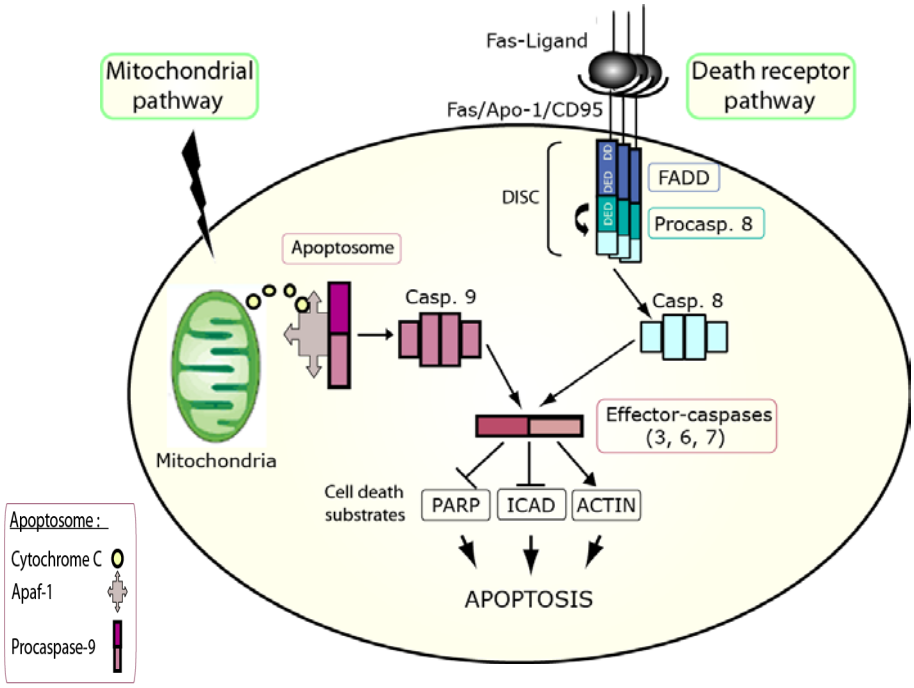

Figure 6. Schematic picture of the two main pathways in apoptosis.
The mitochondrial pathway, which when stimulated leads to the release of cytochrome-C from the mitochondria and activation of the death signal. The second pathway is the death receptor pathway, triggered through the Fas death receptor. Both pathways converge to a final common pathway involving the activation of caspases that cleave regulatory and structural molecules and culminate in the death of the cell.

AIMS OF THE THESIS

- To investigate if the combination of radioimmunotherapy and radiotherapy may improve treatment response in experimental HeLa Hep2 tumours *in vivo* as investigated by:
    - Tumour volume measurements
    - Analysis of the intratumoural morphology
    - Immunohistochemical staining of proliferation markers, apoptotic cells and blood vessels

- To elucidate if apoptosis is of significance in low dose irradiation induced cell death of HeLa Hep2 cells as investigated by:
    - Analysis of distinct apoptotic markers following irradiation
    - Analysis of the involvement of the death receptor and the mitochondrial apoptotic pathways

- To analyse if sequential cell cycle alterations that occur following irradiation are of importance for the subsequent cell death as investigated by:
    - Analysis of DNA-damage checkpoint activation
    - Analysis of mitotic abnormalities
    - Analysis of nuclear alterations

RESULTS AND DISCUSSION

Paper I

The combination of external beam radiotherapy and experimental radioimmunotargeting with a monoclonal anticytokeratin antibody. Cancer. 2002 Feb 15;94(4 Suppl):1314-9
Johansson A, Eriksson D, Ullen A, Löfroth PO, Johansson L, Riklund-Åhlström K, Stigbrand T.

In this paper we have evaluated one potential strategy to improve accretion of radiolabelled antibody in the tumour by combining it with external beam radiotherapy. This study documented improved effects on the accumulated radiation dose to experimental HeLa Hep2 tumours following a combination of radioimmunotargeting using an ^{125}I labelled anti-cytokeratin antibody and external beam radiotherapy. The importance of treatment timing when combining these treatment modalities was also evaluated. The highest accumulated dose, i.e. the highest percentage of injected activity per gram of tumour tissue, as well as the highest accumulated dose per injected activity, was obtained in the group receiving external beam radiotherapy prior to administration of the radiolabelled antibody. This increase in accumulation of the radiolabelled antibody, following pretreatment with external beam radiotherapy, is possibly the result of a decrease in the normally high intratumoural pressure, hyperpermeability of tumour blood vessels and the reduction of cell density in the tumour.
This study indicated that improved treatment effects might be obtained if external beam radiotherapy is given prior to the administration of a radiolabelled monoclonal anti-cytokeratin antibody.

Paper II

Combined low dose radio- and radioimmunotherapy of experimental HeLa Hep 2 tumours.
Eur J Nucl Med Mol Imaging. 2003 Jun;30(6):895-906. Epub 2003 Apr 30.
Eriksson D, Joniani HM, Sheikholvaezin A, Löfroth PO, Johansson L, Riklund Åhlström K, Stigbrand T.

This study was a follow up of paper one. The optimised treatment timing for combined external beam radiotherapy and experimental

radioimmunotargeting obtained from that study was again applied. In this study antibodies were labelled with ^{131}I instead of ^{125}I and furthermore a second monoclonal antibody specific for PLAP was included.
Significant tumour growth retardation was obtained in all treatment groups. The treatment response in the group receiving external beam radiation was transient and after approximately one week the tumours continued to grow quite rapidly. In the three groups receiving radioimmunotherapy alone (^{131}I-anti-PLAP antibody, ^{131}I-anti-cytokeratin antibody, or a combination of both these antibodies) more pronounced and longer lasting tumour growth retardations were observed. Giving radioimmunotherapy alone, the best results were obtained in the group in which both ^{131}I-anti-PLAP antibody and ^{131}I-anti-cytokeratin antibody were administered simultaneously. This is probably because of the homogenous distribution of radiolabelled antibodies throughout the tumour as ^{131}I-anti-cytokeratin antibodies will bind to necrotic areas and ^{131}I-anti-PLAP antibodies to viable regions of the tumour. The most impressive results, however, were detected in the groups receiving external beam radiotherapy in combination with subsequent administration of ^{131}I-labelled antibodies and in these groups a significant reduction in tumour volume could be demonstrated.
Several major treatment related histological alterations were also detected following therapy. When the relative distribution of viable tumour tissue, necrotic tissue, and connective tissue was quantified, a significant decrease in viable and necrotic tissue was detected in the combination treatment group and inversely a pronounced increase of connective tissue was detected. Also in the group given external beam radiotherapy alone, a significant increase in connective tissue was detected. When the relative amounts of tumour cells and cysts were quantified in areas recognised as viable tumour tissue, a significant decrease in tumour cell density and a significant increase of cysts were observed in all treatment groups, again with the most pronounced effects seen in the combination treatment group. Furthermore the most pronounced deviation in cell morphology was observed in the combination treatment group with a marked polymorphism in both cytoplasmatic and nuclear size and shape. Apoptotic cells could be detected in all groups including the control group. In the treated tumour sections, cells positively stained for apoptosis often showed a cytoplasmic swelling and an obvious enlargement of the nuclei.
In conclusion, when external beam radiotherapy is given prior to radioimmunotherapy, efficient treatment is obtained with convincing and long-lasting tumour reduction. This is reflected in a highly aberrant and chaotic histology within the tumour. Furthermore, the cell death responsible for the significant tumour growth retardation showed parallel occurrence of both morphological characteristics of necrosis and molecular biological

features of apoptosis, a phenomenom defined as secondary necrosis or mitotic catastrophe.

Paper III

Cell cycle disturbances and mitotic catastrophes following 2.5-10 Gy of ionizing radiation. Manuscript.
David Eriksson, Per-Olov Löfroth, Lennart Johansson, Katrine Åhlström Riklund, Torgny Stigbrand.

In order to achieve efficient radioimmunotherapy of solid tumours it is important to understand the way tumour cells respond to different doses of radiation at the molecular and cellular level and also the time dependency of these mechanisms. In this study we examined the induced sequential events in Hela Hep2 cells exposed to 2.5-10 Gy of ionizing radiation, with special emphasis on cell cycle arrest, mitotic aberrations and finally cell death. This study shows that a transient G2/M cell cycle arrest is induced in Hela Hep2 cells following irradiation. The cells then reenter the cell cycle prematurely before completely finishing reparation of the DNA damages. This premature entry is followed by disturbed mitoses with an increased frequency of anaphase bridges, lagging chromosomal material and multipolar mitotic spindles. Furthermore a dose dependent significant increase in centrosome numbers in mitotic cells was documented. This hyperamplification of centrosomes might be the cause or a consequence of a failure to complete cytokinesis following irradiation and may be a critical event contributing to the radiation induced cell death now observed. The mitotic disturbances yielded a number of nuclear abnormalities. Irradiated cells often contained one or several micronuclei, formed by nuclear membrane formation around lagging chromosomes or chromosomal material. Furthermore an enhancement of the fraction of cells with several nuclei, probably a consequence of cytokinesis failure, as well as abnormally shaped multilobulated nuclei, could be identified in irradiated cells. An increased fraction of polyploid cells was obtained following irradiation, especially in the treatment group receiving the highest dose of radiation indicating that HeLa Hep2 cells have no functional polyploidy checkpoint, which would otherwise arrest the cell in the subsequent G1 after a failure to complete cytokinesis.

In conclusion this paper elicits the transient activation of the G2/M DNA damage checkpoint, followed by a sequence of disturbed mitoses, the formation of multinuclear polyploid cells and cells with multiple micronuclei, and finally death via mitotic catastrophe and delayed apoptosis,

which occurs in HeLa Hep2 cells following 2.5-10 Gy of ionizing radiation. This indicates that the growth inhibitory effects exerted by radioimmunotherapy may be geared by cell cycle alterations, mitotic catastrophes and delayed apoptosis.

Paper IV

Apoptosis in HeLa Hep2 cells is induced by low-dose, low-dose-rate radiation. Radiat Res. 2002 Nov;158(5):634-40.

Mirzaie-Joniani H, Eriksson D, Johansson A, Löfroth PO, Johansson L, Åhlström KR, Stigbrand T.

This study was performed to elucidate if radiation doses and dose rates of a level comparable to radioimmunotherapy are capable to induce apoptosis in HeLa Hep2 cells *in vitro*. Cells were exposed to defined radiation doses (1, 2, 5, 10, or 15 Gy) with two different dose-rates (0.80± 0.032 Gy/min or 0.072± 0.003 Gy/min) from a ^{60}Co radiation therapy source. Using a ^{137}Cs source, the cells were furthermore irradiated with three different doses (2, 5, or 10 Gy) with a dose rate that was reduced to 0.045 Gy/h. Following radiation, apoptosis was detected and quantified using either fluorescein-labelled Annexin V followed by flow cytometry or DNA ladder analysis. Compared to control cells a pronounced induction of apoptosis was induced after treatment with 5-10 Gy of irradiation. All dose-rates induced significant apoptosis, which was most marked 72-168 hours following irradiation. It can be concluded that low dose, low dose rate radiation is able to induce significant apoptosis in HeLa Hep2 cells *in vitro* and thus may explain a mechanism by which pronounced tumour growth retardation of HeLa Hep2 tumours previously has been obtained by radioimmunotherapy.

Paper V

Apoptotic signalling pathways induced in HeLa Hep2 cells following 5 Gy of ionizing radiation. Manuscript.
David Eriksson, Homa Mirzaie-Joniani, Per-Olov Löfroth, Lennart Johansson, Katrine Åhlström Riklund, Torgny Stigbrand.

The purpose with this study was to elucidate the apoptotic signalling pathways involved in the delayed type of apoptosis that was observed in

HeLa Hep2 cells following low dose irradiation (**Paper IV**). The activation of caspases was studied and several were shown to be induced including initiator caspases-2, -8, -9, and effector caspase-3. This delayed apoptosis-like cell death seems to rely on an interplay between components from the extrinsic (caspase-8) as well as the intrinsic (caspase-2 and caspase-9) signalling pathways. An increased expression of the Fas receptor could be detected following irradiation and correlated well in time with the increased activation of caspase-8. PARP, a protein that is known to facilitate DNA repair and also is known as a caspase-3 cell death substrate, was found to be inactivated by cleavage. Furthermore, cycloheximide a protein synthesis inhibitor completely inhibited the caspase activation and the associated apoptosis, while a caspase-2 inhibitor, increased the survival and partly inhibited the radiation induced caspase activation in HeLa Hep2 cells following irradiation.

In conclusion, activation of the apoptotic signalling pathways following irradiation of HeLa Hep2 cells include components from the intrinsic as well as the extrinsic pathways and seem to require caspase-2 activation and *de novo* protein synthesis.

GENERAL DISCUSSION

Although significant progress has been made in treatment of tumour diseases, current therapies fail to efficiently cure several common types of malignant diseases. Novel therapeutic strategies are required and include approaches in which the tumour cells are specifically targeted and efficiently killed. Monoclonal antibodies have the potential to do so and available clinically useful antibodies typically use a combination of mechanisms to exert cytotoxic effects on tumour cells (*62, 91, 112-115*). Most monoclonal antibodies are able to interact and activate components of the immune system via the Fc-domain. This includes ADCC, a process at which antibodies will bind to their target antigen on tumour cells and the antibody Fc-domain engage Fc receptors on the surface of immune effector cells (NK-cells, monocytes/macrophages and granulocytes). Cross-linking of the receptors triggers the release of cytoplasmic granules containing perforin and granzymes, which lyse the tumour cell. Furthermore complement dependent cytotoxicity is another effector mechanism, which might be activated when monoclonal antibodies bind to antigen on tumour cells. The binding causes exposure of binding sites on the monoclonal antibody for proteins important for initiation of the complement cascade. This leads to release of chemotactic factors and recruitment of more immune effector cells to the tumour and also to formation of the membrane attack complex, which promotes target-cell lysis. Besides activating the immune system, some monoclonal antibodies target growth factor receptors on tumour cells and block ligand binding and downstream signalling events that normally would induce cellular proliferation and resistance to cytotoxic agents. Finally, monoclonal antibodies can function as carriers of chemotherapeutics, enzymes, toxins or radionuclides.
In radioimmunotherapy, labelling of antibodies with radionuclides can potentiate the effect of the antibody alone and increases the tumour response (*116*). Currently, there are several major challenges in radioimmunotherapy:

- to increase the poor accumulation of radiolabelled antibody within the tumour,
- to reduce the background radioactivity from circulating non-targeted antibody
- to specifically modulate the cell cycle and the cell death pathways to increase the radiation induced cell death.

Several strategies to improve radioimmunotherapy in each of these areas have been investigated and will be discussed below.

Modulation of the cell cycle and the cell death pathways

To characterise the type of cell death caused by radioimmunotherapy is a complex mission, since new characteristics of the different types of cell death continuously arise and often overlap earlier definitions. Cell death induced by ionizing radiation has been described to occur directly or indirectly by several mechanisms including necrosis, rapid or delayed apoptosis and mitotic catastrophe with or without apoptosis (*107, 117, 118*). Classical apoptosis is rapidly induced in the interphase within hours (*118*) following irradiation, and it usually occurs in cells highly sensitive to radiation, as those from the lymphoid and myeloid lineages (including lymphomas). The most frequent mode of cell death, however, is mitosis-linked death and although morphologically distinct from apoptosis, it may include activation of the apoptotic machinery (*107, 117, 118*). This cell death is a result of disturbed mitoses in which failure of accurate chromosome segregation and failure in cytokinesis induce formation of micronuclei or binucleated giant cells respectively (*107*). These damaged cells do not necessarily die immediately, but may continue through several cycles of cell divisions, acquiring an increasing chromosomal as well as genomic instability, finally causing cell death.

This thesis documents that an apoptosis related cell death occurs in HeLa Hep2 cells, both *in vitro* (**paper III, IV, V**) and *in vivo* (**paper II**), following low dose, low dose rate radiation. Compared to classical apoptosis, this cell death is delayed and most easily observed 3-7 days instead of hours, following irradiation. It is accompanied by cellular swelling instead of cellular shrinkage and is a postmitotic, not a premitotic, phenomenon.

The kinetics of this death correlates well in time with the mitotic disturbances that follows treatment with ionizing radiation eventually generating the mitotic catastrophe in HeLa Hep2 cells. Mitotic catastrophes were observed to follow after a transient G2 arrest, subsequent entry into mitosis, and hyperamplification of the centrosome number (**paper III**). Centrosome hyperamplification is probably either the cause of the subsequent cytokinesis failure, or a consequence of the same. Hyperamplification of centrosomes might be an essential activating mechanism, which induces the sequential mitotic catastrophes and cell deaths following irradiation. This abnormal amplification of centrosomes occurred approximately at the same time as an increased fraction of cells with multipolar mitotic spindles, lagging chromosomal material, and anaphase bridges were seen.

Anaphase bridges may be generated when broken chromosomes, either induced by irradiation or triggered by abnormal shortening of the telomeres, fuse. If several chromosomal fragments contain a centromere, the force during mitosis on such chromosomes will be substantial when the mitotic spindle pulls these centromers in opposite directions. Anaphase bridges consisting of chromatin bridges will be generated and they usually resolve by new chromosomal breakages generating secondary DNA damages. Radiation and DNA damage have earlier been shown to induce the intrinsic apoptotic pathway, mainly via caspase-9 activation. Lately caspase-2 has gained increased interest as the most apical of the caspases involved in the intrinsic pathway(*119-121*). Caspase-2 is also important for the apoptosis-related cell death, which follows mitotic catastrophes (*119*), which we also observed in **paper V**. There are several possible mechanisms of caspase-2 activation following DNA damage including dimerisation of procaspase-2 and a subsequent autocatalytic cleavage that promotes its stable dimerisation and further enhances its catalytic activity (*121*) (Figure 8). Another possibility is that caspase-2 becomes activated in a complex similar to the apoptosome (Figure 6). The PIDDosome is such a complex, which includes PIDD, a p53-inducible death domain-containing protein, that binds pro-caspase-2 (*121*) (Figure 8).

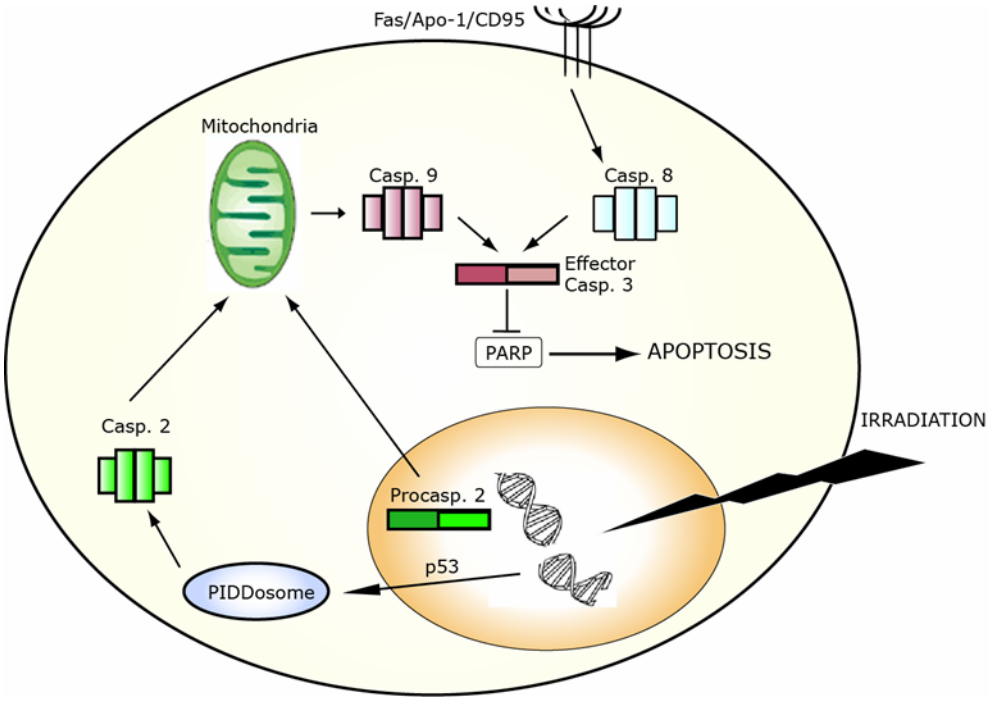

Figure 8. The apoptotic signalling cascade in HeLa Hep2 following ionizing radiation (**paper V**). Two potential ways to caspase-2 activation are visualised.

Furthermore an induction of caspase-8 was observed that correlated well in time with an increased expression of Fas (**paper V**), which can be upregulated by the tumour suppressor p53 following DNA damage. Increased Fas expression following radiation probably activates caspase-8 in a Fas-ligand independent clustering of the Fas receptor. Caspase-8 may also be activated when the intrinsic pathway has been activated, via an amplification loop.

Cell cycle arrests and apoptotic execution are two of the most important determinants of sensitivity to ionizing radiation. Such radiation induces DNA damages that can be detected by the cell and initiate signals that activate either DNA damage checkpoints, which increase the time for DNA repair, or execution pathways, which cause cell death. The activation of both the regulatory cell cycle pathway and the apoptotic signalling pathway involves several key proteins and an increased understanding of the kinetics of this response could enable specific modulation of defined signalling pathways. Several strategies in which these key molecules or the kinetics of the radiation induced cell response have been modulated are currently investigated. These studies will be discussed below in connection to the results obtained in this thesis.

Cell cycle modulation

Tumour cells often comprise an impaired G1/S cell cycle checkpoint, still with the G2/M cell cycle checkpoint intact, which seems to be a reality also in HeLa Hep2 cells (**paper III**). A radiation induced G2/M-arrest is normally observed in tumour cells and this arrest may provide the cell with extra time to repair damaged DNA before entering mitosis. Agents that abrogate this G2/M checkpoint have been shown to sensitize tumour cells to ionizing radiation. These G2/M checkpoints abrogators include caffeine and staurosporine analogs including UCN-1, which have been shown to enhance tumour cell radiosensitivity (*122, 123*).

Several studies have demonstrated that cells respond differently to radiation depending on which cell cycle phase they are in. Usually, cells that are in the M-phase or G2-phase of the cell cycle are considered to be most radiosensitive, opposite to cells in the S-phase which are considered radioresistant (*106, 124*). This fact has been explored in a number of combination therapy studies, in which tumour cells have been synchronised and arrested in cell cycle phases in which these cells are more

radiosensitive. Chemotherapeutic agents have been used for this purpose with the taxanes, paclitaxel and docetaxel included, which block cells at the radiosensitive G2/M phase of the cell cycle (*125, 126*). Similarly, radiation synchronises and arrest tumour cells in G2/M of the cell cycle and might be one rationale behind the efficacy of fractionated radiotherapy (*127, 128*). Furthermore if radiotherapy is combined with radioimmunotherapy, this phenomenon can be explored and taken advantage of. For synergy to occur, the appropriate timing of radiotherapy in relation to radioimmunotherapy is crucial. Radiotherapy should be given so that the maximal fraction of tumour cells is synchronised in G2/M when the radiolabelled antibody starts to accumulate in the tumour. Concurrent administration of the therapies or administration of radioimmunotherapy 1-2 days prior to radiotherapy is probably optimal to fully explore this strategy. This is also supported by Sun et. al. (1997) combining radioimmunotherapy with external beam radiotherapy in human colon carcinoma xenografts and they found that best tumour responses were obtained when both therapies were applied simultaneously. Cells within a tumour however are extremely heterogenous with regard to their cell cycle characteristics compared to cells at controlled culture conditions. Furthermore the timing of synchronisation may vary between different cell lines, which make the optimal synchronisation almost impossible to achieve clinically. When we combined radiotherapy and radioimmunotherapy in **paper II**, the radiolabelled antibodies were administered several days after the radiotherapy, which means that the cell fraction still arrested in the G2/M checkpoint is small when these antibodies start to accumulate in the tumour (see **paper III)**. This may indicate that the convincing treatment results obtained in **paper II** most likely are not the result of cell synchronisation in a radiation sensitive cell cycle phase. Instead other potential positive combination effects can explain this as described below.

Modulation of Apoptosis

Modulation of the apoptotic response by interfering with the specific signal transduction pathways may enhance the therapeutic effect of radiation. Furthermore it may be possible to use radiation to make tumour cells more sensitive for defined pro-apoptotic stimuli.

Modulation of the apoptotic threshold, by inhibiting the epidermal growth factor (EGFR) known to activate anti-apoptotic signalling pathways and increase radiation resistance, has been extensively studied (*129-131*). An antibody against EGFR (C225/cetuximab) increased the radiation induced apoptosis and various Phase-I, -II and -III studies testing this combination have been initiated (*131, 132*). A large fraction of human tumours display mutations in the p53 gene or lack p53 expression or function. P53 has been

shown to be important but not crucial for the induction of radiation induced apoptosis. Loss of early apoptosis is balanced by a delayed wave of p53-independent apoptosis, which presumably is a consequence of mitotic catastrophes (*133*). However, certain tumours that have p53 mutations may evade radiation induced apoptosis and restoration of wild-type p53 function may therefore potentiate the radiation response of these tumours (*134-137*).
Another approach to modulate the apoptotic threshold is by inhibition of the nuclear transcription factor NF-κB, which is known to be constitutively activated in a variety of human malignancies. NF-κB controls the expression of anti-apoptotic and cell survival proteins and is activated in response to cellular stress including radiation (*138*). Studies in which NF-κB activity has been inhibited using a selective inhibitor (PS-341) indicates improved therapeutic tumour response when combined with radiotherapy (*139-142*).
TRAIL, a member of the death ligand family has been shown to induce substantial apoptosis in many tumour cells (*143, 144*). Radiation can be used to sensitise tumour cells to TRAIL and combination treatment has so far been promising with additive and also synergistic effects (*145-147*). Upregulation of the TRAIL receptor has been observed following irradiation but may not be the sole mechanism by which sensitisation to TRAIL following irradiation is induced (*145, 148*). A similar upregulation has been observed also for Fas, another cell death receptor, following irradiation (*149-151*). This upregulation has been shown to initiate a Fas Ligand-independent Fas receptor trimerisation and a subsequent activation of the Fas death pathway (*150, 152*). Ionizing radiation has also been used to sensitise tumour cells to Fas–induced apoptosis and has generated promising results (*151*). This approach is attractive and is currently being evaluated in our group, as we also observed an increased expression of the Fas receptor following irradiation (**paper V**).
TNF-α is an attractive therapeutic protein, which has a broad range of anticancer effects, including direct tumour cell cytotoxicity, antiangiogenic properties, and enhancement of antitumour immunity (*153*). TNF-α expression has been shown to be increased after cellular exposure to ionizing radiation (*154*). TNF-α furthermore has been shown to enhance the cytotoxic effects of radiation both in tumour cells and in experimental tumours (*154-158*). When given systemically, however, TNF-α is not well tolerated and cause severe side-effects (*159, 160*). Connecting TNF-α to a recombinant antibody specifically delivering TNF-α to the tumour would probably reduce the cytotoxic side-effects. We are currently investigating the therapeutic effects of a TNF-α scFv conjugate as a single treatment modality and also as a combination with antibodies labelled with

radionuclides. As TNF-α has been shown to induce necrosis in the centre of tumours (*161*), an interesting approach would be to combine TNF-α scFv with a ^{131}I-anti-cytokeratin antibody, which target the necrotic regions of tumours.

How to improve antibody accumulation within the tumour?
The efficacy in treatment of solid tumours with radioimmunotherapy has been limited by inability of the monoclonal antibodies to reach all regions of a tumour *in vivo* in adequate quantaties. To reach a malignant cell within a tumour, the administered antibody has to make its way into the blood vessels of the tumour. Within the tumour extravasation occurs and the antibodies are transported into the interstitium via diffusion and convection. Finally, the antibodies migrate via the interstitium to target the tumour cells. One significant problem in radioimmunotherapy is that tumours often develop and expand in ways that hinder these steps, which leads to a poor localisation of radiolabelled antibodies within the tumour (*162*). The factors affecting this include heterogenous blood supply, which will severely decrease delivery of the antibodies to regions of the tumour not well perfused (*102, 163, 164*). An elevated interstitial pressure is also often found in solid tumours, which reduces the extravasation of antibodies from the vessels into the tumour tissue (*102, 163, 164*). Furthermore, large transport distances in the interstitium increase the time required for slowly moving intact antibodies to reach distal regions within a tumour (*102, 163, 164*). Finally, low accumulation might also depend on heterogeneity in the expression of tumour associated antigens. If strategies, which could increase the fraction of antibody which targets the tumour can be developed, one important step towards establishing radioimmunotherapy as a more general treatment modality is taken.

Antibody/Radionuclide cocktails
Heterogeneity in antibody and radiation dose distribution within the tumour following radioimmunotherapy is a known phenomenom. This problem relates to heterogeneity in antigen expression, with some tumour cells expressing no antigen and others being weakly or strongly positive. Selected mixtures of antibodies to different antigens are therefore likely to react with more cells than single antibodies, giving a more homogenous dose distribution and a more effective therapy. This approach has been shown to be more efficient than using either antibody alone (*165-167*). This is also in agreement with the results obtained in **paper II** in which radioimmunotherapy given alone, generated the best results in the group in which both ^{131}I-anti-PLAP antibodies and ^{131}I-anti-cytokeratin antibodies were administered simultaneously. This is probably because of the

homogenous distribution of radiolabelled antibodies throughout the tumour as ^{131}I-anti-cytokeratin antibodies will bind to necrotic areas and ^{131}I-anti-PLAP antibodies to viable regions of the tumour.
A combination of radionuclides with properties optimal for primary tumour treatment (β-particle emitters) or treatment of micrometastases (α-particle emitters, Auger-electron emitters) has the potential to improve the dose distribution and serve to improve the therapeutic ratio at radioimmunotherapy.

Combination treatment
In order to obtain additive or even synergistic therapeutic effects, radioimmunotherapy can be combined with other treatment modalities. Such an approach, explored in several studies in both animals (*168-172*) and patients (*173-175*), is to combine radioimmunotherapy with external beam radiotherapy. The potential advantages include spatial cooperation since clinically undetectable micrometastases outside the external beam radiation field can be specifically targeted with radiolabelled antibodies. The major tissue toxicity following radioimmunotherapy appears in the bone marrow with myelosuppression and for external beam radiotherapy negative side effects occur in the normal tissues closest to the tumour. Since radioimmunotherapy and external beam radiotherapy present different tissue toxicity due to differences in dose distribution, the overlap of the toxicities from radioimmunotherapy and external beam radiotherapy will be minimal. This may lead to a relative sparing of normal tissue or that an increased radiation dose can be delivered to the primary tumour without increasing toxicity significantly. Finally there are several hypotheses why a combination of these treatment modalities potentially should be able to induce a synergistic treatment response, if the optimal sequence of administration is used (figure 7).

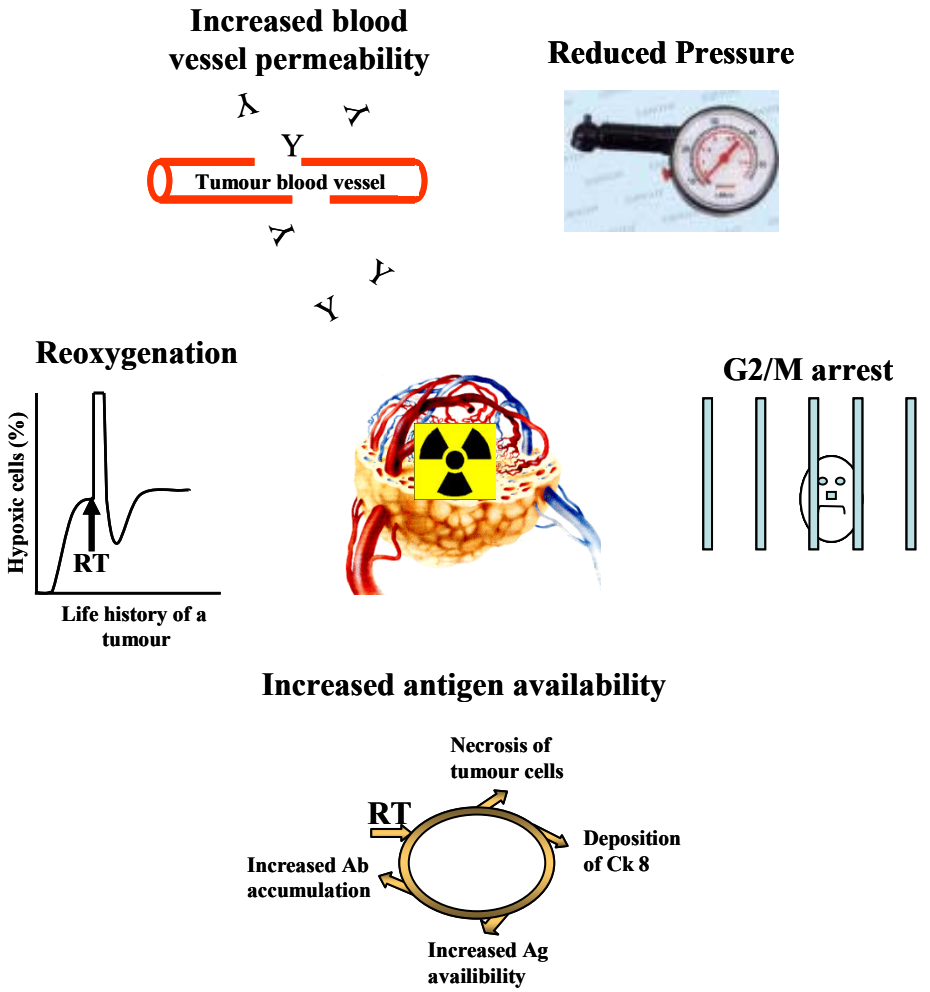

Figure 7. Mechanisms explored when radioimmunotherapy is combined with radiotherapy.

External beam radiotherapy has been shown to reduce the interstitial fluid pressure in tumours and if given prior to radioimmunotherapy an increased antibody accretion can be obtained (*176, 177*). External beam radiotherapy also induces hyperpermeability in tumour blood vessels, which would further enhance the antibody accretion (*101, 169, 171*). This increase in blood flow is however short-lived and usually restricted only to regions of the tumour, which are well vascularised. If a primary dose of external beam radiotherapy induces necrotic cell death, in which the intracellular content is released within the tumour, an increased deposit of cytokeratin 8 will occur. Using antibodies with specificity for this antigen, like TS1, means that there may be increased antigen available for these antibodies, if they are administered after external beam radiotherapy. Besides increasing antibody accretion, combining radioimmunotherapy with external beam radiotherapy

may affect other parameters that potentially increase the therapeutic response. Due to insufficient blood supply, tumours often contain hypoxic regions. The proportion of hypoxic cells in a tumour will influence its response to irradiation as hypoxic cells are less radiosensitive than well oxygenated. If a tumour is treated with external beam radiotherapy, the proportion of hypoxic cells will increase as the major part of the dying cells are well-oxygenated. However after some time there will be an increased availability of oxygen for these hypoxic cells, following reduced consumption by damaged cells, and an improved circulation due to tumour shrinkage (*106*). If radioimmunotherapy is applied in such a way that the radiolabelled antibodies target the tumour when this reoxygenation has occurred, an increased treatment response will be obtained. Furthermore, if the tumour is treated with external beam radiotherapy, tumour cells may be forced into and partly synchronised in a G2/M-arrest, which is a phase of the cell cycle in which cells are known to display an increased sensitivity for radiation. Again radioimmunotherapy has to be administered at an optimal time point so that antibody accumulation occurs at the time when tumour cells are still arrested in this radiosensitive phase.
In order to explore these potential combination effects optimally, the sequence of administration is important and has been studied in several works (*170, 171*) (**paper I**). Differences in the optimal administration sequences probably reflect the different tumour models used and that the above mentioned parameters are more or less important in these systems for an optimal combination treatment design.

In **paper I** the highest antibody accretion was obtained in HeLa Hep2 tumours when external beam radiotherapy was given prior to radioimmunotherapy. In **paper II** we used this optimised combination treatment design to treat nude mice bearing HeLa Hep2 solid tumours on their back. Significant reduction in tumour volume could be demonstrated and a pronounced deviation in cell morphology with a marked polymorphism in both cytoplasmatic and nuclear size and shape.
In **papers III, IV,** and **V** we observed that in HeLa Hep2 cells, death occurs 3-7 days following radiation. This is one observation that might explain the improved antibody accretion and therapeutic tumour response obtained when a radiolabelled antibody is administered days after treatment with external beam radiotherapy. During this time period a substantial amount of tumour cells will die, be cleared by phagocytic cells, with simultaneous appearance of cysts (**paper II**). The drop in cell density facilitates antibody penetration into the tumour and also reduces the intratumoural pressure, and increase perfusion of antibodies into the tumour. The use of a combination of radioimmunotherapy and external beam radiotherapy is a novel technique

with great potential that can increase the tumour treatment response if the timing of administration can be optimised.

Optimizing accretion by antibody engineering
One approach to achieve a high accretion and deep and more homogenous penetration of radiolabelled antibody into the tumour is to use smaller antibody fragments. However if these antibodies should be successful in radioimmunotherapy, they must be able to retain high affinity, not be immunogenic, since repetitive or continous administration will be needed to compensate for their rapid clearance from blood.

How to reduce the background radioactivity from circulating antibody?
Delivery of radiation doses sufficient to induce significant tumour growth retardation is one of the major challenges in radioimmunotherapy. Only a fraction of the injected antibody will target the tumour, and as little as 0.1 % of the injected antibody will be taken up per gram of tumour tissue in man (*178, 179*). High doses of radiolabelled antibody therefore need to be administered, increasing the risk for radiation mediated toxicity, especially of the bone marrow, thereby limiting the dose of radiolabelled antibody that can be used. These side-effects are caused by slow blood clearance of the antibody-radionuclide complex, resulting in a long circulation time in the vasculature, which reduces the tumour to normal tissue ratio. Several strategies to reduce background radioactivity from circulating antibody are currently being investigated and will be discussed below.

Antibody engineering for increased clearence
One strategy to reduce the circulation time of the antibody-radionuclide complex is to develop small antibody fragments, which retain their ability to target the tumour, but are cleared more rapidly from the circulation. Antibody fragments like $F(ab)_2$ and Fab' are produced by enzymatic cleavage and have been used in several clinical and experimental studies (*180-186*). These fragments demonstrated excellent tumour penetration, more rapid clearance, but also a reduced tumour residence time compared to intact IgG antibodies, but still they were able to produce good therapeutic results. Smaller scFv constructs can be produced by recombinant DNA technology in various forms and include scFv, $(scFv)_2$ and diabodies. ScFv are probably not optimal for radioimmunotherapy as their size and their high positive charge affect the filtration rate and excretion through the kidneys, which leads to a rapid renal clearance and therefore a small total tumour uptake (*187-189*). The tumour penetration is however excellent and the rapid clearance makes these small antibody derivates suitable for

radioimmunolocalisation of tumours. Larger recombinant antibody derivates like $(scFv)_2$ and diabodies have molecular weights above the range for rapid renal clearance. They are also bivalent, which enable them to retain their affinity, and have good tumour penetration when compared to intact antibodies and therefore should be suitable for delivering payloads to tumours (*184, 190*). We are currently evaluating a $(scfv)_2$ antibody against PLAP for its tumour targeting capacity and the results are encouraging with a significant accumulation of the antibody in the tumour, good tumour residence time, and still rapid clearance of circulating non-bound antibody (data not published).

Removal of circulating non-targeting antibody

Another way to reduce the background radioactivity is by using various techniques to increase the kinetics by which circulating non-bound antibody is cleared.

Pretargeting

In order to increase the tumour to normal tissue ratio in radioimmunotherapy, pretargeting techniques have been developed, based upon bi-specific immunoconjugates that target tumour antigen and a small molecule carrying the radionuclide (*191, 192*). Two main approaches can be distinguished. The first approach takes advantage of the high-affinity binding between streptavidin/avidin and biotin and several approaches using this strategy have been investigated. This includes a two step approach, in which the first administration consists of antibodies labelled with biotin or streptavidin. When these antibodies have cleared from the circulation this is followed by administration of radiolabelled-streptavidin or radiolabelled-biotin respectively. In a three step procedure biotinylated antibodies are cleared from the circulation by administration of streptavidin or avidin that will also bind to the biotinylated antibodies localised in the tumour. Finally radiolabelled biotin is administered. The second main approach utilises bi-specific anti-tumour/anti-hapten antibodies as a pretargeting step, which is followed by a second step in which a radiolabelled bivalent hapten is administered.

Secondary antibodies

Secondary antibodies can be used as clearing agents for circulating non-tumour targeted anti-tumour antibodies and have demonstrated efficiency in

several experimental studies, generating an increased tumour to normal tissue ratio (*193, 194*). The secondary antibodies bind to the primary anti-tumour antibodies and form immune complexes. These complexes are rapidly deposited in the liver, an organ in which the antibody-radionuclide complex is metabolised, followed by excretion of free radionuclide via the urine. Secondary antibodies as clearing agents were introduced by Spar et. al. already in 1964 when he used polyclonal antibodies to clear primary radiolabelled antibodies (*195*). Since then several studies using polyclonal antibodies as clearing agents have been conducted, but with the introduction of monoclonal syngenic anti-idiotypic antibodies as clearing agents, the specificity and the precision by which non-tumour targeting antibodies can be cleared has significantly increased (*77, 194, 196*). Lately we were able to show that in addition to intact anti-idiotypic IgG also anti-idiotypic $(Fab')_2$ and Fab fragments are able to clear idiotypic antibodies from circulation (*197*).

Extracorporeal immunoadsorption

There is a possibility to reduce the level of non-tumour-targeted antibodies by immunoadsorption *ex vivo*. This method is called extracorporeal immunoadsorption (ECIA or ECAT) and makes use of an elimination strategy that clears the circulation from non-tumour-binding antibody by passage of the blood through an adsorptive column. This system exploits immobilised anti-species antibodies or if used in a pretargeting system affinity interaction between avidin and biotin. Extracorporeal immunoadsorption has been tested and evaluated for its efficacy both for imaging and therapy in animals and patients (*198-203*).

FUTURE ASPECTS

Although intense research has been conducted over the last 30 years in efforts to establish radioimmunotherapy as a new treatment modality of malignancies, there is still a need for significant improvements in this area. Radioimmunotherapy has so far had most success in the treatment of hematological malignancies and in January 2006 two radioimmunotherapeutics have been approved for clinical use. When radioimmunotherapy has been used for treatment of solid tumours, the progress has been much slower, and targeting minimal and micrometastic disease appears at present to be the optimal objective for this therapy. However, a number of putative improvements are currently being investigated in order to improve the radiation dose delivered and to achieve a more uniform distribution of ionizing radiation to the tumours.
Engineering of antibodies with reduced immunogenicity (human, humanised, chimeric) eliminates the HAMA response, making it possible to treat solid tumours with multiple administrations of radiolabelled antibodies. Construction of engineered antibody formats with optimised properties, such as tumour penetration, clearence and binding affinity have also been given considerable attention and we are currently evaluating a collection of recombinant antibody derivates for their targeting capacity.
Several promising pretargeting strategies that reduce the systemic radiation caused by circulating non-targeted radiolabelled antibody have been developed in order to improve tumour to normal tissue ratios. Improved understanding of the activation of cell-cycle checkpoints, the induction of cell cycle disturbances, and the initiation of signalling pathways involved in the radiation induced cell death will improve the strategy by which radioimmunotherapy can be applied. Understanding the effector mechanisms responsible for the execution of radiation induced cell death makes it possible to combine radioimmunotherapy with agents that enhance/reduce the cascade of molecular events required for tumour cell death following continous low dose radiation from radioimmunotherapy.
It is my personal belief that in the future, radioimmunotherapy of solid tumours will not be made by use of a single radiolabelled antibody. Instead cocktails of antibodies, binding to different regions of the tumour will be administered leading to a more homogenous distribution of ionizing radiation throughout the tumour.
Furthermore, antibodies probably will not be labelled with only one radionuclide. Instead a mixture of radionuclides with different properties can be used so that both the larger primary tumour and micrometastic disease can be targeted by antibodies labelled with radionuclides with appropriate characteristics for efficient tumour eradication. Combining

radioimmunotherapy with radiotherapy or chemotherapy might prove to be essential for complete eradication of the tumour and as we show in this thesis, it is important to establish the useful time frames available for treatment.

CONCLUSIONS

- Treatment of malignant tumors by delivering radiotherapy prior to radioimmunotherapy is a new approach to improve antibody accretion within the tumor and to improve the therapeutic response for HeLa Hep2 tumours at experimental conditions.

- Dramatic changes in tumour morphology were observed, with significantly reduced cell density and appearance of necrotic regions, as well as an increase of connective tissue, cysts and giant cells.

- These giant cells displayed heterogeneity in cytoplasmic and nuclear size and shape, with some cells staining positive for apoptotic markers.

- *In vitro* studies on HeLa Hep2 cells showed that ionizing radiation induced a transient G2/M arrest, followed by hyperamplification of the number of centrosomes, disturbances of the mitosis and induction of subsequent mitotic catastrophes and delayed apoptosis.

- This delayed apoptosis-like cell death was induced following absorbed doses and dose rates similar to those obtained by radioimmunotherapy

- Caspases belonging to both the intrinsic and the extrinsic apoptotic pathways were activated and caspase-2 was found to be important for this delayed apoptosis.

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REFERENCES

1. N. A. Lokker, C. M. Sullivan, S. J. Hollenbach, M. A. Israel and N. A. Giese, Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. *Cancer Res* **62**, 3729-3735 (2002).
2. D. George, Targeting PDGF receptors in cancer--rationales and proof of concept clinical trials. *Adv Exp Med Biol* **532**, 141-151 (2003).
3. A. Ostman, PDGF receptors-mediators of autocrine tumor growth and regulators of tumor vasculature and stroma. *Cytokine Growth Factor Rev* **15**, 275-286 (2004).
4. R. Board and G. C. Jayson, Platelet-derived growth factor receptor (PDGFR): a target for anticancer therapeutics. *Drug Resist Updat* **8**, 75-83 (2005).
5. B. L. Ziober, J. K. Willson, L. E. Hymphrey, K. Childress-Fields and M. G. Brattain, Autocrine transforming growth factor-alpha is associated with progression of transformed properties in human colon cancer cells. *J Biol Chem* **268**, 691-698 (1993).
6. D. Jiang, H. Yang, J. K. Willson, J. Liang, L. E. Humphrey, E. Zborowska, D. Wang, J. Foster, R. Fan and M. G. Brattain, Autocrine transforming growth factor alpha provides a growth advantage to malignant cells by facilitating re-entry into the cell cycle from suboptimal growth states. *J Biol Chem* **273**, 31471-31479 (1998).
7. D. Wang, W. Li, W. Jiang, L. E. Humphrey, G. M. Howell and M. G. Brattain, Autocrine TGFalpha expression in the regulation of initiation of human colon carcinoma growth. *J Cell Physiol* **177**, 387-395 (1998).
8. A. Zaczek, B. Brandt and K. P. Bielawski, The diverse signaling network of EGFR, HER2, HER3 and HER4 tyrosine kinase receptors and the consequences for therapeutic approaches. *Histol Histopathol* **20**, 1005-1015 (2005).
9. J. R. Grandis and J. C. Sok, Signaling through the epidermal growth factor receptor during the development of malignancy. *Pharmacol Ther* **102**, 37-46 (2004).
10. B. Aunoble, R. Sanches, E. Didier and Y. J. Bignon, Major oncogenes and tumor suppressor genes involved in epithelial ovarian cancer (review). *Int J Oncol* **16**, 567-576 (2000).

11. D. Hanahan and R. A. Weinberg, The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
12. A. M. Helt, J. O. Funk and D. A. Galloway, Inactivation of both the retinoblastoma tumor suppressor and p21 by the human papillomavirus type 16 E7 oncoprotein is necessary to inhibit cell cycle arrest in human epithelial cells. *J Virol* **76**, 10559-10568 (2002).
13. M. Hollstein, D. Sidransky, B. Vogelstein and C. C. Harris, p53 mutations in human cancers. *Science* **253**, 49-53 (1991).
14. T. Soussi and C. Beroud, Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer* **1**, 233-240 (2001).
15. T. Soussi and G. Lozano, p53 mutation heterogeneity in cancer. *Biochem Biophys Res Commun* **331**, 834-842 (2005).
16. M. A. Blasco, Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet* **6**, 611-622 (2005).
17. D. Gilley, H. Tanaka and B. S. Herbert, Telomere dysfunction in aging and cancer. *Int J Biochem Cell Biol* **37**, 1000-1013 (2005).
18. J. W. Shay and S. Bacchetti, A survey of telomerase activity in human cancer. *Eur J Cancer* **33**, 787-791 (1997).
19. J. Rak, J. L. Yu, G. Klement and R. S. Kerbel, Oncogenes and angiogenesis: signaling three-dimensional tumor growth. *J Investig Dermatol Symp Proc* **5**, 24-33 (2000).
20. O. Lopez-Ocejo, A. Viloria-Petit, M. Bequet-Romero, D. Mukhopadhyay, J. Rak and R. S. Kerbel, Oncogenes and tumor angiogenesis: the HPV-16 E6 oncoprotein activates the vascular endothelial growth factor (VEGF) gene promoter in a p53 independent manner. *Oncogene* **19**, 4611-4620 (2000).
21. R. S. Kerbel, Tumor angiogenesis: past, present and the near future. *Carcinogenesis* **21**, 505-515 (2000).
22. B. Vailhe and J. J. Feige, Thrombospondins as anti-angiogenic therapeutic agents. *Curr Pharm Des* **9**, 583-588 (2003).
23. V. Chiarugi, L. Magnelli and O. Gallo, Cox-2, iNOS and p53 as play-makers of tumor angiogenesis (review). *Int J Mol Med* **2**, 715-719 (1998).
24. V. Chiarugi, L. Magnelli, M. Cinelli and M. Ruggiero, Oncogenes, p53, and tumor angiogenesis. *J Cancer Res Clin Oncol* **124**, 523-525 (1998).
25. A. E. Aplin, A. Howe, S. K. Alahari and R. L. Juliano, Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev* **50**, 197-263 (1998).

26. S. Hirohashi and Y. Kanai, Cell adhesion system and human cancer morphogenesis. *Cancer Sci* **94**, 575-581 (2003).
27. M. J. Duffy, Cancer metastasis: biological and clinical aspects. *Ir J Med Sci* **167**, 4-8 (1998).
28. K. Nabeshima, T. Inoue, Y. Shimao and T. Sameshima, Matrix metalloproteinases in tumor invasion: role for cell migration. *Pathol Int* **52**, 255-264 (2002).
29. P. Ehrlich, *in The Collected papers of Paul Ehrlich*. Pergamon Press, London, 1957.
30. D. Pressman and G. Keighley, The zone of activity of antibodies as determined by the use of radioactive tracers: the zone of activity of nephrotoxic antikidney serum. *J. Immunol* **59**, 141-146 (1948).
31. D. Pressman and L. Korngold, The in vivo localization of anti-Wagner-osteogenic-sarcoma antibodies. *Cancer* **6**, 619-623 (1953).
32. P. Gold and S. O. Freedman, Specific carcioembryonic antigens of the human digestive system. *J. Exp. Med.* **122**, 467-481 (1965).
33. P. Gold and S. O. Freedman, Demonstration of tumor-specific antigens in human colonic carinomata by immunological tolerance and absorption techniques. *J Exp. Med.* **121**, 439-462 (1965).
34. J. P. Mach, S. Carrel, C. Merenda, B. Sordat and J. C. Cerottini, In vivo localisation of radiolabelled antibodies to carcinoembryonic antigen in human colon carcinoma grafted into nude mice. *Nature* **248**, 704-706 (1974).
35. D. M. Goldenberg, D. F. Preston, F. J. Primus and H. J. Hansen, Photoscan localization of GW-39 tumors in hamsters using radiolabeled anticarcinoembryonic antigen immunoglobulin G. *Cancer Res* **34**, 1-9 (1974).
36. G. Kohler and C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495-497 (1975).
37. E. J. Postema, O. C. Boerman, W. J. Oyen, J. M. Raemaekers and F. H. Corstens, Radioimmunotherapy of B-cell non-Hodgkin's lymphoma. *Eur J Nucl Med* **28**, 1725-1735 (2001).
38. T. E. Witzig, Radioimmunotherapy for patients with relapsed B-cell non-Hodgkin lymphoma. *Cancer Chemother Pharmacol* **48 Suppl 1**, S91-95 (2001).
39. C. A. White, J. R. Berlfein and A. J. Grillo-Lopez, Antibody-targeted immunotherapy for treatment of non-Hodgkin's lymphoma. *Curr Pharm Biotechnol* **1**, 303-312 (2000).
40. D. M. Goldenberg, The role of radiolabeled antibodies in the treatment of non-Hodgkin's lymphoma: the coming of age of radioimmunotherapy. *Crit Rev Oncol Hematol* **39**, 195-201 (2001).

41. W. A. Bethge and B. M. Sandmaier, Targeted cancer therapy using radiolabeled monoclonal antibodies. *Technol Cancer Res Treat* **4**, 393-405 (2005).
42. S. V. Govindan, G. L. Griffiths, H. J. Hansen, I. D. Horak and D. M. Goldenberg, Cancer therapy with radiolabeled and drug/toxin-conjugated antibodies. *Technol Cancer Res Treat* **4**, 375-391 (2005).
43. R. M. Sharkey and D. M. Goldenberg, Perspectives on cancer therapy with radiolabeled monoclonal antibodies. *J Nucl Med* **46 Suppl 1**, 115S-127S (2005).
44. D. M. Goldenberg, Advancing role of radiolabeled antibodies in the therapy of cancer. *Cancer Immunol Immunother* **52**, 281-296 (2003).
45. J. G. Jurcic, S. M. Larson, G. Sgouros, M. R. McDevitt, R. D. Finn, C. R. Divgi, A. M. Ballangrud, K. A. Hamacher, D. Ma, et al., Targeted alpha particle immunotherapy for myeloid leukemia. *Blood* **100**, 1233-1239 (2002).
46. J. M. Burke and J. G. Jurcic, Antibody therapy in acute myeloid leukemia: current status and future directions. *Clin Lymphoma* **2 Suppl 1**, S12-18 (2002).
47. J. G. Jurcic, P. C. Caron, T. K. Nikula, E. B. Papadopoulos, R. D. Finn, O. A. Gansow, W. H. Miller, Jr., M. W. Geerlings, R. P. Warrell, Jr., et al., Radiolabeled anti-CD33 monoclonal antibody M195 for myeloid leukemias. *Cancer Res* **55**, 5908s-5910s (1995).
48. R. D. Alvarez, E. E. Partridge, M. B. Khazaeli, G. Plott, M. Austin, L. Kilgore, C. D. Russell, T. Liu, W. E. Grizzle, et al., Intraperitoneal radioimmunotherapy of ovarian cancer with 177Lu-CC49: a phase I/II study. *Gynecol Oncol* **65**, 94-101 (1997).
49. M. Tempero, P. Leichner, J. Baranowska-Kortylewicz, K. Harrison, S. Augustine, J. Schlom, J. Anderson, J. Wisecarver and D. Colcher, High-dose therapy with 90Yttrium-labeled monoclonal antibody CC49: a phase I trial. *Clin Cancer Res* **6**, 3095-3102 (2000).
50. M. Tempero, P. Leichner, G. Dalrymple, K. Harrison, S. Augustine, J. Schlam, J. Anderson, J. Wisecarver and D. Colcher, High-dose therapy with iodine-131-labeled monoclonal antibody CC49 in patients with gastrointestinal cancers: a phase I trial. *J Clin Oncol* **15**, 1518-1528 (1997).
51. D. J. Macey, E. J. Grant, L. Kasi, M. G. Rosenblum, H. Z. Zhang, R. L. Katz, P. T. Rieger, D. LeBherz, M. South, et al., Effect of recombinant alpha-interferon on pharmacokinetics, biodistribution, toxicity, and efficacy of 131I-labeled monoclonal antibody CC49 in breast cancer: a phase II trial. *Clin Cancer Res* **3**, 1547-1555 (1997).
52. T. Mulligan, J. A. Carrasquillo, Y. Chung, D. E. Milenic, J. Schlom, I. Feuerstein, C. Paik, P. Perentesis, J. Reynolds, et al., Phase I study

of intravenous Lu-labeled CC49 murine monoclonal antibody in patients with advanced adenocarcinoma. *Clin Cancer Res* **1**, 1447-1454 (1995).
53. R. F. Meredith, M. B. Khazaeli, D. J. Macey, W. E. Grizzle, M. Mayo, J. Schlom, C. D. Russell and A. F. LoBuglio, Phase II study of interferon-enhanced 131I-labeled high affinity CC49 monoclonal antibody therapy in patients with metastatic prostate cancer. *Clin Cancer Res* **5**, 3254s-3258s (1999).
54. S. Welt, C. R. Divgi, N. Kemeny, R. D. Finn, A. M. Scott, M. Graham, J. S. Germain, E. C. Richards, S. M. Larson, et al., Phase I/II study of iodine 131-labeled monoclonal antibody A33 in patients with advanced colon cancer. *J Clin Oncol* **12**, 1561-1571 (1994).
55. M. R. McDevitt, E. Barendswaard, D. Ma, L. Lai, M. J. Curcio, G. Sgouros, A. M. Ballangrud, W. H. Yang, R. D. Finn, et al., An alpha-particle emitting antibody ([213Bi]J591) for radioimmunotherapy of prostate cancer. *Cancer Res* **60**, 6095-6100 (2000).
56. L. H. Pai-Scherf, J. A. Carrasquillo, C. Paik, O. Gansow, M. Whatley, D. Pearson, K. Webber, M. Hamilton, C. Allegra, et al., Imaging and phase I study of 111In- and 90Y-labeled anti-LewisY monoclonal antibody B3. *Clin Cancer Res* **6**, 1720-1730 (2000).
57. H. P. Kalofonos, T. R. Pawlikowska, A. Hemingway, N. Courtenay-Luck, B. Dhokia, D. Snook, G. B. Sivolapenko, G. R. Hooker, C. G. McKenzie, et al., Antibody guided diagnosis and therapy of brain gliomas using radiolabeled monoclonal antibodies against epidermal growth factor receptor and placental alkaline phosphatase. *J Nucl Med* **30**, 1636-1645 (1989).
58. M. W. Brechbiel and T. A. Waldmann, Anti-HER2 radioimmunotherapy. *Breast Dis* **11**, 125-132 (2000).
59. S. Welt, C. R. Divgi, A. M. Scott, P. Garin-Chesa, R. D. Finn, M. Graham, E. A. Carswell, A. Cohen, S. M. Larson, et al., Antibody targeting in metastatic colon cancer: a phase I study of monoclonal antibody F19 against a cell-surface protein of reactive tumor stromal fibroblasts. *J Clin Oncol* **12**, 1193-1203 (1994).
60. A. M. Scott, G. Wiseman, S. Welt, A. Adjei, F. T. Lee, W. Hopkins, C. R. Divgi, L. H. Hanson, P. Mitchell, et al., A Phase I dose-escalation study of sibrotuzumab in patients with advanced or metastatic fibroblast activation protein-positive cancer. *Clin Cancer Res* **9**, 1639-1647 (2003).
61. L. Li, C. A. Wartchow, S. N. Danthi, Z. Shen, N. Dechene, J. Pease, H. S. Choi, T. Doede, P. Chu, et al., A novel antiangiogenesis therapy using an integrin antagonist or anti-Flk-1 antibody coated

90Y-labeled nanoparticles. *Int J Radiat Oncol Biol Phys* **58**, 1215-1227 (2004).
62. M. Harris, Monoclonal antibodies as therapeutic agents for cancer. *Lancet Oncol* **5**, 292-302 (2004).
63. D. M. Goldenberg, Targeted therapy of cancer with radiolabeled antibodies. *J Nucl Med* **43**, 693-713 (2002).
64. A. M. Scott and C. Renner, "Tumour Antigens Recognised by Antibodies." In: Encyclopedia of Life Sciences, Nature Publishing Group, Macmillan, London, UK, WWW.els.net. (2001).
65. R. B. McComb, G. N. Bowers and S. Posen, *Alkaline Phosphatase plenum*. New York, 1979.
66. G. De Groote, P. De Waele, A. Van de Voorde, M. De Broe and W. Fiers, Use of monoclonal antibodies to detect human placental alkaline phosphatase. *Clin Chem* **29**, 115-119 (1983).
67. P. A. Holmgren, T. Stigbrand, M. G. Damber and B. von Schoultz, A double antibody solid phase radioimmunoassay for placental alkaline phosphatase. *Clin Chim Acta* **83**, 205-210 (1978).
68. K. Hirano, U. M. Domar, H. Yamamoto, E. E. Brehmer-Andersson, B. E. Wahren and T. I. Stigbrand, Levels of alkaline phosphatase isozymes in human seminoma tissue. *Cancer Res* **47**, 2543-2546 (1987).
69. A. A. Dabare, A. M. Nouri, H. Cannell, T. Moss, A. K. Nigam and R. T. Oliver, Profile of placental alkaline phosphatase expression in human malignancies: effect of tumour cell activation on alkaline phosphatase expression. *Urol Int* **63**, 168-174 (1999).
70. J. F. Telfer and C. D. Green, Placental alkaline phosphatase activity is inversely related to cell growth rate in HeLaS3 cervical cancer cells. *FEBS Lett* **329**, 238-244 (1993).
71. R. Makiya and T. Stigbrand, Placental alkaline phosphatase has a binding site for the human immunoglobulin-G Fc portion. *Eur J Biochem* **205**, 341-345 (1992).
72. I. Stefaner, A. Stefanescu, W. Hunziker and R. Fuchs, Expression of placental alkaline phosphatase does not correlate with IgG binding, internalization and transcytosis. *Biochem J* **327 (Pt 2)**, 585-592 (1997).
73. W. H. Fishman, N. I. Inglis, L. L. Stolbach and M. J. Krant, A serum alkaline phosphatase isoenzyme of human neoplastic cell origin. *Cancer Res* **28**, 150-154 (1968).
74. W. H. Fishman, N. R. Inglis, S. Green, C. L. Anstiss, N. K. Gosh, A. E. Reif, R. Rustigian, M. J. Krant and L. L. Stolbach, Immunology and biochemistry of Regan isoenzyme of alkaline phosphatase in human cancer. *Nature* **219**, 697-699 (1968).

75. P. H. Lange, J. L. Millan, T. Stigbrand, R. L. Vessella, E. Ruoslahti and W. H. Fishman, Placental alkaline phosphatase as a tumor marker for seminoma. *Cancer Res* **42**, 3244-3247 (1982).
76. A. Ullen, K. Riklund Ahlstrom, R. Makiya and T. Stigbrand, Syngeneic anti-idiotypic antibodies eliminate excess radiolabeled idiotypes at experimental radioimmunolocalization. *Cell Biophys* **27**, 31-45 (1995).
77. P. Sandstrom, A. Johansson, A. Ullen, S. Rathsman, K. Riklund-Ahlstrom and T. Stigbrand, Idiotypic-anti-idiotypic antibody interactions in experimental radioimmunotargeting. *Clin Cancer Res* **5**, 3073s-3078s (1999).
78. C. Kosmas, H. P. Kalofonos, V. Hird and A. A. Epenetos, Monoclonal antibody targeting of ovarian carcinoma. *Oncology* **55**, 435-446 (1998).
79. E. Fuchs and D. W. Cleveland, A structural scaffolding of intermediate filaments in health and disease. *Science* **279**, 514-519 (1998).
80. F. C. Ramaekers and F. T. Bosman, The cytoskeleton and disease. *J Pathol* **204**, 351-354 (2004).
81. R. A. Quinlan, D. L. Schiller, M. Hatzfeld, T. Achtstatter, R. Moll, J. L. Jorcano, T. M. Magin and W. W. Franke, Patterns of expression and organization of cytokeratin intermediate filaments. *Ann N Y Acad Sci* **455**, 282-306 (1985).
82. R. Moll, W. W. Franke, D. L. Schiller, B. Geiger and R. Krepler, The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* **31**, 11-24 (1982).
83. W. W. Franke, D. L. Schiller, R. Moll, S. Winter, E. Schmid, I. Engelbrecht, H. Denk, R. Krepler and B. Platzer, Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. *J Mol Biol* **153**, 933-959 (1981).
84. A. Johansson, P. Sandstrom, A. Ullen, A. Erlandsson, B. Sundstrom, K. R. Ahlstrom, L. Johansson, S. O. Hietala and T. Stigbrand, Stability and immunoreactivity of the monoclonal anticytokeratin antibody TS1 after different degrees of iodination. *Acta Oncol* **38**, 329-334 (1999).
85. R. Rossi Norrlund, A. Ullen, P. Sandstrom, D. Holback, L. Johansson, T. Stigbrand, S. O. Hietala and K. Riklund Ahlstrom, Experimental radioimmunotargeting combining nonlabeled, iodine-125-labeled, and anti-idiotypic anticytokeratin monoclonal antibodies: a dosimetric evaluation. *Cancer* **80**, 2689-2698 (1997).

86. R. Rossi Norrlund, A. Ullen, P. Sandstrom, D. Holback, L. Johansson, T. Stigbrand, S. O. Hietala and K. Riklund Ahlstrom, Dosimetry of fractionated experimental radioimmunotargeting with idiotypic and anti-idiotypic anticytokeratin antibodies. *Cancer* **80**, 2681-2688 (1997).
87. A. Ullen, P. Sandstrom, R. Rossi Norrlund, S. Rathsman, L. Johansson, K. Riklund Ahlstrom, S. O. Hietala and T. Stigbrand, Dosimetry of fractionated administration of 125I-labeled antibody at experimental radioimmunotargeting. *Cancer* **80**, 2510-2518 (1997).
88. A. Ullen, P. Sandstrom, K. R. Ahlstrom, B. Sundstrom, B. Nilsson, L. Arlestig and T. Stigbrand, Use of anticytokeratin monoclonal anti-idiotypic antibodies to improve tumor:nontumor ratio in experimental radioimmunolocalization. *Cancer Res* **55**, 5868s-5873s (1995).
89. K. E. Riklund, R. Makiya, B. Sundstrom, O. Back, R. Henriksson, S. O. Hietala and T. Stigbrand, Inhibition of growth of HeLa cell tumours in nude mice by 125I-labeled anticytokeratin and antiPLAP monoclonal antibodies. *Anticancer Res* **11**, 555-560 (1991).
90. B. Sundstrom, B. Johansson, S. O. Hietala and T. Stigbrand, Radio-immunolocalization in nude mice using anticytokeratin monoclonal antibodies. *Tumour Biol* **11**, 158-166 (1990).
91. G. P. Adams and L. M. Weiner, Monoclonal antibody therapy of cancer. *Nat Biotechnol* **23**, 1147-1157 (2005).
92. D. E. Milenic, E. D. Brady and M. W. Brechbiel, Antibody-targeted radiation cancer therapy. *Nat Rev Drug Discov* **3**, 488-499 (2004).
93. W. Y. Hwang and J. Foote, Immunogenicity of engineered antibodies. *Methods* **36**, 3-10 (2005).
94. A. C. Roque, C. R. Lowe and M. A. Taipa, Antibodies and genetically engineered related molecules: production and purification. *Biotechnol Prog* **20**, 639-654 (2004).
95. N. R. Gonzales, R. De Pascalis, J. Schlom and S. V. Kashmiri, Minimizing the immunogenicity of antibodies for clinical application. *Tumour Biol* **26**, 31-43 (2005).
96. J. S. Huston and A. J. George, Engineered antibodies take center stage. *Hum Antibodies* **10**, 127-142 (2001).
97. P. Carter, Improving the efficacy of antibody-based cancer therapies. *Nat Rev Cancer* **1**, 118-129 (2001).
98. M. E. Reff and C. Heard, A review of modifications to recombinant antibodies: attempt to increase efficacy in oncology applications. *Crit Rev Oncol Hematol* **40**, 25-35 (2001).
99. L. F. Mausner and S. C. Srivastava, Selection of radionuclides for radioimmunotherapy. *Med Phys* **20**, 503-509 (1993).

100. D. E. Milenic and M. W. Brechbiel, Targeting of radio-isotopes for cancer therapy. *Cancer Biol Ther* **3**, 361-370 (2004).
101. R. K. Jain, Determinants of tumor blood flow: a review. *Cancer Res* **48**, 2641-2658 (1988).
102. R. K. Jain and L. T. Baxter, Mechanisms of heterogeneous distribution of monoclonal antibodies and other macromolecules in tumors: significance of elevated interstitial pressure. *Cancer Res* **48**, 7022-7032 (1988).
103. M. R. Zalutsky and O. R. Pozzi, Radioimmunotherapy with alpha-particle emitting radionuclides. *Q J Nucl Med Mol Imaging* **48**, 289-296 (2004).
104. B. J. Allen, C. Raja, S. Rizvi, Y. Li, W. Tsui, D. Zhang, E. Song, C. F. Qu, J. Kearsley, et al., Targeted alpha therapy for cancer. *Phys Med Biol* **49**, 3703-3712 (2004).
105. Y. Li, P. J. Russell and B. J. Allen, Targeted alpha-therapy for control of micrometastatic prostate cancer. *Expert Rev Anticancer Ther* **4**, 459-468 (2004).
106. A. H. W. Nias, *RADIOBIOLOGY*. Wiley, New York, 1998.
107. K. M. Prise, G. Schettino, M. Folkard and K. D. Held, New insights on cell death from radiation exposure. *Lancet Oncol* **6**, 520-528 (2005).
108. F. Bunz, A. Dutriaux, C. Lengauer, T. Waldman, S. Zhou, J. P. Brown, J. M. Sedivy, K. W. Kinzler and B. Vogelstein, Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**, 1497-1501 (1998).
109. M. Abend, Reasons to reconsider the significance of apoptosis for cancer therapy. *Int J Radiat Biol* **79**, 927-941 (2003).
110. G. Kroemer, W. S. El-Deiry, P. Golstein, M. E. Peter, D. Vaux, P. Vandenabeele, B. Zhivotovsky, M. V. Blagosklonny, W. Malorni, et al., Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ* **12 Suppl 2**, 1463-1467 (2005).
111. H. Okada and T. W. Mak, Pathways of apoptotic and non-apoptotic death in tumour cells. *Nat Rev Cancer* **4**, 592-603 (2004).
112. H. Mellstedt, Monoclonal antibodies in human cancer. *Drugs Today (Barc)* **39 Suppl C**, 1-16 (2003).
113. M. Z. Lin, M. A. Teitell and G. J. Schiller, The evolution of antibodies into versatile tumor-targeting agents. *Clin Cancer Res* **11**, 129-138 (2005).
114. M. C. Green, J. L. Murray and G. N. Hortobagyi, Monoclonal antibody therapy for solid tumors. *Cancer Treat Rev* **26**, 269-286 (2000).

115. Y. Hinoda, S. Sasaki, T. Ishida and K. Imai, Monoclonal antibodies as effective therapeutic agents for solid tumors. *Cancer Sci* **95**, 621-625 (2004).
116. G. L. DeNardo, Treatment of non-Hodgkin's lymphoma (NHL) with radiolabeled antibodies (mAbs). *Semin Nucl Med* **35**, 202-211 (2005).
117. I. Szumiel, Ionizing radiation-induced cell death. *Int J Radiat Biol* **66**, 329-341 (1994).
118. E. C. Jonathan, E. J. Bernhard and W. G. McKenna, How does radiation kill cells? *Curr Opin Chem Biol* **3**, 77-83 (1999).
119. M. Castedo, J. L. Perfettini, T. Roumier, A. Valent, H. Raslova, K. Yakushijin, D. Horne, J. Feunteun, G. Lenoir, et al., Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy. *Oncogene* **23**, 4362-4370 (2004).
120. C. J. Norbury and B. Zhivotovsky, DNA damage-induced apoptosis. *Oncogene* **23**, 2797-2808 (2004).
121. B. Zhivotovsky and S. Orrenius, Caspase-2 function in response to DNA damage. *Biochem Biophys Res Commun* **331**, 859-867 (2005).
122. A. Maity, G. D. Kao, R. J. Muschel and W. G. McKenna, Potential molecular targets for manipulating the radiation response. *Int J Radiat Oncol Biol Phys* **37**, 639-653 (1997).
123. A. Tenzer and M. Pruschy, Potentiation of DNA-damage-induced cytotoxicity by G2 checkpoint abrogators. *Curr Med Chem Anti-Canc Agents* **3**, 35-46 (2003).
124. W. K. Sinclair, Cyclic x-ray responses in mammalian cells in vitro. *Radiat Res* **33**, 620-643 (1968).
125. H. Choy, Combining taxanes with radiation for solid tumors. *Int J Cancer* **90**, 113-127 (2000).
126. H. Choy, Taxanes in combined modality therapy for solid tumors. *Crit Rev Oncol Hematol* **37**, 237-247 (2001).
127. D. H. Rutgers, I. E. van Oostrum, M. F. Noorman van der Dussen and I. S. Wils, Relationship between cell kinetics and radiation-induced arrest of proliferating cells in G2: relevance to efficacy of radiotherapy. *Anal Cell Pathol* **1**, 53-62 (1989).
128. I. E. van Oostrum, S. Erkens-Schulze, M. Petterson, I. S. Wils and D. H. Rutgers, The relationship between radiosensitivity and cell kinetic effects after low- and high-dose-rate irradiation in five human tumors in nude mice. *Radiat Res* **122**, 252-261 (1990).
129. J. Harding and B. Burtness, Cetuximab: an epidermal growth factor receptor chemeric human-murine monoclonal antibody. *Drugs Today (Barc)* **41**, 107-127 (2005).

130. P. M. Harari and S. M. Huang, Combining EGFR inhibitors with radiation or chemotherapy: will preclinical studies predict clinical results? *Int J Radiat Oncol Biol Phys* **58**, 976-983 (2004).
131. J. Mendelsohn, The epidermal growth factor receptor as a target for cancer therapy. *Endocr Relat Cancer* **8**, 3-9 (2001).
132. B. M. Hougardy, J. H. Maduro, A. G. van der Zee, P. H. Willemse, S. de Jong and E. G. de Vries, Clinical potential of inhibitors of survival pathways and activators of apoptotic pathways in treatment of cervical cancer: changing the apoptotic balance. *Lancet Oncol* **6**, 589-598 (2005).
133. A. V. Gudkov and E. A. Komarova, The role of p53 in determining sensitivity to radiotherapy. *Nat Rev Cancer* **3**, 117-129 (2003).
134. H. Huang, S. Y. Huang, T. T. Chen, J. C. Chen, C. L. Chiou and T. M. Huang, Cisplatin restores p53 function and enhances the radiosensitivity in HPV16 E6 containing SiHa cells. *J Cell Biochem* **91**, 756-765 (2004).
135. E. H. Chang, Y. J. Jang, Z. Hao, G. Murphy, A. Rait, W. E. Fee, Jr., H. H. Sussman, P. Ryan, Y. Chiang and K. F. Pirollo, Restoration of the G1 checkpoint and the apoptotic pathway mediated by wild-type p53 sensitizes squamous cell carcinoma of the head and neck to radiotherapy. *Arch Otolaryngol Head Neck Surg* **123**, 507-512 (1997).
136. N. Issaeva, A. Friedler, P. Bozko, K. G. Wiman, A. R. Fersht and G. Selivanova, Rescue of mutants of the tumor suppressor p53 in cancer cells by a designed peptide. *Proc Natl Acad Sci U S A* **100**, 13303-13307 (2003).
137. D. J. Mazzatti, Y. J. Lee, C. E. Helt, M. A. O'Reilly and P. C. Keng, p53 modulates radiation sensitivity independent of p21 transcriptional activation. *Am J Clin Oncol* **28**, 43-50 (2005).
138. X. Li and G. R. Stark, NFkappaB-dependent signaling pathways. *Exp Hematol* **30**, 285-296 (2002).
139. M. Pervan, F. Pajonk, J. R. Sun, H. R. Withers and W. H. McBride, Molecular pathways that modify tumor radiation response. *Am J Clin Oncol* **24**, 481-485 (2001).
140. A. Goel, A. Dispenzieri, P. R. Greipp, T. E. Witzig, R. A. Mesa and S. J. Russell, PS-341-mediated selective targeting of multiple myeloma cells by synergistic increase in ionizing radiation-induced apoptosis. *Exp Hematol* **33**, 784-795 (2005).
141. M. J. Edelman, The potential role of bortezomib in combination with chemotherapy and radiation in non-small-cell lung cancer. *Clin Lung Cancer* **7 Suppl 2**, S64-66 (2005).

142. P. M. Voorhees, E. C. Dees, B. O'Neil and R. Z. Orlowski, The proteasome as a target for cancer therapy. *Clin Cancer Res* **9**, 6316-6325 (2003).
143. G. Fiorucci, S. Vannucchi, M. V. Chiantore, Z. A. Percario, E. Affabris and G. Romeo, TNF-related apoptosis-inducing ligand (TRAIL) as a pro-apoptotic signal transducer with cancer therapeutic potential. *Curr Pharm Des* **11**, 933-944 (2005).
144. S. Bouralexis, D. M. Findlay and A. Evdokiou, Death to the bad guys: targeting cancer via Apo2L/TRAIL. *Apoptosis* **10**, 35-51 (2005).
145. P. Marini, A. Schmid, V. Jendrossek, H. Faltin, P. T. Daniel, W. Budach and C. Belka, Irradiation specifically sensitises solid tumour cell lines to TRAIL mediated apoptosis. *BMC Cancer* **5**, 5 (2005).
146. P. Marini and C. Belka, Death receptor ligands: new strategies for combined treatment with ionizing radiation. *Curr Med Chem Anti-Canc Agents* **3**, 334-342 (2003).
147. C. Belka, A. Betsch, P. Marini, V. Jendrossek, M. Bamberg and W. Budach, [Death inducing ligands in combination with ionizing radiation: objective and current knowledge]. *Strahlenther Onkol* **179**, 141-151 (2003).
148. S. Shankar, T. R. Singh and R. K. Srivastava, Ionizing radiation enhances the therapeutic potential of TRAIL in prostate cancer in vitro and in vivo: Intracellular mechanisms. *Prostate* **61**, 35-49 (2004).
149. M. A. Sheard, S. Uldrijan and B. Vojtesek, Role of p53 in regulating constitutive and X-radiation-inducible CD95 expression and function in carcinoma cells. *Cancer Res* **63**, 7176-7184 (2003).
150. M. Kuwabara, K. Takahashi and O. Inanami, Induction of apoptosis through the activation of SAPK/JNK followed by the expression of death receptor Fas in X-irradiated cells. *J Radiat Res (Tokyo)* **44**, 203-209 (2003).
151. I. C. Park, S. H. Woo, M. J. Park, H. C. Lee, S. J. Lee, Y. J. Hong, S. H. Lee, S. I. Hong and C. H. Rhee, Ionizing radiation and nitric oxide donor sensitize Fas-induced apoptosis via up-regulation of Fas in human cervical cancer cells. *Oncol Rep* **10**, 629-633 (2003).
152. O. Micheau, E. Solary, A. Hammann and M. T. Dimanche-Boitrel, Fas ligand-independent, FADD-mediated activation of the Fas death pathway by anticancer drugs. *J Biol Chem* **274**, 7987-7992 (1999).

153. R. R. Weichselbaum, D. W. Kufe, S. Hellman, H. S. Rasmussen, C. R. King, P. H. Fischer and H. J. Mauceri, Radiation-induced tumour necrosis factor-alpha expression: clinical application of transcriptional and physical targeting of gene therapy. *Lancet Oncol* **3**, 665-671 (2002).
154. D. E. Hallahan, D. R. Spriggs, M. A. Beckett, D. W. Kufe and R. R. Weichselbaum, Increased tumor necrosis factor alpha mRNA after cellular exposure to ionizing radiation. *Proc Natl Acad Sci U S A* **86**, 10104-10107 (1989).
155. R. R. Weichselbaum, D. E. Hallahan, M. A. Beckett, H. J. Mauceri, H. Lee, V. P. Sukhatme and D. W. Kufe, Gene therapy targeted by radiation preferentially radiosensitizes tumor cells. *Cancer Res* **54**, 4266-4269 (1994).
156. D. S. Gridley, S. N. Hammond and B. H. Liwnicz, Tumor necrosis factor-alpha augments radiation effects against human colon tumor xenografts. *Anticancer Res* **14**, 1107-1112 (1994).
157. T. J. Herzog, P. K. Nelson, D. G. Mutch, W. D. Wright, M. S. Kao and J. L. Collins, Effects of radiation on TNF alpha-mediated cytolysis of cell lines derived from cervical carcinomas. *Gynecol Oncol* **47**, 196-202 (1992).
158. A. Manetta, J. Lucci, J. Soopikian, G. Granger, M. L. Berman and P. J. DiSaia, In vitro cytotoxicity of human recombinant tumor necrosis factor alpha in association with radiotherapy in a human ovarian carcinoma cell line. *Gynecol Oncol* **38**, 200-202 (1990).
159. D. R. Spriggs, M. L. Sherman, H. Michie, K. A. Arthur, K. Imamura, D. Wilmore, E. Frei, 3rd and D. W. Kufe, Recombinant human tumor necrosis factor administered as a 24-hour intravenous infusion. A phase I and pharmacologic study. *J Natl Cancer Inst* **80**, 1039-1044 (1988).
160. D. E. Hallahan, E. E. Vokes, S. J. Rubin, S. O'Brien, B. Samuels, S. Vijaykumar, D. W. Kufe, R. Phillips and R. R. Weichselbaum, Phase I Dose-Escalation Study of Tumor Necrosis Factor-alpha and Concomitant Radiation Therapy. *Cancer J Sci Am* **1**, 204 (1995).
161. E. A. Carswell, L. J. Old, R. L. Kassel, S. Green, N. Fiore and B. Williamson, An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A* **72**, 3666-3670 (1975).
162. R. K. Jain, Delivery of molecular and cellular medicine to solid tumors. *Adv Drug Deliv Rev* **26**, 71-90 (1997).
163. R. K. Jain, Haemodynamic and transport barriers to the treatment of solid tumours. *Int J Radiat Biol* **60**, 85-100 (1991).

164. R. K. Jain, Physiological barriers to delivery of monoclonal antibodies and other macromolecules in tumors. *Cancer Res* **50**, 814s-819s (1990).
165. D. J. Buchsbaum, Experimental approaches to increase radiolabeled antibody localization in tumors. *Cancer Res* **55**, 5729s-5732s (1995).
166. S. M. Andrew, J. G. Teh, R. W. Johnstone, S. M. Russell, R. H. Whitehead, I. F. McKenzie and G. A. Pietersz, Tumor localization by combinations of monoclonal antibodies in a new human colon carcinoma cell line (LIM1899). *Cancer Res* **50**, 5225-5230 (1990).
167. R. D. Blumenthal, R. Kashi, R. Stephens, R. M. Sharkey and D. M. Goldenberg, Improved radioimmunotherapy of colorectal cancer xenografts using antibody mixtures against carcinoembryonic antigen and colon-specific antigen-p. *Cancer Immunol Immunother* **32**, 303-310 (1991).
168. F. Buchegger, A. Rojas, A. B. Delaloye, C. A. Vogel, R. O. Mirimanoff, P. Coucke, L. Q. Sun, S. Raimondi, J. Denekamp, et al., Combined radioimmunotherapy and radiotherapy of human colon carcinoma grafted in nude mice. *Cancer Res* **55**, 83-89 (1995).
169. H. Kalofonos, G. Rowlinson and A. A. Epenetos, Enhancement of monoclonal antibody uptake in human colon tumor xenografts following irradiation. *Cancer Res* **50**, 159-163 (1990).
170. S. Ruan, J. A. O'Donoghue, S. M. Larson, R. D. Finn, A. Jungbluth, S. Welt and J. L. Humm, Optimizing the sequence of combination therapy with radiolabeled antibodies and fractionated external beam. *J Nucl Med* **41**, 1905-1912 (2000).
171. L. Q. Sun, C. A. Vogel, R. O. Mirimanoff, P. Coucke, D. O. Slosman, J. P. Mach and F. Buchegger, Timing effects of combined radioimmunotherapy and radiotherapy on a human solid tumor in nude mice. *Cancer Res* **57**, 1312-1319 (1997).
172. C. A. Vogel, M. C. Galmiche and F. Buchegger, Radioimmunotherapy and fractionated radiotherapy of human colon cancer liver metastases in nude mice. *Cancer Res* **57**, 447-453 (1997).
173. M. Garkavij, M. Samarzija, S. B. Ewers, M. Jakopovic, S. Tezak and J. Tennvall, Concurrent radiotherapy and tumor targeting with 111In-HMFG1-F(ab')2 in patients with MUC1-positive non-small cell lung cancer. *Anticancer Res* **25**, 4663-4671 (2005).
174. F. Buchegger, A. Roth, A. Allal, Y. M. Dupertuis, D. O. Slosman, A. B. Delaloye and J. P. Mach, Radioimmunotherapy of

colorectal cancer liver metastases: combination with radiotherapy. *Ann N Y Acad Sci* **910**, 263-269; discussion 269-270 (2000).
175. F. Buchegger, A. S. Allal, A. Roth, J. P. Papazyan, Y. Dupertuis, R. O. Mirimanoff, M. Gillet, A. Pelegrin, J. P. Mach and D. O. Slosman, Combined radioimmunotherapy and radiotherapy of liver metastases from colorectal cancer: a feasibility study. *Anticancer Res* **20**, 1889-1896 (2000).
176. C. A. Znati, M. Rosenstein, Y. Boucher, M. W. Epperly, W. D. Bloomer and R. K. Jain, Effect of radiation on interstitial fluid pressure and oxygenation in a human tumor xenograft. *Cancer Res* **56**, 964-968 (1996).
177. H. D. Roh, Y. Boucher, S. Kalnicki, R. Buchsbaum, W. D. Bloomer and R. K. Jain, Interstitial hypertension in carcinoma of uterine cervix in patients: possible correlation with tumor oxygenation and radiation response. *Cancer Res* **51**, 6695-6698 (1991).
178. S. Potamianos, A. D. Varvarigou and S. C. Archimandritis, Radioimmunoscintigraphy and radioimmunotherapy in cancer: principles and application. *Anticancer Res* **20**, 925-948 (2000).
179. J. P. Mach, S. Carrel, M. Forni, J. Ritschard, A. Donath and P. Alberto, Tumor localization of radiolabeled antibodies against carcinoembryonic antigen in patients with carcinoma: a critical evaluation. *N Engl J Med* **303**, 5-10 (1980).
180. F. Buchegger, A. Pelegrin, B. Delaloye, A. Bischof-Delaloye and J. P. Mach, Iodine-131-labeled MAb F(ab')2 fragments are more efficient and less toxic than intact anti-CEA antibodies in radioimmunotherapy of large human colon carcinoma grafted in nude mice. *J Nucl Med* **31**, 1035-1044 (1990).
181. T. M. Behr and D. M. Goldenberg, Improved prospects for cancer therapy with radiolabeled antibody fragments and peptides? *J Nucl Med* **37**, 834-836 (1996).
182. T. M. Behr, R. D. Blumenthal, S. Memtsoudis, R. M. Sharkey, S. Gratz, W. Becker and D. M. Goldenberg, Cure of metastatic human colonic cancer in mice with radiolabeled monoclonal antibody fragments. *Clin Cancer Res* **6**, 4900-4907 (2000).
183. F. Buchegger, J. P. Mach, S. Folli, B. Delaloye, A. Bischof-Delaloye and A. Pelegrin, Higher efficiency of 131I-labeled anti-carcinoembryonic antigen-monoclonal antibody F(ab')2 as compared to intact antibodies in radioimmunotherapy of established human colon carcinoma grafted in nude mice. *Recent Results Cancer Res* **141**, 19-35 (1996).

184. D. E. Milenic, Radioimmunotherapy: designer molecules to potentiate effective therapy. *Semin Radiat Oncol* **10**, 139-155 (2000).
185. J. C. Kim, H. K. Hong, S. A. Roh, K. H. Koo, I. H. Ka, H. Namgung, K. H. Lee, J. H. Ryu, C. S. Yu, et al., Efficient targeting of gastric cancer cells using radiolabeled anti-carcinoembryonic antigen-specific T84.66 fragments in experimental radioimmunoguided surgery. *Anticancer Res* **24**, 663-670 (2004).
186. A. Boskovitz, G. H. Akabani, C. N. Pegram, D. D. Bigner and M. R. Zalutsky, Human/murine chimeric 81C6 F(ab')(2) fragment: preclinical evaluation of a potential construct for the targeted radiotherapy of malignant glioma. *Nucl Med Biol* **31**, 345-355 (2004).
187. G. Pavlinkova, G. W. Beresford, B. J. Booth, S. K. Batra and D. Colcher, Pharmacokinetics and biodistribution of engineered single-chain antibody constructs of MAb CC49 in colon carcinoma xenografts. *J Nucl Med* **40**, 1536-1546 (1999).
188. D. Colcher, G. Pavlinkova, G. Beresford, B. J. Booth, A. Choudhury and S. K. Batra, Pharmacokinetics and biodistribution of genetically-engineered antibodies. *Q J Nucl Med* **42**, 225-241 (1998).
189. D. E. Milenic, T. Yokota, D. R. Filpula, M. A. Finkelman, S. W. Dodd, J. F. Wood, M. Whitlow, P. Snoy and J. Schlom, Construction, binding properties, metabolism, and tumor targeting of a single-chain Fv derived from the pancarcinoma monoclonal antibody CC49. *Cancer Res* **51**, 6363-6371 (1991).
190. D. J. Buchsbaum, Experimental radioimmunotherapy. *Semin Radiat Oncol* **10**, 156-167 (2000).
191. A. Gruaz-Guyon, O. Raguin and J. Barbet, Recent advances in pretargeted radioimmunotherapy. *Curr Med Chem* **12**, 319-338 (2005).
192. O. C. Boerman, F. G. van Schaijk, W. J. Oyen and F. H. Corstens, Pretargeted radioimmunotherapy of cancer: progress step by step. *J Nucl Med* **44**, 400-411 (2003).
193. R. M. Sharkey, R. D. Blumenthal and D. M. Goldenberg, Anti-antibody enhancement of tumor imaging. *Cancer Treat Res* **51**, 433-455 (1990).
194. A. Ullen, K. R. Ahlstrom, S. Heitala, B. Nilsson, L. Arlestig and T. Stigbrand, Secondary antibodies as tools to improve tumor to non tumor ratio at radioimmunolocalisation and radioimmunotherapy. *Acta Oncol* **35**, 281-285 (1996).

195. I. L. Spar, R. L. Goodland and M. A. Desiderio, Immunological Removal of Circulating I-131-Labeled Rabbit Antibody to Rat Fibrinogen in Normal and Tumor-Bearing Rats. *J Nucl Med* **5**, 428-443 (1964).
196. R. M. Sharkey, O. C. Boerman, A. Natale, D. Pawlyk, M. Monestier, M. J. Losman and D. M. Goldenberg, Enhanced clearance of radiolabeled murine monoclonal antibody by a syngeneic anti-idiotype antibody in tumor-bearing nude mice. *Int J Cancer* **51**, 266-273 (1992).
197. A. Erlandsson, D. Eriksson, L. Johansson, K. Riklund, T. Stigbrand and B. E. Sundstrom, In vivo clearing of idiotypic antibodies with antiidiotypic antibodies and their derivatives. *Mol Immunol* **43**, 599-606 (2006).
198. G. L. DeNardo, S. W. Maddock, G. Sgouros, P. O. Scheibe and S. J. DeNardo, Immunoadsorption: an enhancement strategy for radioimmunotherapy. *J Nucl Med* **34**, 1020-1027 (1993).
199. O. Linden, J. Kurkus, M. Garkavij, E. Cavallin-Stahl, M. Ljungberg, R. Nilsson, T. Ohlsson, B. Sandberg, S. E. Strand and J. Tennvall, A novel platform for radioimmunotherapy: extracorporeal depletion of biotinylated and 90Y-labeled rituximab in patients with refractory B-cell lymphoma. *Cancer Biother Radiopharm* **20**, 457-466 (2005).
200. J. Tennvall, M. Garkavij, J. Chen, H. O. Sjogren and S. E. Strand, Improving tumor-to-normal-tissue ratios of antibodies by extracorporeal immunoadsorption based on the avidin-biotin concept: development of a new treatment strategy applied to monoclonal antibodies murine L6 and chimeric BR96. *Cancer* **80**, 2411-2418 (1997).
201. M. Garkavij, J. Tennvall, S. E. Strand, H. O. Sjogren, C. JianQing, R. Nilsson and M. Isaksson, Extracorporeal whole-blood immunoadsorption enhances radioimmunotargeting of iodine-125-labeled BR96-biotin monoclonal antibody. *J Nucl Med* **38**, 895-901 (1997).
202. Z. Wang, M. Garkavij, T. Ohlsson, S. E. Strand, H. O. Sjogren and J. Tennvall, Biotinylation, pharmacokinetics, and extracorporeal adsorption of humanized MAb 111In-MN14 using an avidin-affinity column in rats. *Cancer Biother Radiopharm* **18**, 365-375 (2003).
203. Z. Wang, M. Garkavij, J. G. Tennvall, T. Ohlsson, S. E. Strand and H. O. Sjogren, Application of extracorporeal immunoadsorption to reduce circulating blood radioactivity after intraperitoneal administration of indium-111-HMFG1-biotin. *Cancer* **94**, 1287-1292 (2002).