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Ultrasound, ions and combined modalities for increased local tumour cell death in radiation therapy

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ABSTRACT

In this thesis, new approaches to improve tumour therapy for patients with inoperable, radiation- and chemoresistant tumours were in focus. Ultrasound and ionising radiation, especially accelerated ions, are both non-invasive agents that have high precision and deep penetration in tissues, enabling increased dose to the tumour while sparing the normal tissues. The first aim was to investigate cell survival and various cell responses of non-thermal ultrasound, both as a single treatment or in combination with radiation or a chemotherapeutic agent, and the second aim was to investigate the effects of accelerated ions.

Treatment of solid tumours using focused high-intensity ultrasound for thermal ablation has been initiated. In the present study lower intensities of ultrasound, not inducing thermal increase but inducing cavitation and production of free radicals were used for investigation of cellular effects. Cells were exposed to 1 MHz continuous mode ultrasound at varying intensities, either alone or in combination with X- or γ-radiation, or the chemotherapeutic agent cisplatin. Intensity-dependent clonogenic cell survival and induction of apoptosis were seen in five different human solid tumour cell lines (lung carcinoma, cervical carcinoma, two gliomas, and melanoma). The variation in ultrasound sensitivity was very small compared to the large variation after photon radiation. Both in a rodent (lung fibroblast) and a human solid tumour (lung carcinoma) cell line synergistic effects after combined exposures to ultrasound and radiation was found. Also, ultrasound enhanced the effect of cisplatin, induced intensity-dependent increase in instant cytolysis, induction in DNA damage, cells with increased membrane permeability and aberrant morphology.

In radiation therapy the dominating modality is external therapy with photons or electrons. Accelerated protons and carbon ions have improved the treatment of inoperable and radioresistant tumours. Other charged particles and radiation qualities may further optimise the treatment. Therefore we investigated the effects of accelerated boron ions at four different linear energy transfer (LET) in a human solid tumor cell line (melanoma). The clonogenic cell survival decreased with increasing LET. Boron irradiation induced higher levels of apoptosis compared to photons at all time points studied although no clear LET-dependence due to the complex time-dependent response pattern. Even intermediate LETs were effective in increasing cell killing, apoptosis, cell cycle delay and reducing DSB rejoining.

Both ultrasound and boron ions were effective in tumour cell elimination. By combining different treatment modalities cell killing was further enhanced due to interacting sublethal damage. This finding, in combination with the minimal variation in sensitivity among human solid tumour cell lines after ion and ultrasound irradiation, suggests some new avenues for treating heterogeneous tumours with varying radiation- and chemoresistance.

Keywords: Focused ultrasound, ionising radiation, high-LET, cisplatin, combined modalities, cell survival, cellular effects

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CONTENTS

LIST OF ABBREVIATIONS

DSB	Double-strand break in DNA
HIFU	High intensity focused ultrasound
LET	Linear energy transfer
PFGE	Pulsed-field gel electrophoresis
PLD	Potentially lethal damage
RBE	Relative biological effectiveness
SF	Survival fraction
SLD	Sublethal damage
SSB	Single-strand breaks in DNA

1 BACKGROUND

1.1 CANCER

Cancer is a heterogeneous disease. There are more than 200 different types of cancer and cancer may originate from almost every tissue type. Metastasises can be significantly different from the primary tumour in many aspects, and even between cells within the same tumour there may be considerable variations. The cell types constituting the tumour can have different genotypes as well as phenotypes due to accumulating genetic alterations and increased genomic instability contributing to the carcinogenesis. Expression of some changes are considered fundamental for malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg 2000). The heterogeneity of a tumour is an obstacle in cancer therapy since the treatment method selected has to have cell killing effect on all tumour cells. Every surviving tumour cell increases the risk of recurrence and metastasis.

1.2 CANCER THERAPIES

In cancer therapy several different treatment modalities are used such as surgery, radiation therapy, chemotherapy, hormone therapy and immunotherapy. The treatment modalities can be given as a single treatment or in combination with others in sequential or concurrent regimens. The option of treatment depends on several factors as for example type, stage and localisation of tumour, known resistance to certain agents, and to varying extent of biological parameters expressed differently by tumour cells relative to normal cells.

1.3 RADIATION THERAPY

Radiation has been used in cancer therapy for more than a century. Today, approximately 50% of the cancer patients in the Western world are given radiotherapy, either as a curative or palliative treatment. The dominating modality is external therapy with photons or electrons. Brachytherapy, using sealed radioactive sources for internal treatment, is the major modality for certain tumour types (Bernier 2004). Moreover, there are facilities for radiation therapy with accelerated charged particles (hadron therapy) such as protons (over 20 located all over the world) and carbon ions (two in Japan and one in Germany), and several new centres for ion therapy are proposed or in construction in Europe (Amaldi 2005, Blakely 2004).

In radiation therapy, most tumours could be cured if a sufficiently high dose is given. Unfortunately, this is limited by adverse effects. One has to balance the dose needed to cure versus the tolerance of normal tissue. The tumour response to radiation therapy is influenced by biological factors such as intrinsic cellular radiosensitivity, oxygen status and proliferative potential. The heterogeneity within a tumour can be reflected by difference in radio- and chemosensitivity in biopsies from the same tumour (Allalunis-Turner 1993) and by tumour hypoxia involving development of radioresistant subpopulations (Kaplan 1976, Brown 2000).

1.3.1 Ionising radiation

Ionising radiation is any radiation that can displace orbital electrons from atoms or molecules and thereby produce ions. The localised energy deposition can break strong chemical bonds between molecules. Ionising radiation is generally classified into electromagnetic or particulate. X- and γ-rays (photons) are electromagnetic radiation, whereas neutrons, electrons, protons, α-particles and other heavier charged ions, such as carbon ions, are examples of particle radiations. The different types of ionising radiation differ in the way how their energy is transferred to matter. This can be described by the concept of linear energy transfer (LET), i.e. the density of ionisations; LET is average energy deposited per unit length of track (eV/nm). For low LET radiation, e.g. X- and γ-rays, the ionisations are sparsely due to the fact that their energy deposition occurs via secondary electrons set in motion by the incident photons at all depths in the target matter. However, clustered ionisations can be caused by free radicals (e.g. hydroxyl) from decomposition of water molecules (radiolysis). For high LET radiation, e.g. α-particles, low energy protons and accelerated ions, the energy deposition is more densely and restricted to a limited volume adjacent to the primary particle track. Therefore, with accelerated ions the dose increases with penetration depth from a low dose in the entrance to a sharp maximum at the end of the particle range (Bragg peak). The absorbed dose is measured in Gy (Gray; 1 Gy=1 J/kg) (Hall and Giaccia 2007, Goodhead 1994).

1.3.2 Biological effects of radiation

Biological effects occur when cells and tissues absorb radiation energy. The ionisations of cellular molecules such as DNA may be direct (~30%) or more often indirect (~70%) via the free radicals produced by radiolysis after exposure to low LET radiation. Ionising radiation induces a variety of cellular lesions including damage to DNA, plasma membrane and other subcellular components. The damage induces stress responses leading to a coordinate network of signal transduction pathways involved in cell cycle arrest, activation of repair and death processes. There is extensively and very active ongoing research in radiation oncology, radiation biology and cancer biology. However, since this thesis mainly focuses on cell survival and cellular effects without dealing with the underlying mechanisms, the several known and complex signalling pathways involved in recognition of damage, amplification and transmission of damage signals, initiation and execution of repair or cell death processes is not discussed here. For an introduction in this field see for example Szumiel (1998) and Pouget and Mather (2001).

Cellular stress response may be initiated when physical and chemical alterations in the cells environment occurs: exposure to ionising radiation, cytotoxic agents, mechanical stress (from ultrasound, centrifugation, shaking etc), heat, cold, altered pH or osmolarity, deprivation of nutrients and growth factors, among others. The stress response depends on type, strength and duration of stimuli and may lead to various cellular effects including cell death.

The type and extent of cell damage induced depends on agents used, dose and exposure time. The cellular effects manifested may vary depending on factors such as cell type and specific biological parameters, but also on method and timing for analyses. For many agents the mechanisms of cell inactivation is not fully known, although the general cytotoxic action is clear and can be used in cancer therapy. The

classification of damage may be into lethal and sublethal. Sublethal damage (SLD) is a nonlethal damage that can be repaired or accumulated with further dose to become lethal. Interacting sublethal damage leading to lethal damage may be also induced by different cytotoxic agents.

Different types of radiation can be compared by measuring the relative biological effectiveness (RBE). The RBE is defined as the inverse ratio of the amount of absorbed radiation required to produce a given effect to a standard (or reference) radiation required to produce the same effect (Hall and Giaccia 2007). The RBE values may vary with endpoint. Generally high LET radiation gives higher RBE than low LET for cell survival (Goodhead 1994). The increased RBE with increasing LET for cell survival and other cellular effects (damage) is of clinical value especially for radioresistant tumours.

A cell's response to DNA damage depends on the type of damage. In our cells DNA damage is constantly induced due to external agents such as background radiation and endogenous factors such as free radicals resulting from metabolic processes. Ionising radiation can induce several types of DNA damage: DNA single (SSB) and double strand breaks (DSB) (two closely situated SSBs on opposite strands can form a DSB), locally multiply damaged sites (LMDS), base damage, sugar damage, cross-links between two DNA strands or between DNA and proteins (Ward 1991, Goodhead 1994, Olive 1998). Several DNA repair systems exist (Norbury and Hickson 2001, O'Driscoll and Jeggo 2006). DSB is considered to be the most critical form of DNA damage. The RBE for DSB after high LET radiation is generally lower than corresponding RBE for cell survival. However, the DSB rejoining capacity after high LET radiation is lower (slower rejoining and higher proportion of unrejoined DSB) (Olive 1998, Prise 1998, Pouget and Mather 2001, Karlsson and Stenerlöw 2004). Non-repaired or misrepaired DNA damage may be lethal or lead to genomic instability.

In the history of radiation biology it has long been assumed that the action of ionising radiation is an "all or nothing" stochastic process. This assumption is based on studies of the inactivation of clonogenic cells (Puck and Marcus 1956). It was observed, after irradiation, that some cells irreversible lost their reproductive capacity even though they were still able to go through some "abortive" divisions. Though, at later generations some of the progeny of the irradiated cells failed to divide. Puck and Marcus regarded cells able to produce a clone with more than 50 cells within a certain time (at least 5 doublings) as survivors. But the size distribution of clones varies a great deal after irradiation compared to unirradiated cells. The range of colony size becomes broader as the dose increases with an increasing number of smaller colonies at higher doses. However, changing the scoring criteria to 150 cells does not seem to significantly change the shape of the survival curve (Elkind and Sutton 1959). When small colonies, fulfilling the criteria as survivors, from irradiated cells were investigated further it was shown that they were "slow growing" and had reduced plating efficiency when compared to the unirradiated parental line. This nonlethal damage took some time to be manifested but once expressed it persisted for many divisions. When further investigating the offspring of the large clones, no such characteristics were shown but there was an increase in the heterogeneity in plating efficiency, a change in the number of chromosomes and a temporary increase in chromosome aberrations (Sinclair 1964). This indicates that the effect of irradiation as an "all or nothing" for cell killing is not valid, at least not when considering the survival parameters generally used. Surviving cells seemed to be different from unirradiated

cells. Further investigations have confirmed the existence of clonal heterogeneity in the offspring of cells surviving irradiation.

Radiation-induced genomic instability in mammalian cells can be expressed in long term surviving cells as for example delayed decreased reproductive capacity, chromosomal instability in the form of *de novo* chromosome aberrations, micronucleation or apoptosis (Kadhim 1992, Holmberg 1998, Belyakov 1999).

There are studies showing that irradiated cells can affect non-irradiated cells by for example reducing cell survival, indicating that some signalling factor(s) can be released in response to ionising radiation (bystander effect) (Mothersill 1997).

There are indications on that DNA DSB are the most important lesion induced by radiation which leads to chromosomal aberrations, however, other DNA lesions such as base damage may lead to aberrations as well (Natarajan 1993, Bryant 1997). DNA damage can be repaired to give normal chromosomes at mitosis, it can be misrepaired or misreplicated when still present during the S phase of the cell cycle to form an aberration, or it can remain unrepaired resulting in a terminal deletion. Radiation induces both stable (translocations) and unstable (dicentrics, acentric fragments and rings) chromosome aberrations in a dose-dependent manner.

Micronuclei are chromosomal fragments that become excluded from the main nucleus at cell division and consist predominantly of acentric fragments, caused by chromosomal breakage, or to lesser extent whole, chromosomes when the spindle apparatus is dysfunctional.

Mitotic catastrophe typically occurs during or shortly after a failed mitosis probably caused by mis-segregation of chromosomes and/or cell fusion. Common characteristics are micronuclei, giant cell formation or multinucleate cells (Abend 2003).

Apoptotic, necrotic, mitotic catastrophe, micronucleated and chromosomal aberrant cells are all considered as morphologically aberrant cells (Abend 2003, Brown 2005). Apoptosis, necrosis, autophagy and direct lysis are considered as cell death processes. A cell undergoing senescence is a cell with permanent cell cycle arrest but with still active metabolic processes. In interphase cell death, in contrast to reproductive cell death, the cell dies before entering mitosis, a phenomenon typical for G0 lymphocytes (Schrek 1942).

DNA damage in mammalian cells leads normally to arrest in G1, S and/or G2 phase of the cell cycle (Maity 1994). The insult of low LET radiation usually results in G1 arrest, which is followed by DNA repair and/ or cell death (Fei 2003). Exposure to high LET does not induce cell cycle arrest, but in a G2/M and S accumulation (≥24 h) (Lücke-Huhle 1984, Fournier and Taucher-Scholz 2004, Holgersson 2005).

Apoptotic as well as necrotic cell death can occur either as premitotic (interphase) or postmitotic (reproductive) cell death (Szumiel 1994). Apoptosis is a process for removal of unwanted, damaged and potentially dangerous cells without negatively affecting surrounding cells (Kerr 1972, Wyllie 1980). This process occurs during normal physiological conditions maintaining tissue homeostasis and embryonal development but also after certain stimuli. Damage to nucleus, membrane and other organelles may initiate apoptotic signalling. During the apoptotic process the cell shrinks, exhibit dense chromatin condensation, nuclear fragmentation, cytoplasmic and membrane “blebbing”, and cellular fragmentation into smaller apoptotic bodies. These are phagocytosed by neighbouring cells or macrophages and removed before disintegration. Therefore apoptosis does not generate an inflammatory response. The

genetic and biochemical events responsible for the regulation and execution of apoptosis are intensively studied (Leist and Jäättelä 2001). Control over these mechanisms offers a potential for promising use in tumour therapy with agents that stimulate this specific mode of death. High LET radiation has been reported to be more effective in inducing apoptosis than low LET *in vitro* in cells with different origin and gene status (Tsuboi 1998, Iwadate 2001, Takahashi 2004). In contrast to apoptosis, necrosis is a death process that triggers an inflammatory response. The characteristics of necrosis are loss of membrane integrity, swelling of cytoplasm and mitochondria, resulting in cell lysis.

Not only damage to DNA may be crucial for cell survival. Radiation of both high and low LET can induce membrane damage such as peroxidation and cell lysis (Choudhary 1998). Oxidative damage to cell membranes by lipidperoxidation can be induced by free radical species such as OH· produced through radiolysis (Girotti 1998).

It is often fruitful to use several criteria when assessing biological effects in order to have as a complete understanding of cell death as possible, and it is also important to consider if the endpoint is relevant for the *in vivo* response (Brown 2005, Abend 2003). Therefore, especially the time for analysis and choice of endpoint, as well as handling of the cells, may be of crucial importance when comparing differing effects of different agents amongst different cell types. There are numerous methods to analyze cellular responses and cell death. However, when focus is on inactivation of every single potentially clonogenic and dangerous tumour cell, one must use adequate methods to define when a cell is eliminated. This is not always easy *in vitro*, and even more difficult *in vivo*. One should be aware of the advantageous and limitations of the method used.

1.4 ULTRASOUND IN CANCER THERAPY

1.4.1 Clinical applications of ultrasound

Ultrasound is not only used as a diagnostic tool and for physiotherapy. Focused high-intensity ultrasound (HIFU) is a non-invasive technique for *in situ* thermal ablation of solid tumours (Wu 2003). The interest in new therapeutic applications for ultrasound in medicine is increasing. Several applications for focused ultrasound are under investigation at different research and clinical stages such as for occlusion of blood flow, sealing of bleeding vessel, sonothrombolysis, myocardium revascularization channel creation, opening of the blood brain barrier and transdermal drug delivery (Harvey 2002, Clement 2004, Kennedy 2005, ter Haar 2007). The biological effects of ultrasound may be used not only for complete tissue destruction of a tumour (coagulative necrosis) but also to induce cell death by other mechanisms or for enhancing the cell killing effect of other treatments such as radiation- and chemotherapy. Of particular interest is to increase the uptake of chemotherapeutic drugs or genes in a tumour by locally increase the cell membrane permeability (sonoporation) or by ultrasound-activation of non-toxic compounds (sonodynamic therapy) (Rosenthal 2004, Mitragotri 2005, Paliwal and Mitragotri 2006).

1.4.2 Ultrasound

Ultrasound, considered as a non-ionizing radiation, is a mechanical wave above the threshold for human hearing (20 kHz). There is a wide range in the frequencies and intensities used in medical applications; frequencies from kHz to several MHz and intensities from mW/cm^2 to kW/cm^2. Ultrasound, as well as sound, is expressed in

power (Watt; 1 W=1 Nm/s=1 J/s) or pressure (Pascal; 1 Pa=1 N/m^2). The intensity of sound is expressed in intensity W/cm^2, i.e. I (intensity)=P (power)/A (area).

Mammalian tissues display different sensitivities to damage by agents such as ionising radiation, chemotherapeutic agents and ultrasound. The tissue and cell response to different ultrasound energy exposures are less investigated, although it is well known that the ultrasound wave propagates differently in different media depending on many factors (Barnett 1997, USMB Section 3 2000, Dalecki 2004). The physical aspects of ultrasound are many, and by using different set up for delivering the ultrasound energy the induced chemical and biological effects can vary. Ultrasound can be used for production of hyperthermia and ablation in a tissue. The cell damage induced by ultrasound can be of both thermal and non-thermal origin and is due to the compression and decompression of the molecules through which it traverses. Examples of parameters that can have impact on the biological effects are for example the frequency, intensity, time of exposure, number of and time between exposures, pulsed or continuous wave mode, viscosity and density of the media, gas content, absorbing components and of course the individual sensitivity of the exposed tissue. When an ultrasound wave traverses a cell suspension or tissue, the mechanical energy that is transferred can rise the temperature and/or generate mechanical and chemical damage to biological structures (Kondo and Kano 1988, Riesz and Kondo 1992, Leighton 1994, Worthington 1997). A specific property of ultrasound is that it can produce acoustic cavitation. The definition of cavitation is formation, growth and collapse of small gas (micro-) bubbles which originates from the compression and decompression of the molecules in the media during the influence of an ultrasound field. The cavitation effect is separated into two forms: non-inert (stable) and inert (transient). During non-inert cavitation conditions the micro bubbles may oscillate for a longer period of time and generate shearing and tearing forces, which can cause mechanical damage to cellular structures. During inert cavitation conditions the micro bubbles oscillate only a few acoustic cycles before they implode. When the cavitation bubble collapse the pressure increases rapidly and highly, which leads to local spots with very high temperatures. This energy release causes thermal decomposition (sonolysis) of water and subsequent production of short-lived free radicals such as hydroxyl- and hydrogen radicals as well as long-lived hydrogen peroxide. These chemically reactive molecules are believed to be capable to induce similar damage as those induced by ionising radiation.

1.4.3 Biological effects of ultrasound

In contrast to the extensive knowledge of biological effects induced by radiation, the knowledge of cellular effects induced by ultrasound is rather limited, especially when it comes to subcellular damage and intracellular mechanisms. Therefore the biological effects of ultrasound are discussed in more detail than those of radiation, intending to provide information not presented in the papers included in this thesis.

Ultrasound has been reported to have the capacity to induce cell death in many different types of cells and many different cellular effects depending on exposure levels used (Dalecki 2004, Miller 1996, NCRP 1983, Suslick 1998, WFUMB 1998). Ultrasound under non-hyperthermic conditions may directly kill the cell by the mechanism of cell lysis (the result of irreversible damage to the cell membrane), or inactivate the cell by not yet known mechanisms, the cells die by reproductive cell

death and more recently, ultrasound has been shown to induce apoptosis in various primary and derived normal and malignant hemopoietic cells (Ashush 2000, Honda 2002, Lagneaux 2002, Feril 2003). Ultrasound-induced has been shown to induce both p53-dependent and p53-independent apoptotic pathways. Some of the apoptotic features seen after ultrasound treatment are morphological changes, reduced mitochondrial potential, loss of phosphatedylserine asymmetry, DNA fragmentation, loss of plasma membrane, activation of caspase-3, proteolytic degradation of the caspase substrate PARP and modulation of bcl-2/bax ratio. A decrease of the intracellular glutathione level has been observed, suggesting that oxidative stress is involved. Also, ultrasound induces DNA damage, both DSBs and SSBs (Kondo 1985, Elsner and Lindblad 1989, Miller 1991a, Miller 1991b, Miller 1995). The major ultrasound-induced cytotoxic event is not clear although membrane damage is considered to be of major importance. The damage to the cell membrane can be both reversible and irreversible. The reversible membrane permeabilisation, denoted sonoporesis, is used for increasing the uptake of foreign DNA (gene transfection), proteins or other molecules such as chemotherapeutic agents (Paliwal 2006, Nelson 2002, Tachibana 2000, Miller 1999, Greenleaf 1998, Bao 1997, Johannes and Obe 1997, Wörle 1994). Other membrane effects reported are lipid peroxidation, removal of cell surface receptors/structures, changes in adhesion and migration capacity (Brayman 1999, Alter 1998, Yumita 1996). Transient decrease of mitochondrial activity, morphological damage to mitochondria and mobilisation of Ca^{2+} from intracellular stores have been reported (Suhr 1996, Seitz 1993). It has been shown that low-intensity ultrasound is capable to induce different intracellular responses in cells, for example increased levels of proteins or mRNAs such as IGF, osteocalcin, bone sialoprotein (Naruse 2000), IL-1beta, IL-8, bFGF, VEGF (Doan 1999, Reher 1999) and SP72 (Huang 1999). There is also a report on ultrasound-induced gene expression, using cDNA microarray expression profiling, showing that internal cavitation seems to be needed to have impact on the gene regulation (Tabuchi 2002). Other effects of low-intensity ultrasound are increased cell proliferation, stimulating effects in wound healing and bone matrix formation. Little is known to what extent these effects can be induced by higher intensities of ultrasound.

1.5 CHEMOTHERAPEUTIC DRUGS - CISPLATIN

Therapeutic drugs used in chemotherapy are classified according to their cellular mechanisms of action: alkylating and platinum agents (e.g. cisplatin), antimetabolites (e.g. 5-fluorouracil), topoisomerase inhibitors (e.g. etopside), microtubular-targeting agents (e.g. vincristine) and others (e.g. new target-specific agents).

An impediment in chemotherapy is development of resistance to a specific drug but also to several drugs i.e. multidrug resistance. The major mechanisms for chemoresistance in tumour cells are decreased uptake of drugs that require transporters to enter cells, increased efflux of drug, changes that affect the capability of the drug to kill the tumour cell such as increased DNA repair, reduced apoptosis, and alterations in cell cycle or drug metabolism (Longley 2005).

Cisplatin is an inorganic and highly reactive alkylating hydrophilic platinum compound. Although its cytotoxic effect is believed to mainly depend on induction of DNA damage, other targets have been reported (Wang 2005, Mandic 2003). The cellular uptake and the mechanism(s) are not fully understood. Several reports support

uptake of cisplatin by passive diffusion, however, recent research have provided evidence for active transmembrane transport (Ishida 2002). Cisplatin binds covalently to DNA and proteins, forms DNA adducts leading to DNA-DNA and DNA-protein crosslinks with primarily intrastrand crosslinks. Cellular effects such as inhibition of DNA synthesis, suppression of RNA transcription, effects on cell cycle and induction of apoptosis are reported. Cisplatin is widely used for many malignancies and is one of the most potent anticancer drugs used for several cancer forms e.g. testicular, ovarian, cervical, head and neck and non-small lung cancer. Many tumours have inherent or acquired resistance to chemotherapeutic agents, including cisplatin. Like for most other chemotherapeutic agents the treatment is limited by adverse effects, e.g. nausea, vomiting, neuro- and nephrotoxicity.

1.6 RATIONALE FOR COMBINED THERAPIES

When exploring modalities using combination treatments, the different cellular damage induced may be advantageous from at least two perspectives, namely if the additional treatment selectively can increase the damage to the site of interest, and hereby allow a lower dose or, even better, if the damage induced from the two agents interacts and results in synergistic effects (Wilson 2006) e.g. trimodality therapy (radiation-, chemo- and hyperthermia therapy) (Corry and Armour 2005). By combining treatment modalities with different mechanisms of cellular action it may increase the probability to target heterogeneous tumour subpopulations since the sensitive cells can only be killed once (Hall and Giaccia 2007).

There are several ways to enhance the cell killing effect. One agent may facilitate another agents cytotoxic action e.g. ultrasound-induced membrane permeabilisation facilitates drug uptake. Or, one agent inhibits the repair of the damage induced by another agent e.g. several interacting mechanisms are proposed for hyperthermia such as inhibited repair of radiation-induced DNA damage (Suit 1974, Kampinga 2001).

Both ionising radiation and ultrasound are non-invasive cell killing agents that can be focused and enhanced with various types of agents such as pharmacological drugs and hyperthermia. The therapeutic advantage of sensitizing the malignant cells with ultrasound to the effect of an anticancer agent is the locally increased efficacy of the drug, involving possible dose reductions and hence reduction of side effects.

By knowing the molecular mechanism of the agents better, together with information of specific properties of the tumour and normal tissue, a selective treatment an optimal dose delivery for every cancer patient may be possible.

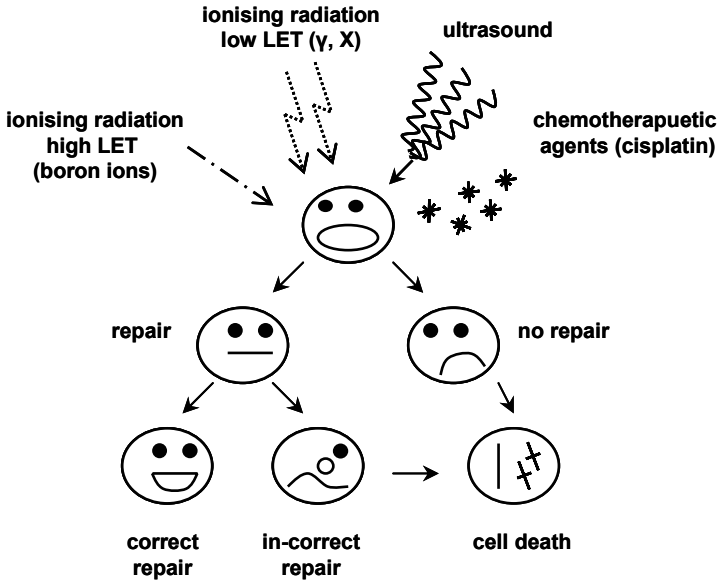

Figure 1. Simplified illustration of a cell's response to harmful agents.

2 THE PRESENT STUDY

2.1 AIM

In this thesis work, the aim was to investigate the cellular effects of ultrasound, both as a single treatment and in combination with radiation or a chemotherapeutic agent, and accelerated ions for new approaches to improve tumour therapy.

Specific aims

- To set up a system for *in vitro* ultrasound exposure of cells

- To study dose-response relationships for a non-malignant solid tissue-derived cell line exposed to ultrasound with respect to cell survival, cell lysis, membrane integrity and DNA damage (paper I).

- To study the intensity-dependence of ultrasound-induced cellular effects in a cell line derived from a human solid tumour with respect to cell survival, cell lysis, membrane integrity, cell cycle distribution effects and morphological characterisations of various modes of cell death or aberrant conditions such as apoptosis or micronucleation (paper II).

- To study if ultrasound could enhance the effect of radiation, and if the combined effect was influenced of intensity, treatment order or time between exposures, and whether the effect was reflected in other endpoints besides cell survival (papers I, II).

- To study if ultrasound could enhance the effect of the chemotherapeutic agent cisplatin, with respect to cell survival and apoptosis (paper III).

- To investigate if the cell killing effect of ultrasound was dependent on tumour type or correlated with sensitivity to radiation, and in addition, if apoptosis was induced (paper IV).

- To study the cell survival of a human tumour cell line after exposure to accelerated boron ions at three different LET and γ radiation in order to estimate the RBE (paper V).

- To investigate and compare the influence of LET, dose and time on the induction of apoptosis in a cell line derived from a human solid tumour after exposure to accelerated boron ions at four different LET and γ radiation. In addition, the effects on cell survival, cell cycle distributions, and induction and rejoining of DNA DSBs were studied (paper VI).

2.2 CELLS

Since we were interested in therapies for inoperable solid tumours, we chose to study the cellular effects of treatment with different agents in cell lines derived from human

solid tumours. However, initially, for the investigation of the potential use of ultrasound in tumour therapy, we started to work with the well established and worldwide used Chinese hamster cell line V79-379A. This was due to the fact that this cell line has been used in numerous studies especially in the field of radiation biology, which enables comparisons of induced cellular effects from a new agent. The tumour cell lines used were U-1690 (small cell lung carcinoma), CaSki (cervical carcinoma), M059J and M059K (gliomas derived from different areas of the same tumour) and AA (melanoma). These cell lines were selected mainly due to the knowledge of their differing sensitivity to radiation, which we also investigated (paper VI) in our laboratory. To avoid that the ultrasound effect was dependent on tumour type, cell lines derived from more than one tumour group were selected. This was an initial selection and the intention was to extend the investigation, if time allowed, with several cell lines representing other tumour types and exhibiting various degrees of radiation- and chemoresistance. The relevance for using a lung cancer cell line, despite the fact that ultrasound treatment of tumours located close to air filled cavities (lung and intestines) at present seems difficult, was the accumulated data in our laboratory of theses cells' response to various toxic agents. All cell lines were grown as monolayer and their plating efficiencies ranged from 30 to 95%.

2.3 ULTRASOUND, RADIATION AND CISPLATIN EXPOSURES

2.3.1 Ultrasound

Two different ultrasound transducers and experimental set ups were used. In the first ultrasound study (paper I) the cells had to be transported by car to the transducer (located at Comair AB in Hjorthagen, Stockholm, Sweden) and the cells were kept on ice during the experiment, except for the 30 s of ultrasound exposure (in room temperature), in order to perform combined treatments with ionising radiation. The cell samples in these combined experiments were considered as "almost" simultaneously exposed. In the following ultrasound studies (papers II-IV) a specially designed transducer (in a smaller design convenient for use at our laboratory) was used. For both ultrasound systems, the frequency was approximately 1 MHz and continuous mode was used. Degassed water (<2.5 mg/l O_2) was used as coupling media. Sonication was performed under non-hyperthermic conditions; no temperature increase was detected after 30 s of exposure in the sample volume measured with 0.1°C precision. Free radical production was measured with Fricke (ferrosulphate) dosimetry to verify the occurrence of cavitation. To be noted is that the intensities given in paper I has been recalculated due to a missing efficiency factor, therefore the correct intensities are achieved by multiplying the electrical powers with 7.21, i.e. 5 W corresponds to 36 W/cm^2 (and not to 63 W/cm^2 which was given).

At the start of the ultrasound project I spent a lot of efforts and time to find a suitable exposure chamber to keep the cells in during exposure. The chamber had to be as acoustically transparent as possible (very thin and of similar acoustic impedance as the surroundings) to avoid interfering with the ultrasound waves, and at the same time have properties acceptable from a cell culturing perspective. The chamber had to endure sterilisation and be possible to seal, and the cells had to tolerate the material. Several different exposure vessels were tested. Plastic vessels such as eppendorf tubes had to thick walls and tubes of glass exploded. Thin condoms were not applicable either due to the problems of handling a small cell volume in it. Also, a small specially

designed cylindrical stainless steel chamber was constructed. However, the expenses for producing the large number of chambers that would be needed in one experiment were too high. Finally, we found disposable cone-shaped soft cases from an ear thermometer suitable for ultrasound exposures and the small beam focus.

With our system the ultrasound waves were focused into a small target volume below the water surface of the bellows attached to the transducer. Exposure of cells in a culture flask or a dish was of obvious reasons not possible. (To note, the detachment of cells must be considered when using such a system, which has been used in several reports on low level ultrasound.) Initially, to mimic a tissue or solid tumour and to keep the cells in place during sonication I embedded the cells in agarose. However, this did not work at all since the agarose was disrupted when ultrasound was applied. Therefore we chose to work with cells in suspension.

2.3.2 Ionising radiation

Photon irradiations (low LET) were performed using X rays (paper I) or γ rays from a ^{60}Co therapy unit (papers II, IV, V, VI) at the Karolinska University Hospital, Stockholm, Sweden. Boron irradiations ($^{10}B^{5+}$, different LET; 40, 80, 125 and 160 eV/nm) were performed using a synchrocyclotron at the The Svedberg Laboratory (TSL) in Uppsala, Sweden.

In the studies exploring a possible combination effect of ultrasound and radiation (papers I, II), a fixed dose of 2 Gy was used in order to be comparable to one fractionation dose used in conventional radiotherapy regimens.

2.3.3 Cisplatin

In order to investigate ultrasound as a potential agent to locally enhance the uptake of chemotherapeutic agents, we concurrently exposed tumour cells to *cis*-diamminedichloroplatinum (II) (cisplatin) and ultrasound. Since several *in vitro* studies have shown an increased effect of various cytotoxic drugs, but not of cisplatin, we thought this drug interesting. Three negative studies were reported (Harrison 1991), however, there are data on synergistic effects reported in an abstract from a conference (Takada 1997) and data on ultrasound-induced increase in intracellular cisplatin uptake in one review (Yu 2006), which unfortunately cannot be used as proper references since there is no or little description of experimental conditions and detailed information about the results. Although ultrasound seems to have a sustained effect on cells, which was seen in our studies with combined ultrasound and radiation regimen as well as in other reports, we believed that in order to obtain a maximal effect of a combined modality, the cells needed to be exposed simultaneously to ultrasound and the drug. Therefore we administered cisplatin immediately before the 30 s ultrasound exposure, to allow for increased drug influx, and for 1 h incubation with drug to allow for drug action, before withdrawal. In a practise, at the clinic, the one hour incubation may not be achievable or optimal, but we chose this time as a starting point after having reviewing our previous experiments with cisplatin (unpublished data).

2.4 COMMENTS ON THE METHODS USED IN THE PRESENT STUDY

2.4.1 Clonogenic cell survival

In all papers included in this thesis, the reproductive capacity of cells was measured by the clonogenic cell survival assay according to Puck and Marcus (1956). Some important advantages with this cytotoxicity assay are that it minimises the short time influence of cell cycle changes and allows cellular response mechanisms to come into full effect. Cells were serially diluted (selected to give approximately 100 colonies per dish) and plated in triplicates. When colonies had grown to appropriate size (8-14 days) they were fixed in formaldehyde and stained with Giemsa. The surviving fraction (SF) was defined as the ratio of the colony forming efficiencies (means of triplicate dishes) of treated and untreated control cells.

2.4.2 Instant cytolysis and membrane integrity

A characteristic effect of high power ultrasound exposure of cells in suspension is its capacity to induce complete cell lysis immediately. Depending on physical parameters of the ultrasound settings and exposure conditions, the degree of cell lysis or detectable membrane effects can vary enormously. Since the purpose with this investigation was not to induce total destruction of cells, and the following intention for tumour therapy was not to induce immediate tissue destruction (as with HIFU for tumour ablation), we focused on using and investigating so-called medium levels of ultrasound in the combined treatments with radiation or cisplatin. For a correct comparison of the cell killing effect induced by ultrasound with that induced by radiation, which does not lead to direct cell lysis (at least not detected in our experiments with photon irradiations), the fraction of cells lost due to the ultrasound exposure itself has to be taken into account. Therefore the remaining fraction of cells not lost by instant cytolysis had to be calculated by counting every sample in the experiments and compare the exposed with the controls.

In addition, to make these experiments even trickier, ultrasound can instantaneously induce both reversible and irreversible disruptions in the cell (plasma) membrane integrity leading to increased membrane permeability to dyes. Since the extent and time course for this event had not been investigated with the cell lines used in this work, cells were seeded after the total number of remaining cells and no correction for cells stained positive for trypan blue dye was made in the clonogenic survival assays.

In studies on the reversible effect, the opening and closure of the membrane pores was shown to be a fast process, occurring within minutes (Schlicher 2006, van Vamel 2006).

2.4.3 Detection of DNA damage

The induction and repair of DNA damage is of crucial importance for cell survival. Without fully and correct repaired DNA damage the risk for of genetic changes such as genomic instability and heritable effects increases for a surviving cell. Since the literature on ionising radiation-induced DNA damage is enormous compared to that on ultrasound-induced, in addition to the knowledge of the importance of DNA damage, we investigated the capability to induce DNA strand breaks with the ultrasound frequency and intensities used for our survival studies. The DNA precipitation

technique in alkali was used to detect both SSB and DSB (Olive 1988), and the pulsed-field gel electrophoresis (PFGE) method for DSB only (Cedervall 1994) (paper I). Graded doses of X-rays were used for comparison of ultrasound-induced DNA breaks. PFGE was also used to measure the induction and rejoining of DSB after photon and accelerated boron ion exposures in order examine the influence of LET (paper VI). In the study on ultrasound-induced DNA damage, it was absolutely necessary to centrifuge and rinse the cells after the ultrasound exposures in order to separate free DNA fragments originating from lysed cells from the DNA fragments of interest still enclosed in non-lysed cells (paper I).

2.4.4 Morphological characterisation of cells

Fluorescence microscope studies were performed on pooled detached and adherent cells (200-500) after being fixed in formaldehyde and dual-stained with Hoechst 33342 (staining nucleus) and acridine orange (staining both nucleus and cytoplasm) to morphologically analyse cellular conditions (papers II, III, IV, VI). Several criteria were used to characterise cells as normal or abnormal (aberrant) such as apoptotic or micronucleated. An general estimation of the ultrasound effects could also be made in fresh cells when counting and staining them with trypan blue, which was performed with every sample after exposure and when collecting cells at different time points. Also applied in some experiments was Türk staining of fresh cells to visualise apoptotic cells (data not shown).

In addition to morphologic characterisation of apoptotic cells, formation of apoptotic bodies were measured by cell size analyses with a Coulter counter with a Channelyzer (sizes verified in Bürker chamber) (paper VI). Interestingly, the induction of apoptosis in the melanoma cells, in contrast to the glioma cell lines M059J and M059K (Holgersson 2003), was not accompanied by the formation of apoptotic bodies (which has also been found in other cellular systems, unpublished data). This was explained by engulfment of the triggered apoptotic cells, see figure 2.

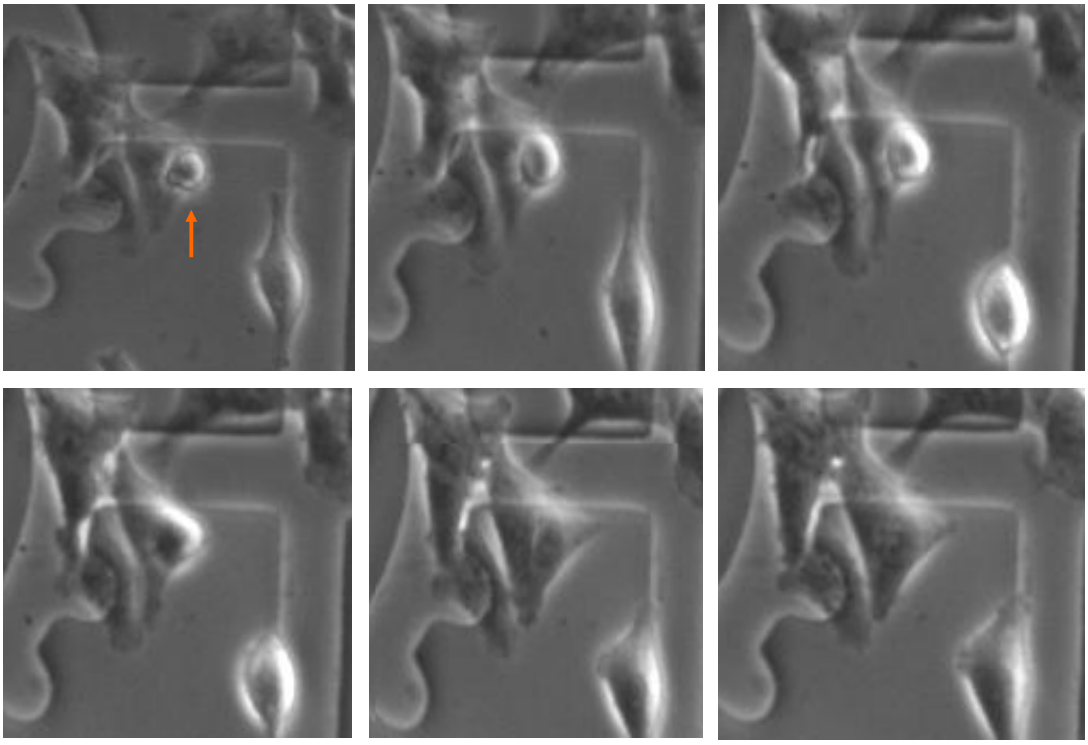

Figure 2. Time-lapse studies of engulfment of an apoptotic cell (arrow). Human melanoma cells (AA) were incubated at 43^{o}C for 30 min, thereafter at room temperature for 30 min, and then incubated at 37^{o}C where pictures were taken every 6th minutes.

The formation of micronuclei was also investigated in the cell morphology analyses (especially in U-1690 cells in paper II). In these analyses, no cytochalsin B-induced inhibition of the cytokinesis (leading to binucleated cells where the micronucleus is trapped) was involved. Instead, an estimation of micronucleation in cells fixed directly after sample collection was made. This method was slightly more difficult, and maybe more uncertain, due to the fact that the analyses were performed with fluorescent dyed cells (not Giemsa) fixed in non-optimal conditions making the separation of micronuclei and nuclei in the space inside the cell smaller, and hence more difficult to distinguish. However, a general estimation of the fraction of micronucleated cells was possible to do.

2.4.5 Cell cycle phase distributions and TUNEL assay

The estimation of cells in different phases of the cell cycle was determined by flow cytometric analyses of the DNA content (papers I, VI) (Heiden 2000). Cells were fixed in formaldehyde, nuclei were released and permeabilized before staining with the fluorescent dye DAPI. For flow analyses 11 000-64 000 (paper II) or 10 000-50 000 (paper VI) nuclei were measured per sample and histogram.

Generally, several methods are recommended in order to verify the induction of apoptosis. Therefore, apoptosis was also quantified with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) technique for *in situ* detection

of DNA fragmentation (paper II). The TUNEL analyses were performed in combination with the flow measurements, where the cell cycle analysis was done in gated TUNEL-negative (i.e. non-apoptotic) cells. In addition, the intensity and specificity of the TUNEL staining were verified in fluorescence microscope.

2.4.6 Cell proliferation

The mean doubling time of all cell lines used in this study and proliferation response of the U-1690 cells after exposure to single or combined exposures of ultrasound and radiation were measured by establishing growth curves (paper II). The adherent cell fraction was counted in a hemocytometer (Bürker chamber) after certain incubation times and used for growth rate calculations.

2.4.7 Chromosomal aberrations - a pilot study

Since there are reports on synergistic effects of combined exposure with radiation and ultrasound on the induction of chromosome aberrations (Burr 1978, Künze-Muhl 1981), I was interested in if this effect would be induced with our experimental conditions (unpublished data) and whether the genetic change was reflected in the cell survival assessed in parallel (paper I). The rodent V79 cells with a low and homogeneous (and stable) modal number of chromosomes were used for this purpose. Cells in exponential growth phase were exposed to ultrasound or radiation alone, or to combined exposures with ultrasound either before or after radiation. After exposure, cells were incubated first without (to let the cells continue through the cell cycle) and thereafter with colcemid (inhibitor of spindle formation during cell division, leading to increased fraction of cells inhibited at the metaphase stage of mitosis), before hypotonic treatment (to make the cells swell and the membrane permeable in order to lose it cytoplasm) and following fixation. After being dropped on slides and stained with Giemsa, microscope analyses of 100 cells per treatment were performed. The criterion of chromosome aberrations was formation of dicentrics. The results are presented in Summary and Discussion.

2.5 SUMMARY OF PAPERS

2.5.1 Paper I

Ultrasound induces intensity-dependent cellular effects and synergistic reduction in cell survival in combination with radiation in non-tumour rodent cells
Aim: To study focused continuous high-intensity ultrasound-induced cellular effects with different methods (clonogenic cell survival, direct cell lysis, membrane damage, DNA damage, DSB and SSB), and to investigate if combined treatments of ultrasound and ionising radiation can enhance the cell toxicity in the widely used V79 hamster lung fibroblast cells.
Results & Conclusion: Ultrasound induced an intensity-dependent reduction in clonogenic survival, increase in cell lysis and cells with lost membrane integrity. Both SSB and DSB were induced. This implies that the ultrasound-induced cell death probably depends on both membrane and DNA damage. In the combined treatments the regimen with ultrasound exposure immediately before, but not after radiation, resulted in a significant synergistic reduction in cell survival (enhancement ratios 1.6 and 1.1, respectively). Addition of ultrasound, which can be delivered to a defined

volume, to radiation therapy may enhance the cell killing effect in a tumour while sparing the surrounding healthy tissue.

2.5.2 Paper II

Ultrasound induces intensity-dependent cellular effects and synergistic reduction in cell survival in combination with radiation in human solid tumour cells
Aim: To study the cellular response of non-hyperthermic focused continuous ultrasound alone and in combination with ionising radiation in a human tumour cell line, and to study the influence of intensity, treatment order and time interval between the exposures after combined treatment. Clonogenic cell survival, immediate cell lysis, loss of membrane integrity, apoptosis, growth and cell cycle distributions (0-96 h) were investigated in U-1690 small cell lung carcinoma cells.
Results & Conclusion: Ultrasound exposure resulted in an intensity-dependent reduction in clonogenic cell survival, cell lysis and loss of membrane integrity. No significant changes in cell cycle distributions were found after ultrasound in contrast to radiation. High intensities of ultrasound induced apoptosis and cells with aberrant morphology. Combined treatment with ultrasound and radiation resulted in synergistic reduction in cell survival (enhancement ratios 1.1-2.9) and was observed even after a 2 h repair-interval between the exposures. The synergistic effect was most pronounced when ultrasound was delivered before radiation. The intensity-dependence was significant, but not treatment order or time interval. High-intensity ultrasound alone is an efficient inducer of cell death, both immediate and delayed. The results show that there could be a benefit by adding high-intensity ultrasound to radiation therapy.

2.5.3 Paper III

Ultrasound induces synergistic reduction in cell survival in combination with cisplatin
Aim: To investigate if ultrasound can enhance the cytotoxic effect of cisplatin in U-1690 small cell lung carcinoma cells measured by clonogenic cell survival and apoptosis.
Results & Conclusion: Combined ultrasound-cisplatin treatment induced a synergistic reduction in cell survival for both concentrations tested (enhancement ratios 1.40 and 1.15, respectively). No major enhancement effect in apoptosis was detected at any of the time points studied (0-72 h). Ultrasound, with low toxicity that can be focused deep in a tissue, could be applied to locally increase the cytotoxic effect of cisplatin in a tumour.

2.5.4 Paper IV

Ultrasound induces similar intensity-dependent reduction in cell survival in different human solid tumour cells
Aim: To study clonogenic cell survival in human cells of different tumour origin (lung carcinoma, cervical carcinoma, two gliomas, melanoma) after exposure to ultrasound and compare with photon radiation. Also, other cellular effects such as instant cytolysis, membrane integrity and induction of apoptosis were investigated.
Results & Conclusion: Ultrasound induced an intensity-dependent reduction in cell survival in all cell lines tested. Although, there were differences in the survival

response, indicating differences in ultrasound sensitivity, the variation was significantly reduced compared to conventional photon radiation. This resembles the response after accelerated ion irradiation. Ultrasound of high intensity induced apoptosis in all cell lines studied (U-1690, M059J, M059K, CaSki). Ultrasound appears to be a potent inducer of cell death for several tumour cell types. This may be of clinical value for using ultrasound as an adjuvant treatment in cancer therapy, especially since the ultrasound energy can be focused into a deep seated tumour.

2.5.5 Paper V

Increasing LET increases the RBE for cell survival
Aim: To compare the difference in relative biological effectiveness (RBE) between boron ions with three different LET with γ radiation in a human melanoma cell line (AA). Two different cell survival models were used.
Results & Conclusion: There was an LET-dependent decrease in clonogenic cell survival; the RBE increased with increasing LET. Different cell survival models may generate different RBE, especially at low doses and high survival levels. Advantages with ion beams are improved precision in dose delivery and increased biological effect i.e. increased damage to the tumour while sparing normal surrounding tissue.

2.5.6 Paper VI

High LET ion radiation induces increased apoptosis compared to low LET photons
Aim: To investigate and compare the influence of LET, dose and time on the induction of apoptosis in a human melanoma cell line exposed to accelerated boron ions at four different LET and photons. Apoptosis and cell cycle distribution was studied up to 9 days after exposure. Clonogenic cell survival and DNA induction and repair were also investigated.
Results & Conclusion: Boron ion irradiation induced higher levels of apoptosis compared to photons at all time points studied. Despite LET-dependent clonogenic cell killing, quantitative measurement of LET-dependent apoptosis was not possible due to different time course of appearance and disappearance of apoptotic cells resulting in a complex response pattern. Even intermediate LETs were effective in increasing cell killing, apoptosis, cell cycle delay and reducing DSB rejoining.

2.6 SUMMARY AND DISCUSSION

In the present investigation the cellular effects, with main focus on reproductive capacity, of non-thermal high-frequency ultrasound alone or in combination with either ionising radiation of low LET or the chemotherapeutic agent cisplatin were studied. In order to examine if there was an intensity-dependent response, and whether this response was cell type specific the cell survival after ultrasound exposure was studied in a number of different human tumour cell lines. The other objective for the present investigation was to study the cellular effects of boron ion irradiation and the influence of radiation quality (LET).

An intensity-dependent reduction in clonogenic cell survival was seen in all cell lines here studied; rodent V79 lung fibroblast cells (paper I) and human tumour cell lines derived from lung, cervix, glioma and melanoma (paper IV). The shapes of the

survival curves showed, after an initial threshold, similar exponential decrease. Hence, the variation in ultrasound sensitivity was significantly smaller compared to the large variation in cell survival of these cell lines seen after exposure to photon radiation. This small variation in ultrasound sensitivity in different tumour cell types is in contrast to the large variation reported by Lejbkowicz and Salzberg (1997). They showed that normal cells were relatively resistant compared to malignant cells to non-thermal low-frequency low-intensity ultrasound for variable periods of time. However, the malignant cells derived from distinct origin showed wide variation in sensitivity. One important factor that may explain these contradictory results in cell survival is the completely different ultrasound qualities used. Our data, based on ultrasound of a higher frequency and intensity, are more similar to that of HIFU (which induces tissue ablation) with respect to the effect not being influenced by tumour origin. Although not buy the same cellular mechanisms, it may be so that the difference in sensitivity resembles that induced by ionising radiation of different qualities. The variation in sensitivity to low LET radiation is reduced for high LET, and, furthermore, the cytotoxicity is not dependent on functional p53 (Takahashi 2004, Iwadate 2001, Tsuboi 1998). Different qualities of a certain agent, as well as dose and time of exposure of stimuli may induce different damage and stress response to the cell.

A characteristic of ultrasound is its capacity to induce instant cytolysis, which may be related to *in vitro* studies using cells surrounded by fluid in a greater extent than cells in a tissue and thereby lowering the cavitation threshold. Intensity-dependent cytolysis was seen in all cell lines studied. Also an intensity-dependent decrease in the fraction of cells with intact cell membrane integrity was seen. The damage to the cell membrane, and subcellular components, can be due to both cavitational effects (free radicals and mechanical effects) as well as mechanical effects (tearing and shearing forces).

Ultrasound induced an intensity-dependent induction of DNA damage, measured as SSB and DSB in rodent V79 cells, reaching a plateau at higher intensities (paper I). Also in the lung cancer cells DSB was induced (unpublished data). To what extent the DNA damage affected the cell survival was difficult to estimate since membrane effects were induced at the same time and it was not possible to separate cells with increased membrane permeability from DNA damaged cells. In addition, neither the rejoining capacity nor the correctness of DSB repair were studied, which could have given further insights of the complexity or severity of the damage.

There are few reports on ultrasound-induced DNA damage, and none on ultrasound in combination with radiation. The synergistic effects in V79 cell survival after combined treatment with ultrasound exposure before radiation was not reflected in the DNA damage induced (paper I). However, in a pilot experiment I studied the induction of chromosome aberrations (dicentrics) after ultrasound and radiation alone or in combination with ultrasound before or after radiation using the same doses and intensities as in paper I. The formation of dicentric fragments was 0.9% in unexposed cells, 2% after 5 W (36 W/cm^2), 7% after 2 Gy, 14% after ultrasound before radiation and 32% after ultrasound after radiation. There are few studies on chromosomal aberrations induced by ultrasound alone or in combination with radiation and data is not consistent (Clarke 1970, Burr 1978, Künze-Muhl 1981). Induction of DNA damage may be indicative of the genotoxic capacity of an agent, but it may not be indicative of later responses such as cell survival. Correct repair of DNA may be crucial and this was not studied here. But the formation of chromosome aberrations reflects that all cells

were not capable to correctly repair the DNA damage induced. The amount of chromosome aberrations formed (and possible to quantify at a certain time point) may be influenced of several factors such as cell cycle arrest since it may take some time for the DNA damage to be manifested or that severely damaged cells die before metaphase is entered.

Ultrasound enhanced the cytotoxic effect of cisplatin (paper III). The mechanism(s) for the synergistic effect may involve both increased uptake of the drug due to ultrasound-induced increased membrane permeability (van Vamel 2006, Schlicher 2006) and interacting sublethal damage. Ultrasound has been shown to induce several different cellular effects from sonoporation, membrane lipid peroxidation (Yumita 1996), changed gene expression (Tabuchi 2007) and DNA damage to cell lysis (paper I). There are a large number of reports on *in vitro* synergistic effects of ultrasound of various qualities and exposures times in combination with several types of chemotherapeutic agents such as adriamycin and 5-fluorouracil (Harrison 1991, Rosenthal 2004) but also *in vivo* with for example 5-fluorouracil and boron compounds (Harrison 1991, Sur 1999). There is a great interest in the potential application of ultrasound energy in combination with chemotherapeutic agents due the obstacle with chemoresistance in tumour therapy. Ultrasound-enhanced chemotherapy with locally increased cytotoxicity due to ultrasound-increased drug uptake (contribution from increased permeability in both tumour cells and endothelial vasculature) (Paliwal and Mitragotri 2006) and interacting damage is a potential combination modality to improve chemotherapy.

The synergistic effects seen in clonogenic cell survival after the combined treatments with ultrasound and radiation or cisplatin were not reflected in the induction of apoptosis. High fractions of apoptotic cells were not seen after single exposures to lower doses of low LET radiation or cisplatin. Therefore one simple explanation may be that the manifestation of cell death after combined exposures occurs through other death processes. A reduced cell growth was seen after radiation in combination with the higher ultrasound intensities (paper II). Various morphological criteria of aberrant cells were investigated in parallel with apoptosis but there was no clear trend for synergistic effect with these endpoints either, except for some doses or time-points. However, since there may be different modes of cell death involved in the response to toxic stimuli, and their time course may differ, a complete picture of the induced cell death may be difficult to obtain due to the complex response pattern shown by different methods of analysis (Abend 2003, Brown 2005).

In figures 3 a, b and c it is shown that different combinations of agents can induce synergistic effects in clonogenic cell survival in the same human tumour cell line (U-1690). Figure 3 a shows ultrasound in combination with photon radiation and illustrates that various intensities and time intervals between exposures as well as both of the treatment orders can result in synergistic interaction (paper II). Figure 3 b shows the ultrasound-enhanced effect of cisplatin (paper III). In an ongoing project we have studied the cell survival after combined irradiations with high LET nitrogen ions before low LET photons (pilot experiment showed no significant difference with reversed treatment order) and a synergistic effect was found (unpublished data) (Figure 3 c). In the literature both additive and synergistic effects have been reported (using different cell types, experimental design, radiation qualities and treatment schedules).

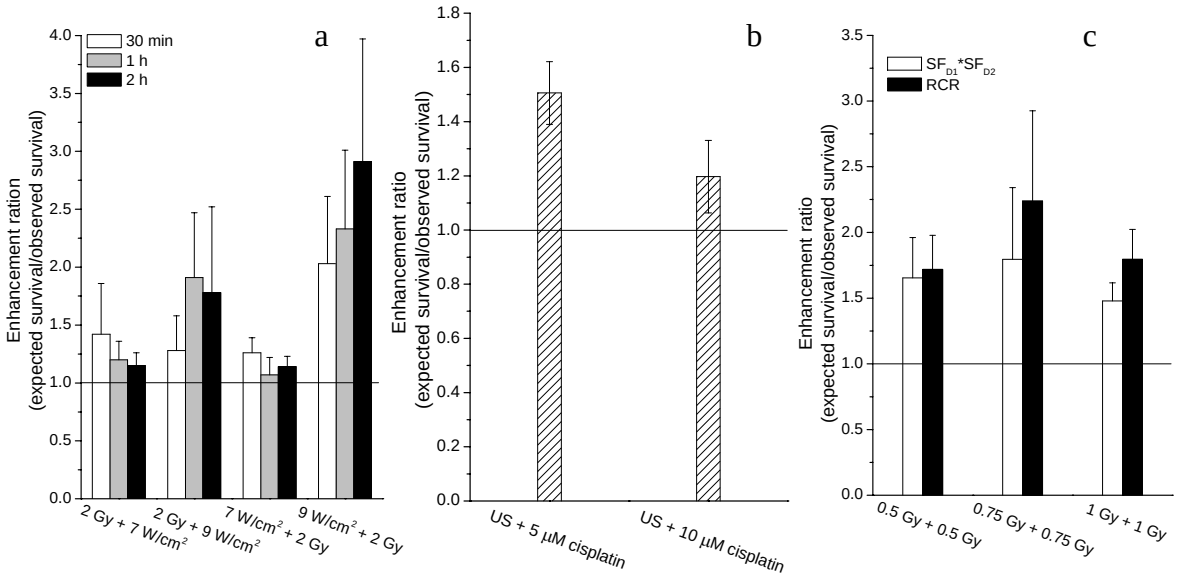

Figure 3. Estimation of synergistic effects after combined treatments. The enhancement ratio ($C_{synergy}$) based on data from the clonogenic cell survival of the small cell lung cancer cell line U-1690 after exposure to a) ultrasound (US) for 30 s and γ radiation (i.e. various ultrasound intensities, treatment orders, time intervals between exposures), b) 7 Wcm^2 for 30 s US and cisplatin (5 or 10 μM for 1 h), and c) high LET nitrogen ions (140 eV/nm) and γ radiation.

High LET radiation is more effective in cell killing relative to low LET (paper V, VI). Higher RBE values after exposure to charged particles (with atomic numbers higher than protons) radiations are obtained for all types of cells, varying LET and ions (Suzuki 2000, Stenerlöw 1995). Figure 4 a shows the cell survival of melanoma cells (AA) after irradiation with accelerated boron ions at different LET (paper VI) and in figure 4 b the cells were irradiated with different ions (unpublished data).

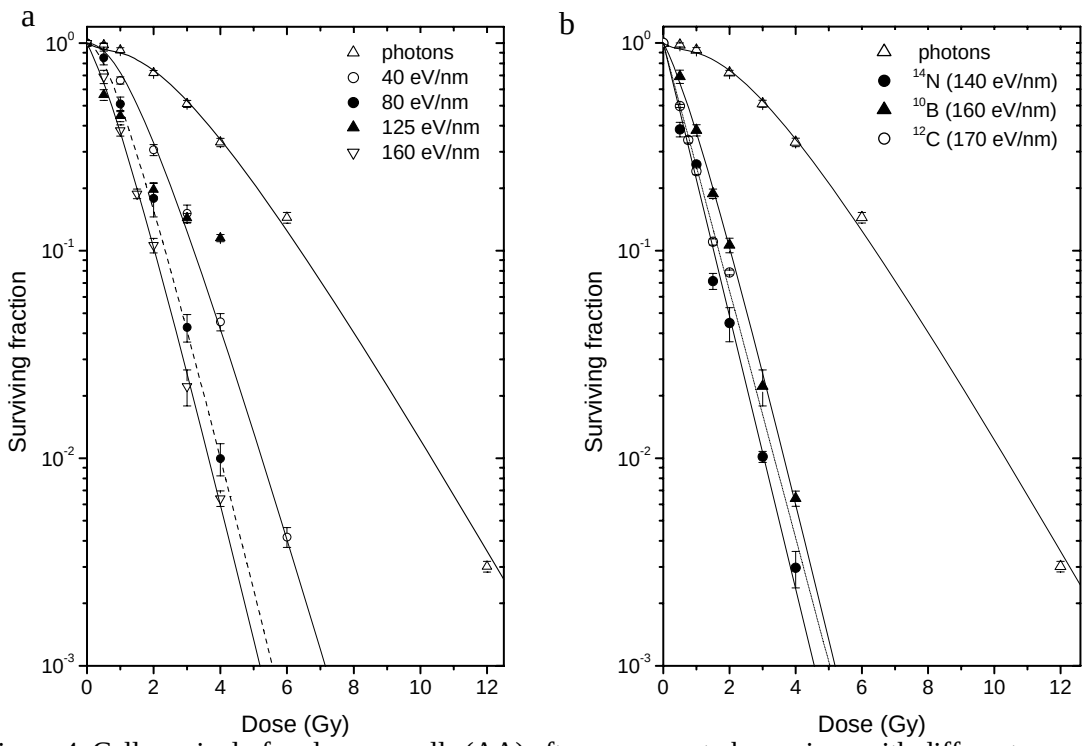

Figure 4. Cell survival of melanoma cells (AA) after exposure to boron ions with different LETs (a) and with different ions (b). Photon irradiations were performed at Radiumhemmet, Stockholm, Sweden, boron and nitrogen at the TSL, Uppsala, Sweden and carbon ions at GSI, Darmstadt, Germany in collaboration with Claire Rodriguez-Lafrasse, Molecular and Cellular Radiobiology Laboratory, Lyon-Sud, France and Gisela Taucher-Scholz, GSI, Darmstadt.

To further investigate the LET effect other endpoints were used (paper VI). The boron irradiations induced increased apoptosis relative photons at all time points studied (up to 216 h). At the early time point (1-5 h) the apoptotic response increased with both dose and LET, but at later time points the apoptotic response pattern was more complex and no clear LET-dependent increase was seen. The biphasic response after high LET irradiation was suggested to be due to two different apoptotic pathways involved, one early and one late response, which have been observed in previous studies (Meijer 2001, Holgersson 2003). Interestingly, no radiation-induced apoptotic bodies or debris could be detected for the melanoma cells, which is an additional method for quantification of apoptosis at later stages. This was later found out to be due to engulfment of apoptotic cells by neighbouring cells; see time laps pictures in figure 2. There was dose-, time- and LET-dependent accumulation of cells in G2/M phase of the cell cycle. The rejoining capacity of DNA DSB was greatly reduced with increasing LET.

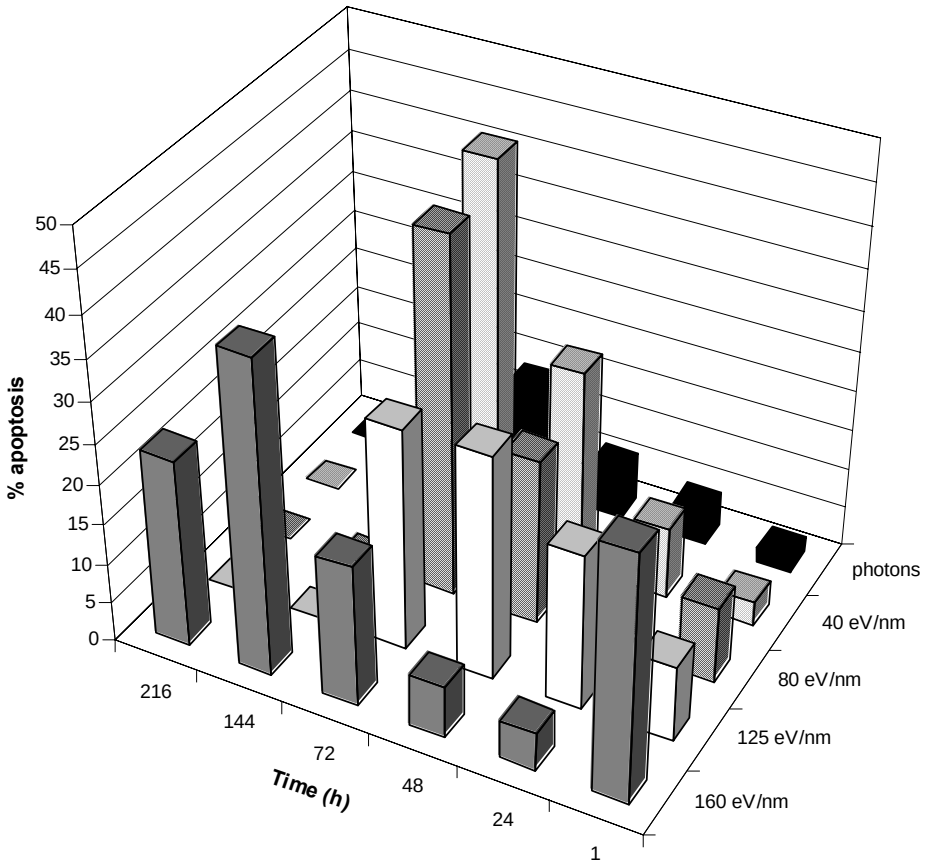

Figure 5. Radiation-induced apoptosis in melanoma cells (AA) exposed 2 Gy photon and boron ions with different LET (only 160 eV/nm were followed longer than 72 h).

Both high LET and low LET induce lesions to the DNA, membrane and subcellular components. Damage to DNA, especially DSB and complex damage being difficult to repair, is considered the main critical target. However, the cellular membrane is also affected upon radiation exposure. For example both photons and heavy ions (lithium and oxygen) induced lipid peroxidation (in microsomes isolated from rat liver) and cell lysis (hemolysis, in erythrocytes) (Choudhary 1998).

The great advantages with charged particle radiation such as the inverse dose profile (increased dose with penetration depth) and increased RBE towards the end of the particle range enables treatment with high dose to the tumour while sparing healthy tissue (Turesson 2003, Kraft 2000). The carbon ion therapy given at the facilities in Japan and Germany, and the ongoing projects for construction of new facilities (for protons as well as other particle radiations), involves increased accessibility not only for patients but also for researchers to further investigate the biological effects *in vitro* and in *vivo*. A better understanding of the cellular effects and underlying mechanisms, especially of factors important for radioresistance in tumours, can lead to a more individual and optimal treatment.

2.7 CONCLUSION

There are numerous ways to kill tumour cells *in vitro*. To selectively eliminate tumour cells *in vivo* without inducing toxicity to surrounding healthy tissues or other adverse effects to the patient is more difficult. Several approaches for improved tumour therapy have been carried out, yet the treatment regimens given at the clinic today will not cure all patients. Therefore there is a need to continue the search for more optimal treatment methods and diagnostic tools. This involves increased understanding of cellular mechanisms as well as development and improvement of therapies targeted to cancer cells. In the present investigation we wanted to integrate cell killing agents that selectively may be given to patients, especially inoperable radiation- and chemoresistant tumours, in order to increase the therapeutic effect. Focused ultrasound and accelerated ions are two non-invasive agents where their qualities of high precision and deep penetration in tissue imply that local energy deposition is possible. Thereby a local treatment by these agents alone, or in combination with other modalities, can increase the damage to the tumour while sparing normal tissue. Indeed, our *in vitro* studies showed that both ultrasound and ions effectively induced cell death. The large variation in sensitivity between different human tumour cell types to conventional photon radiation seen in clonogenic cell survival was greatly reduced and nearly disappeared at higher intensities of ultrasound. This is similar to the effect after ion irradiation. In addition, combined exposures of ultrasound and radiation as well as ultrasound and cisplatin resulted in synergistic effects.

2.8 FUTURE STUDIES

There are yet many discoveries in the field of ultrasound and particle radiation to explore. Investigating combined treatment modalities for enhanced therapeutic effect is a wide and complex field with many factors to consider for a realistic treatment regimen. In addition, the cellular effects seen *in vitro* has to be proven to occur *in vivo* before translated to the clinic. In this thesis, the elimination of cells by different agents was clearly shown by cell survival measurements. Although additional methods were used to detect different damage and cellular responses induced, many of the cellular and molecular mechanisms underlying the cell killing effect are not clear and remain to be elucidated. A general aim is to investigate if the observed effects found in the present investigation are similar in other cell systems i.e. study the survival and other cellular responses in additional cell lines derived from both tumour and normal tissues with various sensitivity to conventional radiation and chemotherapeutic agents. And if positive results are achieved, prove them in *in vivo* systems.

Some specific areas that I find interesting and would like to investigate further are:

Induction and repair of DNA damage. Ultrasound induces DNA damage, both SSBs and DSBs, which was shown in the present investigation (paper 1 and unpublished data) and reported in the literature. However, the type and complexity of DNA damage and DNA repair is very little investigated. Since DNA damage may be crucial for cell survival this needs further understanding.

Late effects of DNA damage. To further study the formation of micronuclei and chromosomal aberrations, and also the induction of genetic changes for a better

understanding of the ultrasound effect and its possible role for genomic instability. This is relevant for risk estimation of ultrasound exposure to man. For example, medical exposure to ultrasound for diagnostic or therapeutic purposes may be applied either alone or in combination with ionising radiation or chemotherapeutic agents as well as contrast agents (microbubbles). Very little is known about these effects and due to the continuous increase of these agents this is an important risk to consider. Also, combined modalities can increase the risk of other adverse effects of which we are not yet fully aware.

Ultrasound source and settings. Two ultrasound systems (transducers) were used in the present study. Using other ultrasound qualities (e. g. frequencies, continuous or pulsed mode) for comparison of cellular response in the same cell system could show which setting is better for certain biological endpoints e.g. modes of cell death.

Methodology. Using spheroids or pieces of tissues in order to better simulate the *in vivo* situation improves the methodology. Especially for combined treatment modalities this would enable studies of ultrasound-increased plasma membrane permeability and drug uptake. And also, for ultrasound alone, and in combination with ionising radiation, the biological effects and access of ultrasound produced radicals could be better investigated.

Sonodynamic therapy. There is a lot of interest and research activity in the field of combined ultrasound and drug treatment. There are several promising *in vitro* reports on increased uptake of various drugs and enhanced biological effects. The great advantage of encapsulation of cytotoxic compounds in microbubbles or microsomes is the locally release and cytotoxic action of drug within and close to the tumour upon ultrasound exposure. Also, administration of non-toxic compounds and activation of them to a cytotoxic form by ultrasound exposure on the site of the tumour (like photodynamic therapy) is an intelligent approach for local tumour therapy. This field is very intriguing and could clearly improve chemotherapy. Sine ultrasound, like radiation, can be delivered deep in a tissue this strategy enhances the possible tumour groups that can be treated.

Free radicals. The production of free radicals by ultrasound and its stress and damage induction in cells would be interesting to study further; especially the amount that is produced at various exposure conditions and ultrasound qualities due to their importance for induction of cellular damage.

Cell signalling. Ultrasound induces cell death. The present investigation showed that that the cell killing was intensity-dependent. Different forms of cell death (e.g. reproductive and apoptotic) and cellular damage (e.g. to plasma membrane and DNA) were induced. The research on ultrasound-induced intracellular mechanisms, e.g. stress responses, damage detection and signalling, repair and death execution, is still relatively new. To understand the mechanisms underlying ultrasound-induced cell death this needs to be further investigated.

Radiation qualities and different ions. The investigation of accelerated ions for improved radiation therapy is an ongoing project in our research group. We have

national and international collaborations and access to hadron facilities, which enables continuous research to better understand and compare cellular effects of different ions and LETs.

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