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TUMOR RADIOSENSITIVITY AND PROLIFERATION AS PARAMETERS FOR OPTIMIZING RADIOTHERAPY

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ABSTRACT

Radiotherapy is a widely used method to treat malignant tumors. However, the sensitivity to the treatment varies between tumors, and local tumor control is not always achieved. The balance between treatment success and the side effects of the treatment affords important information for developing treatment schedules at patient population level. Conversely there are no methods to tailor treatment schedules in an individual patient in clinical practice. The purpose of such methods would be to better balance treatment success with side effects in the individual, hopefully avoiding unnecessary treatments. As some tumors are resistant to radiotherapy doses that may be delivered without severe side effects, finding methods to sensitize tumor cells is of major importance.

In **Paper I** we evaluated a radiobiology model for predicting surviving fraction (SF) in five lung cancer cell lines. The purpose was to see whether it was important to include tumor cell proliferation during fractionated radiotherapy in a predicting radiobiology formula based on radiosensitivity, proliferation and number of tumor cells. When the clonogenic assay is used to establish SF, including proliferation seems to predict SF after fractionated radiation better than using inherent radiosensitivity alone.
In **Paper II** we evaluated the same radiobiology model in a clinical material of head and neck carcinomas. In 18 patients we compared using patient-specific radiobiological parameters with using population averages. Sensitivity in predicting local recurrence and predictive values were both better with individual parameters than with population averages. The accuracy of calculated probability of local control with the patient-specific-parameter model reached borderline statistical significance ($p = 0.07$).
In **Paper III** we investigated the entire material of head and neck carcinoma patients, including those receiving brachytherapy using a tumor control probability (TCP) model based on biologically effective dose (BED). Again we compared patient-specific radiobiological parameters with population averages to calculate individual TCPs. Evaluating the method using an ROC curve demonstrated a statistically significant difference in discriminating between local control or not when using patient specific parameters. This difference was not seen with population averages.
In **Paper IV**, the role of a phosphine gold(I) compound in altering radioresistance in a radioresistant human lung cancer cell line U1810 was investigated. This effect is achieved by shifting the intracellular redox balance by inhibiting TrxR. After a single fraction of clinically relevant radiation doses, a clear radio-sensitizing effect on SF and repopulation was demonstrated. Gene expression analysis demonstrated genetic expression changes in related cellular pathways connected to DNA repair, cellular response to stress, and cell cycle.

LIST OF PUBLICATIONS

I. Fractionated irradiation of five human lung cancer cell-lines and prediction of survival according to a radiobiology model
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IV. Effects of redox modulation by inhibition of thioredoxin reductase on radiosensitivity and gene expression
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LIST OF ABBREVIATIONS

AP-1	Activator protein 1
ASK-1	Apoptosis signal-regulating kinase 1
ATM	Ataxia telangiectasia mutated
BED	Biologically effective dose
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
Chk 1/2	Serine/threonine-protein kinase 1/2
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DSB	Double strand breaks
Gy	Gray
LI	Labeling index
LQ	Linear quadratic
MRN	Mre11, Rad50 and Nbs 1 complex
mRNA	Micro RNA
mTor	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
OER	Oxygen enhancement ratio
p53	Tumor protein 53
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
SF	Surviving fraction
siRNA	Small interfering RNA
SSB	Single strand breaks
TCP	Tumor control probability
Tpot	Potential doubling time
Trx	Thioredoxin
TrxR	Thioredoxin reductase

1 INTRODUCTION

Radiotherapy has been a successful way of treating cancer for the past 100 years. The development of different treatment schedules was driven by clinical experience regarding side effects of the delivered treatment. A major concern when treating a patient is the balance between getting enough dose to the tumor to eradicate all tumor cells but at the same time sparing enough normal tissue cells to preserve functions.

1.1 CLINICAL RADIOTHERAPY

Radiotherapy is used in several different cancers as a curative treatment option. It is used in carcinomas of different origins such as carcinoma of the head and neck, lung cancers and prostate carcinomas (1-4). Clearly, the surrounding tissues differ in structure and function, and knowledge of potential side effects after delivered treatment is essential. This is crucial in determining the total dose that may be delivered in the "target" representing tumor and areas with suspected tumor spread.

The importance of inherent radiosensitivity was proposed by Fertil and Malaise in the early 1980s (5, 6). Tumor cells show a great difference in sensitivity to radiation. This is true for tumor cells of different origin but cancers with the same histology also show a wide range in their sensitivity to radiotherapy (7-10). Thus a sufficient dose to treat a certain type of cancer will be difficult to find as it varies between individuals with that cancer. Currant treatment schedules are adjusted to deliver a maximal dose based on previous experiences while avoiding long-term side effects in normal tissues in the vast majority of patients. This means that any given treatment results in a known risk of side effects (usually fewer than five per cent serious long-term complications) and a known chance of treatment success in a *group* of patients. However for an *individual* patient the dose to achieve treatment success is not known. A lower total radiation dose might be sufficient to eradicate all cancer cells with less risk of side effects in one patient as opposed to the entire patient population. Correspondingly in another patient there is no chance of a cure with the pre-specified radiotherapy dose but the same risk of side effects.

It would obviously be a great advantage for many patients if it was possible to predict outcome in terms of local tumor control after delivery of a specified radiation dose, or to predict the dose required for treatment success. Another valuable tool would be a means to sensitize tumor cells to ionizing radiotherapy while leaving normal tissue unaffected i.e. widening the therapeutic window for radiotherapy, balancing between treatment success and side effects.

The radiosensitivity of tumor cells is controlled by different mechanisms. Inherent radiosensitivity refers to how well a cell can defend itself against radiation damage. Proliferation allows the tumor to grow between and during the treatment. Oxygen tension has a direct impact on radiation effect, with hypoxia and anoxia making the cells up to three times more resistant to ionizing radiation (11). Another factor of demonstrated clinical importance is the size of the tumor, with the trivial explanation that more tumor cells need more treatment (12).

1.2 IONIZING RADIATION

Radiotherapy consists of depositing ionizing radiation in pre-specified regions in the body. Radiation is mostly produced in and delivered by linear accelerators where electrons are accelerated by microwaves and either delivered for treatment or transformed into photons before hitting the target tissue.

Ionizing radiation results in the ejection of electrons that in their turn damage molecules of different origins. This results in an ionizing cluster that when close to the DNA produces damage in the structure of the double helix that is difficult for the cell to handle. This can be expressed as double strand breaks (DSB) in the DNA molecule (13). The depletion into the cell of high energy that reacts with water consequently forms reactive oxygen species (ROS) (13, 14). The production of these free radicals that interact with oxygen and fixes damage to the DNA can be said to be the ultimate goal in radiotherapy. In addition to DSB, ionizing radiation results in single strand breaks (SSB). These, however are normally easier for the cell to repair as the unharmed strand of the DNA can serve as a template. A radiation dose of 1 Gy is believed to result in 20-40 DSB (15). Several defense mechanisms have developed to evade mutations with their possible detrimental consequences and consequently DNA damage to a cell can result in; repair, cell cycle arrest, senescence or cell death.

1.2.1 Repair

A delicate system of sensor proteins and enzymes is constantly checking the DNA for damage in order to initiate repair if needed (16). One repair pathway for DSB is homologous repair. This uses DNA from the opposite chromosome as a template for an exact repair (17). However, this is time-consuming and the cell needs to be in a cell cycle phase where the opposite DNA strand is available. Non-homologous end-joining repair, using recombination, is faster but not exact and can result in mismatch repair, deletion or insertion; but allows the cell to survive (18). As an illustration ataxia telangiectasia mutated protein (ATM) is a part of the sensor system of DSBs together with several other proteins. ATM phosphorylates a protein, histone H2AX, which is then essential for recruiting other proteins necessary for DNA damage response (DDR) (19). Histones are important in packing the DNA and are proteins closely related to the DNA (20). Mutation of ATM as in ataxia telangiectasia normally results in enhanced radiosensitivity caused by the defect in detecting DSBs and consequently poor DDR. The MRN complex is another important protein complex taking part in sensing DNA damage together with ATM. It can interact directly with the damaged DNA and a dysfunctional or lacking MRN complex results in enhanced radiosensitivity in the same way as a defect in ATM does (18). Numerous proteins are consequently activated in response to radiation-induced DSBs and it is thought that ATM is the major protein transmitting these signals (21).

1.2.2 Cell cycle arrest

Activation of cell cycle checkpoints is an important part of DDR and delays the movement of cells through the G1, S and G2 phases (22). This gives the cell more time to initiate repair or decide whether the damage is so severe that it is inconsistent with survival. Inhibition of cyclin-dependent kinases (CDKs), proteins that make the cell move through the cell cycle, is essential for this to occur. This is controlled by phosphorylation or dephosphorylation depending on the CDK (23). As a result of this inhibition, transcription of genes essential for initiating DNA replication and movement through the cell cycle is stopped. ATM also plays a role in this process by inducing among others p53 or Chk 1/2 which play important roles in inhibiting these CDKs (24, 25).

1.2.3 Senescence

Senescence signifies that the cell irreversibly stops to proliferate although it maintains its metabolic biological functions intact (26). A senescent cancer cell is no longer harmful as long as it does not start to proliferate again and it will eventually die for one reason or another. Severe damage to the DNA, especially DSBs, promotes damage-initiated senescence. This stress-induced senescence does not involve shortening of telomeres as in replicative senescence (27). The induction of stress-induced senescence is not fully understood but CDK inhibitors plays a role, and p53 seems to be important in initiating senescence in response to ionizing radiation. As a result the cells enter a sort of permanent cell cycle arrest where chromatin changes and gene silencing might be present (28).

1.2.4 Cell death

Apoptosis is an active process characterized by specific morphological changes: programmed cell death. Enzymatic reactions result in DNA fragmentation, chromatin condensation and finally the cell breaks up into apoptotic bodies (29). Conditions inside the cell as well as external signals reflecting conditions outside the cell can initiate enzyme reactions and lead to apoptosis. Several enzymes are activated as a result of radiation and ultimately the balance of these enzymes controls the cell's fate (30). If there is enough damage to the DNA the systems might act in favor of cell death instead of attempting repair. Activation of p53 by ATM results in induction of BAX, a pro-apoptotic protein that causes release of cytochrome *c* from mitochondria. Consequently activation of a protease, caspase 9, leads to cleaving of cell target proteins. Apoptosis induced by caspase 9 in response to internal cellular damage is described as the intrinsic pathway (31).

Autophagy is a well-controlled cellular process. It is initiated by the formation of autophagosomes containing different intracellular cytoplasmic constituents and their fusion with lysosomes resulting in degradation of the content (32). The process is activated by the inhibition of the mammalian target of rapamycin (mTOR) and can be initiated with rapamycin which inhibits mTOR (33, 34).

Necrosis has been considered as an uncontrollable and accidental form of cell death caused by release of lysosomal degradative enzymes that attack the cell, leading to cell

lysis. Evidence, however, suggests that necrosis can be tightly regulated and follow oxidative stress and ROS generation in a cell (35).

1.2.4.1 Mitotic cell death

The major mechanism causing a clonogenic cell in a solid tumor to die after ionizing radiation is probably what is termed mitotic cell death (36). A tumor cell might continue to proliferate and go through several cell cycles after radiation before dying as a result of the radiation damage. Due to structural damage of the DNA resulting in mutation and chromosomal aberrations, the cell has lost its clonogenic ability. This loss is the result of repair processes that are unfaithful to the genetic information and consequently not functional or in agreement with cell survival. The final process, mitotic catastrophe, is caused by the incapacity of the cell to complete mitosis successfully (37). The cell may subsequently die from various forms of programmed cell death such as apoptosis, autophagy or necrosis (38).

1.2.4.2 Surviving fraction after ionizing radiation

The surviving fraction after ionizing radiation can be expressed as the fraction of clonogenic cells surviving a radiation dose. There is a random nature in cell survival after radiotherapy. In brief, a hundred lethal lesions distributed randomly to a hundred sensitive cells will not kill all cells. Some will get more than one lethal hit but cannot become “more dead” as a result, while others get no hit at all and will consequently survive (39). As a fraction of cells will survive after radiation their number is directly related to the initial number of cells (12). This emphasizes the importance of tumor size for tumor cell survival after radiotherapy. While tumor size does affect outcome after radiotherapy, it is not alone as good an indicator of treatment outcome as might be expected (40, 41). The survival of cells after radiotherapy is, in addition, affected by factors such as DNA damage-response mechanisms, scavenging of free radicals and the cell environment i.e. oxygen tension. These factors differ between tumors and affect cellular radiosensitivity.

1.3 FRACTIONATED RADIOTHERAPY

A course of radiotherapy is as a rule delivered as a number of smaller fractions. This is done primarily to spare the normal tissues as much as possible from side effects: the basis for this will be presented later. However, this practice was developed empirically

when radiotherapy was first introduced as a treatment modality and it was soon discovered that fractionation gave an advantageous balance between treatment effect in the tumor and side effects in normal tissue (42). Cell survival after fractionated radiotherapy is affected by the four "Rs" of dose fractionation as introduced by Withers: repair of sublethal damage, repopulation, redistribution through the cell division cycle and reoxygenation (43). A fifth factor, inherent radiosensitivity, has been proposed as an explanation of different tolerance to fractionated radiation in different tissues (44).

1.3.1 Repair and redistribution

The ability to detect damage to DNA is essential for normal cell development and cell survival. As DNA double-strand breaks are thought to be the major cause of cell sensitivity to radiation the number of unrepaired DSB after radiotherapy correlates with cell survival (45). The capacity to repair damage to the DNA thus affects radiosensitivity and varies between cells. This is evident in patients with mutation in repair enzymes such as the ATM protein as described above, which results in an extraordinary radiosensitivity with a high fraction of cell kill due to impaired detection of DNA damage and repair. The response to damage is crucial for whether the cell is to continue to cycle and proliferate, or die. The "quality" of repair is also important in the context that DNA must be functional after repair or the ability of the cell to continue to divide is lost and it will eventually die. This highlights the importance of cellular systems both detecting damage and then initiating a repair that is correct. The balance in these systems is also important. The detection of DSB by ATM can initiate repair mechanisms but also the activation of the tumor suppressor gene p53 (46). The p53 protein plays an important role in controlling cycle arrest and also acts as a proapoptotic enzyme that by stimulating caspase activity leads to cell death by apoptosis (24, 31). The repair of DSB in the DNA is either by homologous repair where the corresponding unharmed DNA on the *other* sister chromosome is used as a template for the damaged base pairs, resulting in a perfect repair. The alternative is non-homologous end joining repair in which the DNA strings are mended disregarding missing base pairs in the process (47). As a consequence genetic information might be lost or risk misinterpretation (48). Repair of the DNA that is not functional (or if there is no repair), such as certain rearrangements or DNA breaks, can lead to mitotic catastrophe which in turn leads to cell death (see mitotic cell death above).

Progression through the cell cycle is also important when considering radiosensitivity. Cells show great difference in radiosensitivity in different cell-cycle phases, being sensitive in G2-M and resistant in late S-phase (49). One possible explanation of this resistance in the S-phase is that the other chromosome is opened and readily accessible for homologous repair of DSB. After being irradiated, surviving cells are in general in more radioresistant cell-cycle phases and radiosensitivity depends on how the cells progress to more sensitive phases. As mentioned above inhibition of CDKs after radiotherapy is important for the cell to stop cycling and consequently remain in these radioresistant phases (24). The potential impact on radiosensitivity of cell-cycle arrest in more resistant cell-cycle phases after radiation can be hypothesized (50).

1.3.2 Proliferation

Tumor cells divide and proliferate in an uncontrolled manner. When most apparent, tumor growth can be observed in the clinic during a course of radiotherapy (51). The consequence for the patient is obviously severe, with symptoms of tumor progression in addition to side effects of the treatment. This is fortunately uncommon, although continued proliferation during radiotherapy is a clinical problem (52-54). A protracted treatment requires an increased total radiation dose for the same chance of local control as with the original treatment schedule. Withers et al. have proposed a model where an accelerated proliferation kicks in after a lag period of about four weeks without clinically significant tumor growth (54). However, the basis for this assumption has been questioned. Dubben has in the same material shown a lack of a dose-response relationship between tumor control rate and normalized total dose in 2 Gy fractions (55). This indicates that the time effect observed could be related to an equivalent radiation dose i.e. longer treatment schedules deliver higher radiation doses. Bentzen et al. drew yet other conclusions, again studying the same material. They assumed a much shorter lag period, if any, before onset of proliferation or alternatively continuous tumor-cell proliferation with a cell-doubling time of little less than a week (56). Even so, in most relevant clinical radiation schedules proliferation must be accounted for. In the majority of recent papers a delay of three weeks after beginning radiotherapy is often applied before repopulation is considered to begin (57). This limit can be viewed as arbitrary since even if there is a lag period with no proliferation this will probably vary considerably between individuals. The question whether there is a lag period after initiation of radiotherapy before repopulation begins at a certain rate or whether

proliferation at a lower rate exists throughout treatment has, moreover, not been answered.

Tumor proliferation can be measured in many ways. Tpot describes the potential doubling time in a tumor with no cell loss during tumor growth (58). Cell loss during tumor growth is normally caused by diverse factors such as lack of nutrition or oxygen. In clinical materials Tpot has not been proved sufficiently robust to demonstrate a relationship between tumor proliferation and local control after radiotherapy. However, a feasible relationship between highly proliferative tumors expressing an elevated labeling index (LI) and impaired local control has been shown (59). The labeling index represents the mitotic activity in a tissue representing cells in S-phase in relation to the total number of cells (60). Nevertheless Tpot has been considered a better predictor of tumor regeneration than measured tumor growth before radiation. Assuming that cell kill during a course of radiotherapy is caused mainly by radiation damage, and not hypoxia or lack of nutrition, it can be thought that tumor cell repopulation moves towards Tpot. However it has also been suggested that tumors as well as normal tissue can sense damage and initiate accelerated proliferation as a response to this (61).The importance of proliferation is further demonstrated by the potentiating effect of chemotherapy concomitant with radiotherapy. This additive effect can at least partly be attributed to slower proliferation due to the cytotoxic drug (62).

1.3.3 Hypoxia

Hypoxic cells are more radioresistant than well-oxygenated cells. This has been demonstrated *in vitro* by irradiating tumor cells during both well-oxygenated and hypoxic conditions (63, 64). Ionizing radiation readily reacts with intracellular water and in the presence of oxygen creates hydroxyl radicals that in turn damage the DNA and are also able to fixate DNA damage (14, 65). During hypoxic conditions this damage is more likely to be repaired instead. Hydroxyl radicals also react with other molecules in the cell. All these created free radical species have longer half-lives during hypoxic conditions and can react with hydrogen as an alternative to oxygen to restore their structure and form water molecules instead of secondary peroxy radicals (60). Hypoxia in tumors before radiotherapy correlates with a worse prognosis (66, 67). However hypoxia in tumors before surgery has also demonstrated similar results, correlating with poorer prognosis (68). From these results it is clear that hypoxia itself is a bad prognostic factor and has also been related to the tumor’s metastatic capacity.

The reason for this might be selection of tumor cells that support hypoxic conditions better and become resistant to hypoxia-induced apoptosis, for example by mutation of p53 (69). The cells adapt to the hypoxic condition and can stimulate angioneogenesis. Hypoxia has also been claimed to change DNA repair towards a more pronounced malignancy grade with suppressed expression of several DNA repair genes resulting in genomic instability (70).

The effect of radiation thus depends on the oxygen tension in the tumor. The oxygen enhancement ratio is the factor by which tumors respond better to radiation in oxic than hypoxic environments. This ratio has traditionally been set to between two and three (71). However most experiments have been performed with large radiation doses and the enhancement ratio is probably less significant in the dose range used in the clinic for most fractionated regimens (72, 73). In hypofractionated treatment regimens there could theoretically be a significant clinical problem due to the larger radiation doses usually delivered. Experience of stereotactic radiotherapy has nonetheless shown very promising results not indicating a serious problem potentially caused by hypoxia (74, 75). The use of a hypoxic radiosensitizer in combination with primary radiotherapy for carcinoma of the head and neck has, however, showed improved loco-regional control indicating an hypoxic effect in conventional radiotherapy treatment situations (76). Reoxygenation is a continuous process in tumors during fractionated radiotherapy. This is to some extent due to tumor shrinkage getting the remaining tumor cells closer to intra tumoral blood vessels. These blood vessels are, however, often of poorer quality resulting at times in impaired blood perfusion and thereby causing acute hypoxia in tumors (77). The nature of temporary hypoxia in tumors has been demonstrated in several studies (78-80), indicating that most tumor cells are oxic at least in periods during a course of radiotherapy. The clinical impact of hypoxia on tumors during fractionated radiotherapy is therefore difficult to assess, and oxygenation will most likely vary during radiotherapy in most tumor cells. It is clear that hypoxia influences radiosensitivity but the effect of reoxygenation most likely reduces this effect in fractionated radiotherapy and the net effect might be fairly limited (81).

1.4 OXIDATION REDUCTION (REDOX) SIGNALING

Interaction between cellular proteins and DNA is an important mechanism in the cell, controlling functions such as transcription, repair and replication (82). This interaction

regulates gene expression, which in turn controls cell metabolism including cell growth and cell survival. The possibility of interaction between a protein and its target, for example the DNA binding site, depends on the protein structure and its three-dimensional shape (83). This is controlled by post-translational mechanisms such as phosphorylation and methylation. In addition oxidation reduction (redox) of proteins plays a major role in controlling protein function (84). Redox-regulated proteins can sense changes in the oxidation-reduction balance in different ways; one common one being through a reactive cystein residue with a thiol-coordinated zinc site within the protein (85). The agent responsible for the changes in redox balance is reactive oxygen species (ROS), produced both within and outside the cell. As these ROS can be hazardous for the cell in high concentrations - although essential for cellular metabolism at physiological levels - the cell has systems for both production and elimination of ROS (86).

1.4.1 Thioredoxin-thioredoxin reductase

Thioredoxin (Trx), a small protein, contains a dithiol group which is reduced from disulfide in the oxidized state by thioredoxin reductase (TrxR), another small selenoprotein (87, 88). Trx has several important biological functions in its reduced state. Transcription factors such as p53 and activator protein 1 (AP-1) interact with Trx in the nucleus to enable their interaction with DNA (89). A binding between reduced Trx and apoptosis-signal-regulating kinase 1 (ASK-1) prevents downward signaling for apoptosis (90). Another important function is as an antioxidant keeping the intracellular environment reduced by acting as a scavenger for ROS. Trx is a hydrogen donor to ribonucleotide reductase, which highlights its importance in the construction of new DNA and involvement in repair processes. TrxR reduces oxidized Trx in a NADPH-dependent process and is therefore essential for a functional Trx - TrxR system (87). Several gold compounds such as auranofin, a clinically used anti-rheumatic drug, are both selective and effective inhibitors of TrxR and consequently also Trx (91, 92).

1.4.2 Thioredoxin and radiation

Activation of intracellular thiols such as glutathione and Trx by oxidative stress and irradiation affects radiosensitivity (93, 94). Trx is over-expressed in several types of cancers (95), and low levels of ROS have been demonstrated in cancer stem cells and correlate with increased resistance to radiation (96). One relevant function in response to ionizing radiation is to act as a scavenger and reduce oxidized molecules (97) that

otherwise might interact with and damage DNA by causing and fixating strand breaks. Oxidative stress which leads to depletion of reduced Trx can result in apoptosis triggered by the higher levels of free ASK-1 then unbound by Trx. Depleted levels of cellular thiols have been associated with increased radiation-induced apoptosis (98) while increased levels of Trx have been associated with increased radioresistance in a malignancy-induced human cell line (99). As mentioned above, Trx plays a role in stimulating the DNA-binding activity of several transcription factors important in conditions of cellular stress. A role has also been demonstrated for Trx in reducing and thereby activating the Ape1/Ref1 complex that plays a central role in base excision repair (100, 101). Finally, Trx acts as a hydrogen donor to ribonucleotide reductase, emphasizing its role in DNA construction and repair (87).

1.5 MODELING IN RADIATION

In contrast to great improvements of the technical aspects of delivering radiotherapy during the past ten years, no improvement in biological optimization has been achieved. Modeling radiotherapy would be a helpful tool in tailoring individual treatment or deciding whether a person would benefit from a prescribed treatment schedule (102). However, no mathematical model to predict treatment outcome after radiotherapy has been validated so far. For this, several obstacles remain. First what radiobiological parameters are required in a mathematical model has to be verified. Inherent radiosensitivity, tumor-cell proliferation and tumor oxygen tension are considered to be the most important parameters and are therefore the most studied. Inherent radiosensitivity measured as surviving fraction after 2 Gy (SF2) correlates with local control after radiotherapy (103, 104). On the other hand measurements of proliferation using Tpot have demonstrated conflicting results and have not been shown to correlate with clinical outcome; nor have other parameters that measure proliferation come into clinical use (59). A factor in the tumor micromilieu, oxygen tension specifically affects clinical outcome when measured before treatment (67). However, this factor also varies considerably during a course of radiotherapy (105-107). Gene expression analysis might offer yet other ways to consider radiosensitivity (108) as well as analysis of DNA end-binding complex density (109), although no recent papers on these subjects showing clinically applicable results have been presented (110).

Numerous models have been presented over the years in attempts to predict clinical outcome after radiotherapy (111-113). The maybe most widespread model, the LQ formula, is a mathematical adaptation to fit cell-survival curves after radiation with different doses and fractionations. This model has been adapted in many ways (102). The radiobiological parameters used in such models are normally population averages derived from interpreting local control data from clinical studies. In other words the radiobiological tumor characteristics assumed are based on tumor control data and fitted to the different parameters used in the specific model. The usefulness of any of these models in the clinic to guide what treatment schedule to use in an individual patient seems very distant, even though some guidance on the population level in developing radiation schedules for a certain tumor type might be appropriate.
A predictive model that considers individually measured radiobiological data is therefore warranted. This would probably consider several of the radiobiological parameters mentioned above. One factor that has to be considered is the reliability of such measurements *in vivo*. Still, this has to be evaluated in prospective clinical trials. Another and equally important issue is the feasibility of such measurements and further development of easy, and fast methods to acquire individual tumor-specific radiobiological information is essential. Despite these initial remarks mathematical models are in wide clinical use in modern radiotherapy. They have important roles in comparing different dose schedules and in deciding continued treatment after an unexpected treatment pause, to name a few examples. However a prospective model to decide the best treatment schedule for an individual patient is yet to be developed (114).

1.5.1 A radiobiology model

For many physicians a model based on radiobiological parameters reflecting actual tumor-biological features would be a simple and intuitive way to predict radiotherapy outcome. These thoughts were the basis for the following model predicting outcome in the form of surviving cells after fractionated radiotherapy as presented by Brodin (115):

$$C_N = C_0 \times SF^N \times P^{N-1}$$

Number of surviving cells after N fractions (C_N) is related to the initial number of tumor cells (C_0) by surviving fraction (SF) and proliferation (P):

$$P = e^{-ln2\times(t-m)/Td}$$

The doubling time of the cell population (*Td*) in relation to the time between fractions (*t*) subtracting the mitotic delay (*m*) affects the proliferation factor. This model is easy to understand, and the number of surviving cells is an endpoint that is very easy to relate to in terms of achieving local control or not. The number of tumor cells in a tumor is thought to be related to its size and therefore easy to appreciate (60).

1.5.2 The multi-target model

The multi-target model describes an initial slope of the survival curve (D_1) caused by single-hit cell kill, and a final slope (D_0) due to multiple-event cell killing. In this model an extrapolation number (*n*) represents the number of sensitive targets in a cell. This model previously dominated in describing cell survival curves after radiotherapy. D_0 represents the dose that reduces cell survival from 1 to 0.37 (or e^{-1}), and this term is still widely used to describe a radiation dose that reduces cell survival by one natural logarithm. The explanation of this is a dose that delivers an average of one lethal hit per target. This model is not the most used today, maybe because of the difficulty to define what the radiation target actually consists of and the more complex equation compared to the LQ model. However it still represents a good fit to expected cell survival after radiotherapy, especially in very radiosensitive tumors and tissues.

1.5.3 The LQ model

The most commonly used model to describe the shape of the cell survival curve today is the linear-quadratic (LQ) model:

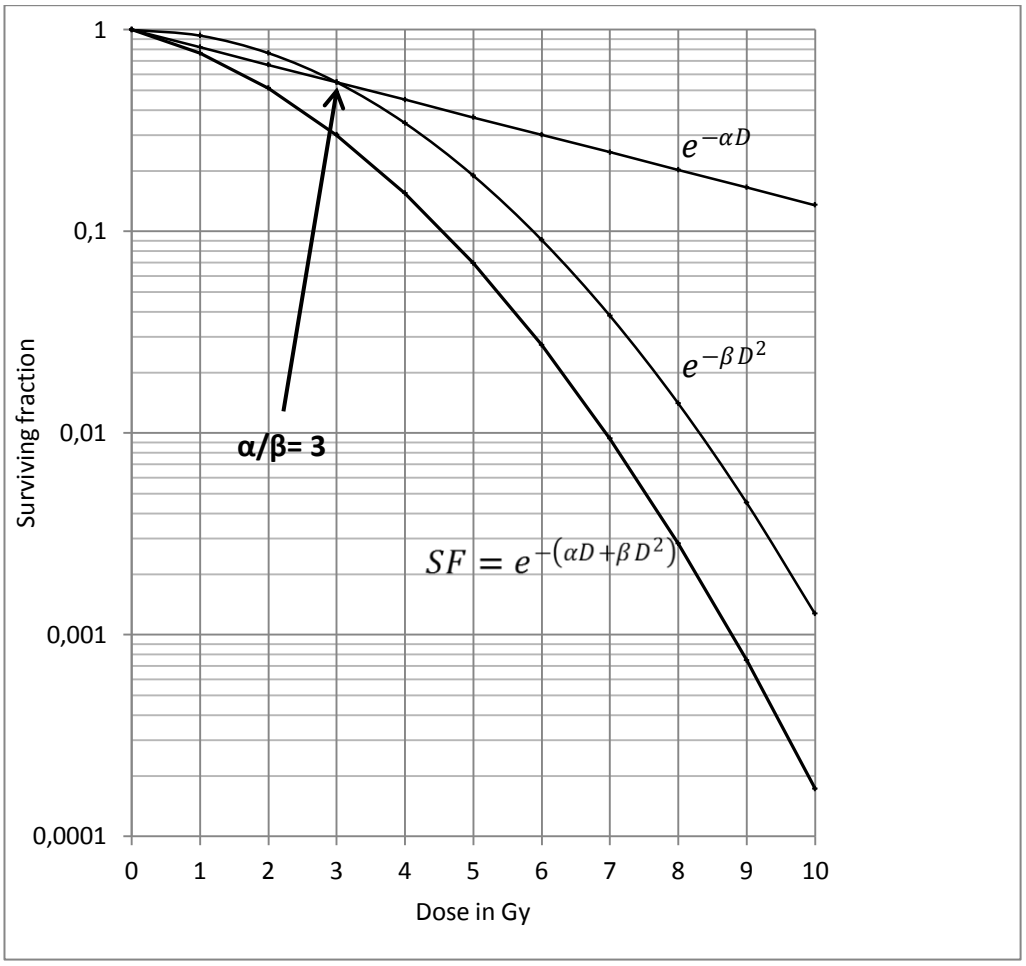
$$-\ln(S) = \alpha D + \beta D^2$$

$$SF = e^{-(\alpha D + \beta D^2)}$$

Cell survival (*S*) is related to dose with a factor α that expresses a linear relationship with dose and a factor β that is related to the square of the delivered dose. The result is a continuously bending response to radiation dose which has proved very useful to describe response in the clinical relevant dose range (0.5 – 6 Gy). The shape of the curve is described by α/β, where a higher rate gives a more linear curve and a lower a "curvier". The model is mathematical to fit the experienced cell survival after radiation (116). However, biological explanations have been proposed. Curtis introduced the idea where the linear α component is due to single-hit lethal lesions and the quadratic β

component depend on repair of sublethal damage that can accumulate to lethal damage (117). Saturation deficit of repair enzymes at high dose ranges is another possible explanation (118). At low radiation doses the α effect dominates and is of major importance when delivering clinically relevant doses of around 2 Gy in fractionated radiotherapy.

Figure 1. Cell survival curve demonstrating contribution of the linear α-component and quadratic β-component to the combined cell survival curve. α/β represents the dose where the components contribute equally to cell kill.



Differences in α/β

The α/β value represents the dose where the linear contribution to cell kill expressed as α equals the quadratic contribution expressed as β (Figure 1). Differences in α/β express differences in the tumor or tissue response to fractionated radiotherapy (119). A low α/β signifies less tissue damage if the radiation dose is delivered in several smaller fractions whereas this difference is less pronounced in tissues or tumors with a high α/β value. This is an advantage in clinical radiotherapy where fractionated radiation spares normal tissue around a tumor more than the tumor itself i.e. a favorable therapeutic

ratio. Several studies have defined the value of α/β in different tissues and in tumors. In general late-reacting tissues have a lower α/β value, in the clinic often set to 3. Highly proliferative, acutely reacting tissues on the other hand have a high α/β of at least 10, which is the most common value applied when performing calculations in a clinical setting (116). Tumors are considered to have α/β values similar to acutely reacting tissues: however, a wide range has been demonstrated probably due to wide differences in tumor biology (120). Exceptions are prostate cancer with an α/β of around 3 or even less (121), while data supports the assumption that carcinoma of the breast has an α/β somewhere between 3 and 10 (122).

1.5.4 Isoeffective dose in fractionated radiotherapy

It has proved very useful to have a model in clinical practice to compare radiation effect in acute and late-reacting tissues when using different fraction sizes and total dose. This difference and possible applications with optimization of dose per fraction was introduced by Thames et al in 1982 (119). A simple isoeffect equation was proposed by Withers in 1983:

$$\frac{D_2}{D_1} = \frac{d_1 + (\alpha/\beta)}{d_2 + (\alpha/\beta)}$$

where (D) is total radiation dose in the different treatments schedules, (d) is the dose per fraction and (α/β) defines the reaction of the tissue to radiation (late- versus early-reacting tissue or tumor) (123).

1.5.5 Biological Effective Dose (BED)

BED is the theoretical total dose to achieve an isoeffective dose in a tissue or tumor using infinitely small doses per fraction and infinitely numerous fractions (124). It demonstrates the relationship between the total dose, dose per fraction and tissue response to radiation:

$$BED = D[1 + d/(\alpha/\beta)]$$

where (D) is the total radiation dose, (d) the dose per fraction and (α/β) defines the shape of the cell surviving curve to ionizing radiation as described above. The addition of a time factor completes the BED formula:

$$BED = nd[1 + d/(\alpha/\beta)] - \ln(2)/\alpha \times (T - T_k)/T_p$$

where (n) is the number of fractions delivered in (*T*) total time, (*Tp*) is the cell doubling time and proliferation start at the time (T_k) (k of kick-off). The clinical use of this equation is evident in comparing the effect of a radiotherapy schedule for tissues with different *α/β*, or estimating what a change in dose per fraction would result in. The minus sign before the time factor emphasizes the disadvantageous effect of prolonging a treatment schedule (if *T* is larger than T_k), reducing the biologically effective dose according to this model (125). For *biologically equivalent doses* divide the BED by a factor in relation to dose and *α/β*. For equivalent doses in 2 Gy divide BED by 1.67 for an *α/β* of 3 (late responding tissues) and by 1.2 for an *α/β* of 10 (tumors and acutely responding tissues). This is explained by this part of the equation: $1 + d/(\alpha/\beta)$.

1.5.6 TCP according to the Poisson model

The probability of tumor control depends on the number of surviving tumor cells, clonogens, which sustain the ability to proliferate after radiotherapy (126). This is useful when considering a group of patients with tumors receiving radiotherapy. The basis is the random nature of cell killing resulting in different numbers of surviving clonogens in the different tumors, from zero (cure) and upward (no cure). Cell survival in a tumor will follow a Poisson distribution after a specified radiation dose i.e. a sigmoid curve (12). The median number of surviving clonogenic tumor cells gives the tumor control probability:

$$TCP = e^{-N \times SF}$$

where (*N*) is the number of clonogenic cells before radiation and (*SF*) is the surviving fraction of cells after radiation. TCP curves for human tumor control are in general shallow even if tumor size is considered, indicating tumor heterogeneity in sensitivity to radiotherapy (127).

1.6 BRACHYTHERAPY

No details of brachytherapy are provided here; only a brief summary is presented. Brachytherpy involves bringing the source of radiation close to the target and using

radiation with a very short range. This permits the delivery of high radiation doses while at the same time sparing surrounding normal tissue. Doses can be delivered in a shorter or a longer time frame. When delivered as low dose rate, i.e. during a longer time, continuous repair can take place and the tumor survival curve is defined mainly by α. Therefore when considering brachytherapy delivered at lower dose rates in a mathematical model the continuous repair has to be accounted for.

2 AIMS OF THE STUDY

2.1 OVERALL AIM

The overall aim of the work presented in the present thesis was to increase understanding of the predictive capacity of the *individually* measured radiobiological parameters inherent radiosensitivity, proliferation and number of tumor cells on treatment outcome after radiotherapy. How changing the cellular redox balance affects inherent radiosensitivity and repopulation after ionizing radiation was also investigated.

2.1.1 Specific aims

Specific aims were

- to evaluate the usefulness of two radiobiological models based on inherent radiosensitivity, proliferation and the number of tumor cells to predict outcome after radiation,

- to establish whether individually measured radiobiological parameters are better in predicting clinical outcome than average population based parameters, and

- to investigate the possibility of affecting inherent radiosensitivity by altering the redox status in tumor cells using a gold compound, and the corresponding affect on gene expression.

3 MATERIAL AND METHODS

3.1 MATERIAL

Established lung cancer cell lines were used in papers I and IV and previously published data originating from patients treated for head and neck carcinomas at Sahlgrenska University Hospital were used in papers II and III.

3.1.1 Cell lines

Five well-characterized human lung cancer cell lines were used in paper I: three small cell lung cancer cell lines; U-1285, U-1906E, U-1906L, one squamous cell carcinoma cell line U-1752 and one large cell carcinoma cell line U-1810 (128, 129). All had well-defined radiobiological characteristics. In paper IV, U-1810 was used due to its known radioresistant features and U-1906L was used as a radiosensitive control.

3.1.2 Patient-related data

Previously published data originating from patients treated for carcinoma of the head and neck at the Oncology Department at Sahlgrenska University Hospital in Gothenburg was used (130). Tumor material had been collected from numerous patients and tumor biological characteristics had been investigated. In studies II and III we used data where all the following parameters were known; inherent radiosensitivity expressed as SF2, tumor proliferation (Tpot), tumor size, external radiation dose and duration of treatment. In study III information regarding brachytherapy i.e. dose-rate and duration, was also used.

3.2 METHODS

3.2.1 Radiosensitivity assays

Clonogenic assay (paper I)

The clonogenic assay is considered the “gold standard” for characterizing inherent radiosensitivity. A cell suspension is prepared and plated in a cell culture medium (131). The cell cultures are then irradiated or kept as control and incubated for a period of time. The ability to form viable colonies is then investigated. The numbers of colonies formed, in treated versus untreated plates, are compared and the difference

will represent the surviving fraction. In this study cells were suspended on agarose mixed cell-culture medium.

Extrapolation method (papers I and IV)

An extrapolation method has been proposed as an alternative in cell cultures with low clonogenic capacities and consequently are unsuited for the clonogenic assay (132). The extrapolation method explores the cells ability to proliferate after treatment. The cells, treated or untreated, are kept in cell culture medium for a period of time after treatment. They are sub-cultivated and the medium changed repeatedly to keep culture conditions optimal and cells are counted at the same time. The number of cells on each occasion (corrected for the dilution factor) is plotted on a logarithmic scale and is finally extrapolated back to the intersection with the y-axis, representing number of cells at the time of treatment. The difference between treated and untreated cell cultures represents the "surviving fraction" (Figure 2). For the method to be successful, the irradiated cells must proliferate at the same rate as the control in the exponential growth phase and all cell loss must be attributable to the radiation.

3.2.2 Radiation

Radiation was performed using a linear accelerator, a cobalt machine or a Gamacell Exactor.

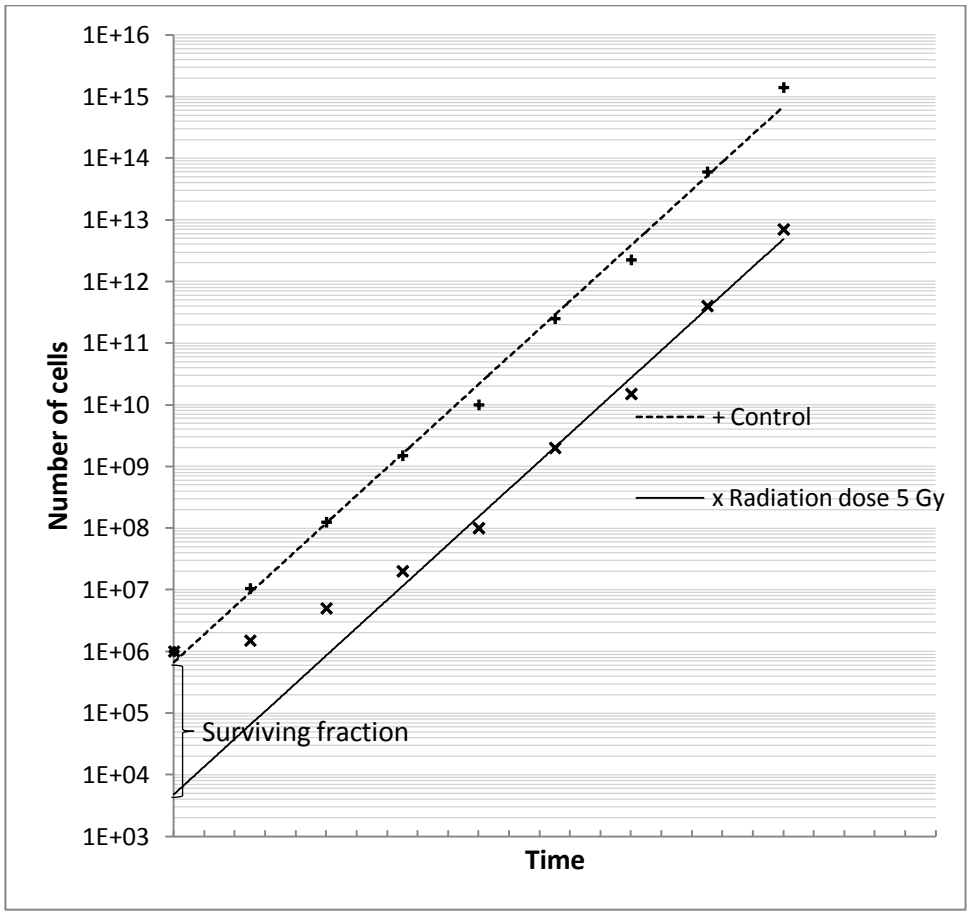
Linear accelerator (paper I)

A linear accelerator produces photon radiation by accelerating electrons and then causing them to halt abruptly. A bolus is used to get a sufficient dose superficially in a tissue or, as in the present studies, in the cell suspension. We used 8 or 16 MeV with a dose rate of 4 Gy/min. Changing the duration of radiation results in different total radiation doses.

Gamacell exactor (paper I)

We used a Gamacell 40 Exactor where the radiation was produced by a Cs^{137} source. The dose rate was 1.29 Gy/min in the dose range 0-1 Gy and 1.23 Gy/min for 2 Gy and above. Varying the duration of radiation results in different radiation doses.

Figure 2. The growth curve of a cell line after irradiation and the corresponding extrapolation from the exponential part of the curve.



Cobalt-60 machine (paper IV)

Cell lines were irradiated with a machine using an ionizing Cobalt source with a dose rate of 0.50 – 0.51 Gy/min. The duration of radiation affects the delivered dose.

3.2.3 Laboratory work in paper IV

Inhibition of TrxR

A gold compound [Au(SCN)(PEt_3)] was used and cell lines were incubated with 2.5 µM of the compound 24 hours prior to treatment and kept for 24 hours after treatment. siRNA are short double-strand RNAs able to prohibit the expression of a gene and consequently the corresponding gene product or protein. Suppression of TrxR1 as described in this paper was achieved by reverse transfection using 10 nm TXNRD1 Silencer® Pre-designed siRNA, ID:111302 and Silencer® Negative Control siRNA #1 (Ambion). The transfection reagent used was siPORT™ NeoFX™ (Ambion).

Measurements of TrxR

The amount of active TrxR was established with insulin as a substrate (133). The activity of reduced Trx is measured by its ability to reduce protein disulfides in insulin. An excess of Trx is added and the Trx-dependent formation of SH groups in insulin is determined by comparison to a standard curve with known concentrations of human TrxR measured by absorbance at 412 nm.

Real-time qPCR

Reversed transcriptase produces cDNA from mRNA for subsequent use in the PCR. In paper IV SYBR green was used to detect double-stranded DNA during the amplification process. SYBR green binds non-specifically to double-stranded DNA and then emits green light (fluorescence), permitting quantification of amplified DNA. The number of cycles needed to achieve a specified fluorescence threshold is directly related to the amount of nucleic acid originally present in the sample. This is because the more nucleic acid copies originally put in the original sample the fewer cycles are needed to reach the threshold.

Western Blot

The Western blot method detects protein in cell extracts using protein-specific antibodies. Western blot was used in paper IV to detect TrxR. First proteins are separated according to size using gel electrophoresis. After being transferred to a filter, antibodies against the protein of interest are probed. Finally a secondary antibody is added, in this case a HRP-conjugated antibody that is detected using chemiluminescence.

Cell cycle analysis

Flow cytometry is used to identify cells in different phases of the cell cycle. This is a method where cells pass a beam of light, usually laser, and detectors pick up scattered and fluorescent light reflecting both cell volume and cell content. To perform cell-cycle analysis the cells are made permeable and treated with a fluorescent dye, in paper IV propidium iodide. This stains DNA quantitatively and consequently the fluorescence intensity will correlate to the DNA amount in the cell, which in turn reflects the different phases of the cell cycle.

RNA isolation

For RNA isolation, cell solutions are lysed in the presence of a buffer that inactivates RNases. The total amount of RNA containing 200 bases or more will then bind to a silica-gel-based membrane and this selectivity enriches the mRNA content since shorter forms of RNA are excluded.

Gene expression profiling

The microarray technique employs a large set of oligonucleotide probes attached to a surface, for instance a silicon chip. A sample containing nucleic acid is subsequently added which results in hybridization between the probe and sample nucleic acid, for example mRNA (cDNA). Non-specific bonds are washed off. The amount of target-sample binding will be reflected by fluorescence. This allows a gene expression analysis of the sample to be made. Due to the large amount of data produced by microarrays it is important to manage it in a correct way. Background noise has to be accounted for and the data is normalized to minimize statistical errors. In study IV the Affymetrix GeneChip scanner (Santa Clara, Ca, USA) was used to measure changes in gene expression in cell lines U-1810 and 1906L after radiation and exposure to the gold compound[Au(SCN)(PEt_3)]. The data so produced was processed with an Affymetrix expression console.

3.2.4 Radiobiological models

In papers I and II a model including inherent radiosensitivity, proliferation and tumor size was used, see radiobiology model above:

$$C_N = C_0 \times SF^N \times P^{N-1}$$

where C_N is the number of cells after N irradiation treatments, C_0 equals the original number of cells immediately before first treatment, SF is the surviving fraction of cells for each dose and cell line and P is the proliferation factor describing exponential growth:

$$P = e^{\ln 2(t-m)/Td}$$

where *t* is the time interval between fractions, *m* is the mitotic delay set to 1.5 h/Gy and *Td* is the doubling time of the cell population. This might imply the potential doubling time if all cell kill is afforded by irradiation.

In study II the Poisson model was used to calculate tumor control probability (TCP) after radiotherapy according to the following equation:

$$TCP = e^{-C_N}$$

In study III the biologically effective dose (BED) was calculated using the following equation for external beam radiotherapy:

$$BED_{EBRT} = nd\left(1+\frac{d}{\alpha/\beta}\right)$$

where *n* is the number of fractions, *d* the dose per fraction and α/β defines the shape of the cell surviving curve. For Brachytherapy the following equation was used to calculate BED:

$$BED_{BT} = D\left\{1+\frac{2D}{(\alpha/\beta)\mu T}\left[1-\frac{1}{\mu T}\left(1-e^{-\mu T}\right)\right]\right\}$$

where *D* is the total radiation dose, *T* is the total treatment time and μ represents the repair coefficient of sub-lethal damage during brachytherapy.

Most patients received combined therapy and the total BED was calculated after possible proliferation had been subtracted according to the following equation:

$$BED_{tot} = \sum_i BED_i - \frac{\ln(2)}{\alpha}\frac{T_{treat}-T_k}{T_p}$$

where T_{treat} is the overall treatment time, T_k is the lag time for the onset of proliferation, T_p is the effective doubling time during proliferation and α is the linear parameter of the

LQ model. Note that no correction for proliferation is needed if the overall treatment time is shorter than T_k.

In study III the Poisson-LQ model was used to calculate the TCP according to the following equation:

$$TCP = \exp\left\{-N_0 \cdot \exp\left[-\alpha \cdot EQD_2 \cdot \left(1+\frac{2}{\alpha/\beta}\right)\right]\right\}$$

where N_0 is the initial number of clonogenic cells in the tumor. The EQD_2 is the equivalent dose in 2 Gy per fraction of the total biological effective dose received by the patient and calculated as follows:

$$EQD_2 = \frac{BED_{tot}}{1+\frac{2}{\alpha/\beta}}$$

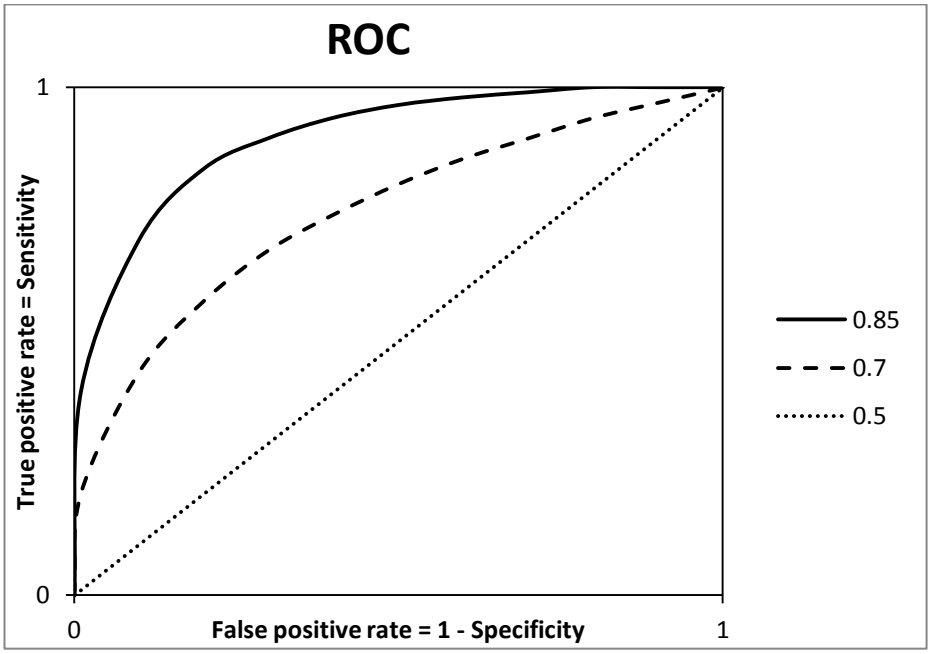
3.2.5 Statistical methods

The following well-established statistical methods were applied accordingly: Student's *t* test, the Chi-square test, the Mann-Whitney U test and Wilcoxon's matched pairs test. They are referred to in THE respective paper. Statistical analysis of microarray data were analyzed according to protocol using Student's *t* test for gene expression levels and Pearson correlation for relationship between each treatment category and are presented in study IV.

Receiver operating characteristic (ROC) analysis was used in study III. ROC curves may be used to present the sensitivity of the test versus its false-positive rate. ROC analysis determines how well a system (or method) can answer a question and distinguish a difference in a studied object or population (134, 135). The area under the curve represents the accuracy of the test, the greater the area the better the result. In general, a straight line where the area under the curve equals 0.5 represents a worthless test and an area over 0.9 an excellent test. Comparing two methods, the method with a better true positive fraction for the same true negative would be considered better. An optimal test and cutoff value with 100% sensitivity and 100%

specificity would thus be plotted in the upper left corner (sensitivity = 1 and 1-specificity = 0) (Figure 3).

Figure 3. Examples of ROC curves, for details see text.



4 RESULTS AND DISCUSSION

4.1 STUDY I

The usefulness of radiobiological parameters in predicting clinical outcome after radiotherapy has not been fully clarified. Study I sought to evaluate a radiobiological model by comparing predicted and experimentally found effects of incorporating proliferation with inherent radiosensitivity in five tumor cell lines to see whether this resulted in better accuracy in predicting radiation responsiveness.

Radiosensitivity after fractionated radiation differed in the five investigated human tumor cell lines, the most sensitive cell line showing a greater response to fractionated radiation than the more radioresistant cell lines. The latter were comparatively more sensitive to hypofractionation. In the three cell lines where SF was evaluated with the clonogenic assay, adding proliferation to single-dose SF seemed to get a prediction of SF after fractionated radiation that came closer to the experimentally achieved "fractionated" SF. This was not seen in the two cell lines where SF was measured with the extrapolation method. Interestingly in these two cell lines, prediction of SF and the experimentally measured SF after fractionated radiation were very similar in each fractionation regime whether proliferation was added to single dose SF or not. The methodology of the extrapolation method permitted establishment of the observed doubling time of the two cell lines. This clearly showed that proliferation took place during and after fractionated radiation: it even accelerated as the measured doubling time was reduced after the first two weeks. Our conclusion is that the extrapolation method was not useful in this experiment. There might be several reasons for this. As mentioned above cell kill must be caused by the irradiation for the method to be reliable. In this experiment with repeated radiation sessions and frequent sub-cultivations this might not be the case. This may also have affected the cell doubling time after radiation. We did observe a difference in the changes of the cell doubling time after radiation in the two cell lines compared to control. This implies that the proliferation rate in the exponential part of the cell growth curve may have differed between cell lines and control, making the experiment difficult to interpret.

The extrapolation method and clonogenic assay have previously been compared in achieving SF after a single dose of radiation. No major difference was found (128). It might be hypothesized that the risk of introducing other causes of cell loss and the proliferation effect is minor in relation to a single fraction; but when multiplied as is the case in fractionated treatment schedules, the effect gets more pronounced. This model for predicting radiosensitivity after fractionated radiation using the number of tumor cells and earlier established SF, and including Tpot, seemed to give a better prediction in three cell lines than the exclusion of proliferation did. In two of the cell lines the effect of proliferation was difficult to evaluate. No lag time before onset of proliferation after first radiation was observed in these cell lines. These results suggest that it is important to include proliferation in calculation of SF after fractionated radiation when using the clonogenic assay to establish SF.

4.2 STUDY II

The main objective of study II was to assess the usefulness of the radiobiological parameters inherent radiosensitivity, proliferation and number of tumor cells on treatment outcome after radiotherapy. A material of head and neck carcinomas was used. The hypothesis was that individually measured parameters are better for predicting treatment outcome than population averages are.

As stated previously, inherent radiosensitivity expressed as SF, measured with the clonogenic assay, correlates with local control (103, 104). However not all studies have found the same correlation although they used smaller patient material (136, 137). Proliferation has not been firmly demonstrated as an independent prognostic marker of treatment outcome (59). The combination of these two parameters, inherent radiosensitivity and proliferation, was evaluated in study II in a simple equation together with tumor size translated into number of tumor cells. To assess the robustness of individually measured radiobiological parameters, these were tested against population-derived averages. In the available material we evaluated 18 of 46 patients with individual radiobiological parameters measured. These 18 patients remained for an extensive analysis after we decided to exclude patients who had received brachytherapy since the effect of this treatment was not accounted for in the equation we used. The model's ability to predict treatment outcome after radiotherapy in this material of head

and neck carcinoma patients reached borderline significance (p=0.073), using patient-specific parameters even though only 18 patients remained in the final analysis. Both sensitivity and negative predictive value were better (75% versus 38% and 100% versus 67%, respectively) when patient-specific parameters were used. Note that the predictive values in this study concern local recurrence. Considering, instead, local control with a predictive cutoff value of 95% (the same as in study III) gives a sensitivity of 40% for both individual and average data. However, both specificity and positive predictive value are 100% for patient-specific parameters versus 75% and 57%, respectively, for population averages. An ROC analysis to compare population based versus individually derived radiobiological parameters shows a slightly better result using individually measured parameters with AUC = 0.7 versus AUC = 0.6 for population-derived parameters in this small patient material.

The reliability of individually measured radiobiological parameters must be addressed. Heterogeneity within a tumor is to be expected and can possibly affect the robustness of measurements and raise questions regarding how to interpret the results (138). Well established and easy-to-use methodologies are essential if they are to be used in clinical practice with a high ratio of conclusive results. The inherent radiosensitivity cell-survival curve of a tissue and the effect after fractionated radiotherapy are reflected in the LQ formula by the α/β ratio. Higher values are normally seen in tissues with pronounced early side effects after ionizing radiation, which also demonstrates less sparing by dose fractionation. This also applies to most carcinomas. We anticipated an α/β ratio of 15 in the equations in study II. However this assumption is tentative, albeit based on the fact that head and neck carcinomas normally have a high α/β ratio (139). We tested different α/β ratios in the equations (table 1) but this did not have any major affect on the results. Proliferation was assumed to be equal to Tpot measured before radiotherapy (59). This implies that all tumor-cell kill was achieved by radiotherapy. This can be questioned especially in fast-growing and radioresistant tumors. Tumor cells die from lack of nourishment and oxygen during normal growth conditions. This prolongs the tumor doubling time, making the clinical doubling time longer than the potential doubling time. Although it is unlikely that this form of tumor cell death ceases as soon as antitumoral treatment is given it is reasonable to assume that as tumors regress during therapy a greater extent of tumor kill is gradually achieved by the treatment. How fast this process occurs probably depends on several factors such as how quickly after radiation the tumor cells die and also the radiosensitivity of the

tumor. The idea of a lag period before onset of accelerated proliferation in tumor cells after radiation was not considered in the radiobiology model used (54, 56). Proliferation was assumed during the entire course of radiotherapy with a tumor cell doubling time represented by Tpot. This might be a weakness of the present work and most studies include a lag period when considering proliferation after radiotherapy even if it varies in time. However no solid data on the precise length of such a lag period before onset of proliferation can be considered to exist, and this is an area where further research is warranted. Calculations of TCP using a lag period of three weeks before onset of proliferation and an α/β of 15 are, however, presented in table I.

Table I. Predicted and actual local control in 18 carcinomas of the head and neck for different α/β values and depending on whether a lag period before proliferation is introduced.

Patient	Q α/β=15	Q α/β=10	Q α/β=5	Q α/β=3	Q T_k 22d.	Local Control
2	0	0	0	0	***1.00***	Yes
11	***0***	***0***	***0***	***0***	***0***	-
13	***1.00***	***1.00***	***1.00***	***0.98***	***1.00***	Yes
18	0	0	0	0	0.33	Yes
20	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***	Yes
24	0.05	0	0	0	***1.00***	yes
25	***0***	***0***	***0***	***0***	***0.05***	-
29	0	0	0	0	0	Yes
33	0	0	0	0	0	Yes
35	***1.00***	***1.00***	***1.00***	0.54	***1.00***	Yes
39	0	0	0	0	0	-
42	***0***	***0***	***0***	***0***	0.98	-
47	***0***	***0***	***0***	***0***	***0.01***	-
50	***0***	***0***	***0***	***0***	***0***	-
54	***0.15***	***0***	***0***	***0***	***0.54***	-
55	***0.73***	***0.35***	***0***	***0***	0.95	-
56	0.57	0.06	0	0	***0.95***	Yes
57	***1.00***	***1.00***	***1.00***	***1.00***	***1.00***	Yes

Q = Probability of local control where 1 equals 100 %

A limited clonogeneity of the tumors is not included in the model but all tumor cells were considered as potentially clonogenic. However, to test the model calculations were performed considering 10 % of the tumor cells as clonogenic (calculations not shown). This did not alter the results considerably. As expected, the probability of local control did increase in the tumors with a probability between 5% and 95% in the original calculations. Still only one of these patients reached a probability of local control above 95%.

Another factor of importance for radiation effect is the oxygenation status of the tumor. It has been demonstrated for head and neck carcinomas that oxygen tension in the tumor before start of treatment correlates with treatment outcome (67). Whether this is an effect of enhanced radioresistance or a more aggressive tumor phenotype is not fully understood. An unfavorable effect of hypoxia on radiation effect after a single fraction is well established, as presented earlier (11, 140). The oxygen enhancement ratio (OER) represents the dose difference required to achieve a certain cell kill when oxygen is introduced to anoxic or hypoxic cells. The effect of hypoxia on fractionated radiotherapy is more complicated and reoxygenation is an important factor in treatment outcome (81). To test the supposed impact of hypoxia on the current results we introduced an imaginary fraction of hypoxic cells (10% or 30%) in the tumors. SF was then recalculated with an OER of 2 representing the presumed effect of hypoxia in these cells. The number of surviving cells in the oxic and hypoxic fractions in each tumor was accounted for and the probability of cure was calculated for each tumor with 10% or 30% of hypoxic cells respectively. The results are presented in table II. As shown, even a small fraction of hypoxic cells has theoretically a major impact on predicted treatment outcome after fractionated radiation, making it very difficult to accomplish complete tumor cell kill and local control. Clinical experience does not, however, confirm such an important impact on treatment outcome. Although a certain oxygen effect might be present even in fractionated radiotherapy, it is probably difficult to quantify and measure this correctly to fit into an equation. To do so you would probably require a model that accounts not only for continuous variations in hypoxia levels but also for how large a volume of the tumor is hypoxic at each fraction.

Table II. Probability of local control with different ratios of tumor hypoxia.

Patient	Q, 30 % hypoxia	Q, 10 % hypoxia	Q, Oxic	Local control
2	0	0	0	Yes
11	0	0	0	-
13	0	0.05	1.00	Yes
18	0	0	0	Yes
20	0.94	1.00	1.00	Yes
24	0	0	0.06	Yes
25	0	0	0	-
29	0	0	0	Yes
33	0	0	0	Yes
35	0	0	1.00	Yes
39	0	0	0	-
42	0	0	0	-
47	0	0	0	-
50	0	0	0	-
54	0	0	0.15	-
55	0	0	0.72	-
56	0	0	0.57	Yes
57	0	0.16	1.00	Yes

Q= Probability of local control where 1 equals 100 %

The results of study II support the idea that individually measured radiobiology parameters are robust enough to be used in models to predict treatment outcome after radiotherapy. Further, individual radiobiological measurements are better than population-based averages at prediction of treatment outcome after radiotherapy. Predictive radiotherapy modeling is an area well merited for further investigation.

4.3 STUDY III

Study III was designed to further evaluate the role of individually measured radiobiological parameters in the same material of head and neck carcinomas that was presented in paper II. At the same time another mathematical model for evaluating

tumor control probability (TCP) based on biologically effective dose (BED) was evaluated. This enabled the patients that received brachytherapy to be included in the TCP analysis, allowing more data to be evaluated.

Forty-six patients had defined tumor material, with 35 tumors demonstrating local control and 11 local relapse. Forty-three patients were evaluable. In three tumors a BED could not be defined. In two of these it was negative due to a large proliferation coefficient and in the third SF was determined to 1, rendering a definition of α impossible. There was a trend towards increasing probability of local control with a higher BED both when calculated with tumor-specific radiobiological parameters and also when using population average parameters. However when TCP was established by introducing number of tumor cells estimated by tumor volume before treatment, the two groups appeared to separate more clearly. TCP based on tumor-specific radiobiological parameters seems to give a better prediction of actual tumor control than corresponding average data, which greatly underestimates cure with less than a 10% prediction of tumor control. Assuming that a calculated TCP of 95% is the threshold for accurate prediction of local tumor control, the sensitivity, the specificity and the positive and the negative predictive values of the different approaches for calculating the TCP were determined. Sensitivity and specificity for tumor-specific parameters were 63% and 80%, respectively. The corresponding values for population-based averages were 0% and 91%, respectively. Positive predictive value was 92% when tumor-specific parameters were used compared to 0 % for population-based. An ROC analysis was done to distinguish the predictive values of the different methods of establishing TCP (134). The ROC curve obtained, based on patient-specific α and Tpot, had an AUC of 0.7 with a p-value of 0.01, showing that the area under the ROC curve differed significantly from 0.5. There is therefore evidence that this method is able to distinguish between a group of patients presenting local control and a group that does not. The same advantageous result was not shown when calculations were based on population averages. These resulted in an AUC of 0.5 and consequently a non discriminatory p-value.

These results support the idea of combining individual radiobiological data - as in this case SF and proliferation - and also of considering number of tumor cells to establish a prediction of tumor control. Previous reports have not been able to firmly establish a relationship between radiobiological parameters, except that greater T stage and

increasing tumor volume indicate worse prognosis (41, 141), and the papers on SF2 mentioned previously indicate a role of inherent radiosensitivity on local control (103, 104). The combination of individual data that seems fruitful might be explained by the fact that if only one factor is considered the others can interfere as confounders and conceal a potential relationship. Note that the relationship between tumor size and local control in this material was reversed, with a median tumor volume of 14 cm^3 when local control was achieved compared to 10 cm^3 in tumors that relapsed. The present study result is therefore not likely to merely reflect the impact of tumor size on TCP. Some assumptions regarding radiobiological parameters differed in this study from those stated in paper II and referred to in the previous chapter. The main reason is that numerous recent papers (57, 142-144) have evaluated BED and the possible impact on tumor control after treatment. In doing so they used different radiobiological assumptions. We used assumptions similar to the ones in those papers regarding α/β and proliferation. In general we set tumor α/β to 10 as in a recent paper evaluating head and neck carcinomas (57). Those authors also specified a lag period before start of proliferation to 22 days and we did the same in the present study. As already stated, no definite time point when proliferation should be considered to begin after start of radiotherapy is established. One reason for using a lag period is that it might take a while before the net proliferation of tumor cells during radiation reaches the Tpot level measured before start of radiotherapy - presuming that it in fact does so. This lag period would thus account for the uncertainties regarding proliferation during radiotherapy, allowing the use of Tpot as a substitute for tumor doubling time – even though Tpot might underestimate doubling time after radiotherapy, at least initially. To summarize: this study shows that TCP based on BED using individually measured radiobiological parameters is superior to TCP based on BED and average radiobiological parameters. This further supports the idea of a favorable impact of individually-measured radiobiological parameters when predicting outcome after radiotherapy.

4.4 STUDY IV

In study IV the role of the thioredoxin (Trx) - thioredoxin reductase (TrxR) system and the cellular redox balance on radioresistance was investigated. A gold(I) compound, $[Au(SCN)(PEt_3)]$, that selectively inhibits TrxR, was evaluated in a radioresistant cell line, U-1810. The effect on radiation sensitivity was assessed as were gene expression changes following radiation in combination with the gold compound.

The use of gold compounds in clinical practice is an attractive approach since gold is readily used in the clinic in inflammatory diseases i.e. rheumatoid arthritis (145). The inhibitory effect of the gold(I) compound on expression of TrxR in U1810 was confirmed and it decreased by about two-thirds after cultivation with 2.5 µM [Au(SCN)(PEt_3)] for 24 and 48 hours. Continuous cultivation of U1810 cells in 0.05 µM gold(I) compound maintained the TrxR at similar low levels. qPCR was used to measure the inhibition of siRNA on TrxR mRNA expression and this expression decreased over time from 60 % of original values at 24 hours after transfection to 35 % at 72 hours. Western blot confirmed an inhibitory effect on TrxR expression at protein level. Proliferation of cell line U1810 was not affected after a shorter exposure of up to 48 hours of the gold(I) compound alone. However, continuous exposure to the lower level of 0.05µM seriously affected the ability of the cell line to proliferate. Suppression of TrxR with siRNA confirmed this negative effect on long-term cell proliferation.
In addition, exposure of U1810 cells to ionizing radiation with a single fraction of 2 Gy and 5 Gy was evaluated. This radioresistant cell line only demonstrated impaired repopulation after exposure to 5 Gy, a dose that also reduced the surviving fraction according to the extrapolation method (132). The lower, but perhaps clinically more relevant, dose of 2 Gy did not impair surviving fraction or repopulation significantly. Radiosensitive cell line U1906, used as a control, was sensitive to both 2 Gy and 5 Gy. Exposure to radiation in combination with [Au(SCN)(PEt_3)] in U1810 reduced the surviving fraction and repopulation rate significantly after 2 Gy as well as after 5 Gy. These findings indicate the possibility of sensitizing radioresistant cells to clinically relevant doses of radiation by inhibiting TrxR. It would be of great interest to evaluate whether normal cells are also sensitized to ionizing radiation by inhibition of TrxR. If not, an advantageous therapeutic ratio between tumor cells and surrounding normal tissue might be explored. Low levels of ROS have been related to radioresistance and depletion of ROS scavengers would consequently sensitize cells to ionizing radiation (96). The intracellular redox balance is often altered in malignant cells and the expression of Trx - TrxR elevated (95). This suggests that inhibition of TrxR would affect malignant cells but not necessarily normal cells in the supporting stroma and surrounding tissues, at least not to the same extent, possibly giving a selective tumor sensitizing effect to ionizing radiation. The use of flow cytometry showed that exposure to the gold(I) compound affected the cell cycling in U1810 cells. Relatively more cells traversed the cell cycle into 2nd cycling with a continuously larger fraction associated

with longer exposure times. It is possible that this also affects the sensitivity of the cells to ionizing radiation with more cells cycling and therefore in potentially more radiosensitive cell cycle phases than if they had become quiescent or remained in G_1 (146). However exposure to the gold compound alone did not accelerate proliferation: as noted earlier it was unaffected. Instead, with a continuous low exposure, there was reduced proliferation. This can seem to contradict the findings on the effect of cell cycling. One explanation could be that there was an arrest in finalizing the cell cycle, with relative more cells in the ultimate cell cycle phase. Exposure of the gold compound alone to the U1810 cell line demonstrated 20 differentially up-regulated genes connected to the cell cycle in the gene expression analysis. This is well in line with these findings.

The use of gene expression analysis in the clinical setting has yet to be verified. A few clinical studies evaluate the importance of gene expression patterns to discriminate outcome after radiotherapy in different patients but further clinical evaluations are needed before clinical use can be established (147, 148). Gene expression analysis after exposure to [Au(SCN)(PEt_3)] in combination with radiation demonstrated a much stronger effect than exposure to radiation or the TrxR inhibitor alone. 181 genes were uniquely up-regulated and 95 down-regulated after the combined treatment. This was not seen after exposure to radiation alone or to the gold(I) compound where only a few genes were uniquely affected. The Gene Ontology vocabulary was used for functional classification and enrichment testing in order to achieve a more comprehensive survey of the affected genes. When these were subjected to enrichment testing using the Gene Ontology Tree Machine (GOTM), several GO terms emerged as significantly enriched. Four different clusters of related enriched GO categories were identified: DNA-damage, repair and replication, DNA and chromatin organization, cell cycle and finally cytokine and chemokine activities. These findings correlate well to corresponding genes that must be involved in cellular response to ionizing radiation. The interesting finding is that only combined treatment gives significant alterations in gene expression. This indicates a pronounced effect on cellular response to ionizing radiation when cells are treated with the gold(I) compound, which is reflected in the altered repopulation and decreased surviving fraction.

5 CONCLUSIONS

The present study was performed to investigate the value of using models based on radiobiological parameters to predict clinical outcome after radiotherapy and the possibility to affect the radiobiological parameter inherent radiosensitivity by altering the intracellular redox balance. The following conclusions are made:

Radiobiological models based on inherent radiosensitivity, proliferation and number of tumor cells have the possibility to predict clinical outcome after radiotherapy. Individual derived radiobiological parameters are better to use than population derived averages in predicting clinical outcome after radiotherapy when used in radiobiological models.

The radiobiological parameters SF2 and Tpot are robust enough to be used in models to predict individual treatment outcome after radiotherapy.

Different models to predict treatment outcome may be used if they consider variations in radiobiological parameters between individual tumors.

The findings support the idea of combining different radiobilogical parameters in a model to predict clinical outcome after radiotherapy.

Altering the redox balance in tumor cells by inhibition of TrxR reduces the inherent radiosensitivity and decreases repopulation after exposure to inonizing radiation. The effect on inherent radiosensitivity is supported by gene expression analysis demonstrating effect on DNA –damage, -repair, cell cycle and chromatin organization.

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