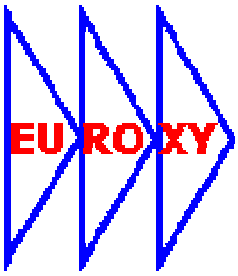
ELIMINATION OF LOW DOSE HYPER-RADIOSENSITIVITY IN T-47D CELLS

The significance of dose-rate and oxygen level

Nina Frederike Jeppesen Edin

Centre for Education and Research
in Radiation Oncology (SUFS)
Institute for Cancer Research
The Norwegian Radium Hospital

Department of Physics
Faculty of Mathematics and Natural Sciences
University of Oslo



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Abbreviations and terms

ATM	Ataxia telangiectasia mutated
Chk1/2	Cell cycle checkpoint kinase
DNA-PK	DNA protein kinase
DSB	DNA double strand break
HRS	Low dose hyper-radiosensitivity
γ-H2AX	Phosphorylated histone 2AX
H3	Histone 3
HDR	High dose-rate, in this thesis 40 Gy/h
HRR	Homologous recombination repair
IMRT	Intensity modulated radiation therapy
IRR	Increased radioresistance
LDR	Low dose-rate, in this thesis 0.3 Gy/h
LET	Linear energy transfer
NHEJ	Non-homologous end joining
p53	Protein 53 kilo Dalton
PARP	Poly(ADP-ribose) polymerase
pRb	Retinoblatoma protein

Early G2-phase checkpoint: ATM-dependent G2-checkpoint activated early in response to doses above ~0.4 Gy (dependent on cell line)

Sinclair checkpoint: Late responding ATM-independent, dose-dependent G2-accumulation of cells

List of papers

I. Recovery of low-dose hyper-radiosensitivity following a small priming dose depends on priming dose-rate
Edin, N.F.J., Olsen, D.R., Stokke, T. and Pettersen, E.O.
International Journal of Low Radiation 4, 69-86 (2007)

II. The elimination of low-dose hyper-radiosensitivity by transfer of irradiated cell conditioned medium depends on dose-rate
Edin, N.F.J., Sandvik, J.A., Olsen, D.R. and Pettersen, E.O.
Radiation Research, in press

III. Mechanisms of the elimination of low dose hyper-radiosensitivity in T-47D cells by low dose-rate priming
Edin, N.F.J., Olsen, D.R., Stokke, T., Sandvik, J.A., Ebbesen, P. and Pettersen, E.O.
Submitted to International Journal of Radiation Biology

IV. Low dose hyper-radiosensitivity in T-47D cells is eliminated by chronic moderate hypoxia but returns after reoxygenation
Edin, N.F.J., Olsen, D.R., Sandvik, J.A., Malinen, E. and Pettersen, E.O.

1. Introduction

During recent years, it has become evident that while the number of ionizations induced by γ-radiation or X-rays shows a linear relationship to dose, the biological responses to small doses of radiation are a more complex affair and can not be predicted on the basis of knowledge acquired from irradiation with higher doses. Genomic instability, adaptive responses, bystander effects and low dose hyper-radiosensitivity/increased radioresistance are radiobiologic phenomena observed after low-dose irradiation with the traits in common that not all cell and tissue models show the same response, and that there is no "true" dose-response - the effect is either on or off (*1*). However, the mechanisms involved are different and understanding the biologic effects of low dose irradiation requires separate study of each of these low dose phenomena. In this thesis, the main topic is low dose hyper-radiosensitivity (HRS).

1.1 Low dose hyper-radiosensitivity

There appear to be two principal types of protection of tissue against low doses of X- or γ-radiation. One is to keep cells alive and functioning by preventing or repairing DNA damage. The other is to eliminate potentially mutagenic cells by terminal differentiation, immune responses, or inducing cell death.

Low-dose hyper-radiosensitivity (HRS) is a manifestation of the latter. Cells exhibiting HRS are characterized by a high sensitivity to radiation doses below ~0.5 Gy (cell line dependent) which is followed by a more radioresistant response per unit dose in the dose range ~ 0.5-1 Gy. This transition towards radioresistance is generally described by the term "increased radioresistance" (IRR) (figure 1).

HRS was first identified in vitro in 1993 (*2*) after having been observed in mouse skin in 1986 (*3*) and in mouse kidney in 1988 (*4*). HRS has been observed in cells given acute proton and pi-meson irradiation(*5-7*) as well as in cells given high LET neutrons at a low dose-rate (*8*) and appears to be the default response for all radiation qualities in both tumour and normal cell lines (only ~20% of the >50 tested cell lines failed to exhibit HRS (*9-11*)). IRR, on the other hand, is only observed after low LET irradiation and only in repair-competent cells lines (*12*).

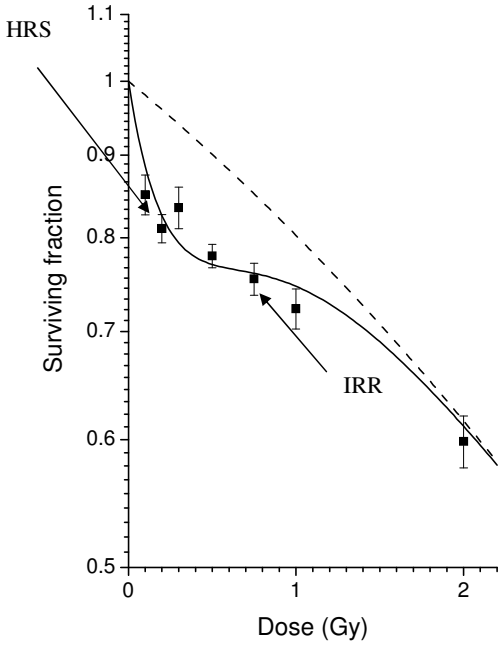

Figure 1: HRS/IRR in T-47D cells. Dashed line represents the fit using the Linear Quadratic (LQ) model, solid line the Induced Repair (IR) model

Ionizing radiation causes DNA damage as well as mitochondria-dependent generation of reactive oxygen species (*13*). DNA single strand breaks and base damages are proficiently repaired by error-free mechanisms while unrepaired (or misrepaired) DNA double strand breaks (DSBs) are believed to be the prime cause of radiation induced cell death (*14*). Radiation induced DSBs are chiefly repaired by the efficient process of non-homologous end joining (NHEJ) though homologous recombination (HRR) contribute to repair of breaks during the S- and G2-phase of cell cycle (*15*). A core component of mammalian NHEJ is DNA protein kinase (DNA-PK) (*16*). A marked decrease in DNA-PK activity was observed in response to a low dose of 0.2 Gy in six cell lines exhibiting HRS, whereas DNA-PK activity was increased in the four HRS-negative cell lines which indicates a role for NHEJ in the transition from HRS to the IRR survival response (*17*). However, inhibition of DNA-PK did not produce HRS in HRS-negative U373 astrocytoma cells in the low-dose range (*18*) but this was explained by a back-up pathway for NHEJ utilizing Ligase III and PARP-1 proposed by Wu et al., who found that the putative contribution of HRR to the repair in IR-induced DSBs in G2-phase cells was undetectable (*19*). While HRR requires extensive homology and normally uses sister chromatid strands

as template for repair, NHEJ is used when no template is available. Thus, NHEJ is intrinsically error prone because, unless the two ends are compatible, the necessary enzymatic modifications of the ends may generate base substitutions, insertions or deletions (*15*). Since NHEJ repair of DSBs thus implies a risk of misrepair, which, if not lethal, might lead to potentially cancerous mutations, it may be better for the tissue as a whole to sacrifice damaged cells as long as the number is not so large that it impedes the function of the tissue/organism.

1.1.1 The early G_2-phase checkpoint

HRS has been associated with failure to induce the early (active 0-2 h post-irradiation) and transient (lasting ~12 h) G_2-phase checkpoint (*20*), in which cells irradiated in G2-phase are arrested for doses >~0.4 Gy in order to provide time for repair. Low dose radiation damaged G2-phase cells are thus allowed to enter mitosis with unrepaired injuries resulting in cell death.

Contrary to the dose-dependent mechanisms by which cells irradiated in G1- or S-phase accumulate in G2 (Sinclair checkpoint), the "early" G2-checkpoint is ATM-dependent (*20*) and independent of dose over the range of 1 to 10 Gy, with a distinct activation threshold of ~0.4 Gy (*11, 21-23*). Activation of the critical damage sensor molecule ATM by an autophosphorylation event at serine 1981 is detectable at doses of 0.1 Gy with a gradual increase until phosphorylation of more than 50% of the ATM molecules in the cell after a dose of 0.5 Gy and saturated expression at larger doses (*24*). Thus, the HRS/IRR transition appears to be coincident with both the induction of the early G_2-phase checkpoint and activation of ATM.

One of the earliest detectable downstream targets of ATM is the histone H2A variant, H2AX, which is phosphorylated by ATM at Ser-139 (*25, 26*). Foci of γ-H2AX (a phosphorylated histone), detected by immunofluorescence, has been shown to be quantitatively the same as DNA DSBs (*27*) and this assay was used to demonstrate that HRS does not reflect a failure of DSB recognition (*28*). In addition, it was demonstrated that HRS-negative cell lines have the same ATM activation pattern as cells with HRS whereas they show an early G2-arrest even after low radiation doses that produce insufficient damage to induce full ATM activation (*11*). It was suggested by Krueger et al. that the dose-dependent ATM regulatory control is evaded by aberrant early G_2-checkpoint response in HRS-negative cell lines caused by dissociation between ATM activity and early G_2-checkpoint function (*11*).

The existence of HRS appears in some cell lines to be associated with an elevated level of caspase-3 mediated apoptosis after low-dose exposures (*29, 30*) suggesting that the radiation-damaged G_2-phase cells that evade the early G_2-checkpoint are disposed of by this mechanism when entering mitosis. However, also cell lines deficient in TP53 induction after irradiation or with mutated TP53 have been shown to display HRS/IRR and no increase in apoptosis in response to HRS-inducing doses was observed in BMG-1 cells with wild-type TP53 (*31*). Also Vaganay-juery et al. reported no connection between HRS and apoptosis in the six HRS-competent cell lines investigated measured by DNA-PKcs as early apoptosis marker (*17*). This corroborates the hypothesis that the transition from HRS to IRR primarily is related to induction of the "early" G_2-checkpoint and that the death process of the cells entering mitosis with damages is cell line dependent.

1.2 Phenomena with relations to HRS/IRR

1.2.1 Adaptive response

The radiation-induced adaptive response was first described by Olivieri et al. in 1984 (*32*) as the reduced sensitivity to a challenge irradiation induced by a previous small priming dose. Radio-adaptive responses have been observed in vitro and in vivo using various end points, such as cell lethality, chromosomal aberrations, mutation induction, radiosensitivity, and DNA repair (*33*). Adaptation is most efficiently induced by doses of 0.01-0.5 Gy at dose-rates from 0.01-1.0 Gy/min (*33*) with challenge doses in the range of 0.5-2 Gy (*1*). The protective effect has been reported to last for about three generations following the priming irradiation (*34*).

Both IRR and adaptive response can be inhibited by cycloheximide in V79 hamster cells (*35*), demonstrating the need for protein synthesis in both phenomena. However, adaptive response and IRR are not homologous phenomena. Firstly, activation of poly(ADP-ribose) polymerase (PARP) is required for adaptation (*34, 36*) while inhibition of PARP1 induced marked radiosensitization of V79, CHO, and exponentially growing T98G cells in the 0.05-0.3 Gy range, but not in confluent T98G cells or U373 cells neither of which displays HRS (*37*). Secondly, the adaptive response has been shown to be independent of ATM (*38*), while ATM is a key factor for activation of the early G_2-phase checkpoint which is believed to be requisite for IRR (*22*). Thirdly, the time courses for

activation and deactivation is generally much shorter for IRR than for adaptive responses (*10*).

However, a priming dose of 0.2–0.5 Gy given at a high dose-rate (HDR, *i.e.*, acute irradiation) has been shown to temporarily abolish HRS (*35, 39-41*) but with no significant effect on the response to higher doses (*40*). The time courses for full induction of IRR and for full return of HRS appear to be cell line dependent, but take in the order of hours. For the T-47D cells used in this study, we found that HRS was abolished 6 h after a HDR priming dose of 0.3 Gy, but 24 h after the priming dose, HRS had returned (paper I).

1.2.2 Dose-rate effects

For x- or γ-rays (low LET), dose-rate is one of the principal factors that determine the biological consequences. Sublethal damages only become lethal (DSBs) if not repaired before a second (in itself also sublethal) injury is inflicted. Prolonged radiation time of a given absorbed dose results in a lower density of sublethal damages which in combination with repair decreases the probability of coincidence with reduced biological effects as a consequence (*42*). Decrease in cell kill as a result of lowering the dose-rate of a given dose is termed the classical dose-rate effect and is very important in radiotherapy because it usually is more pronounced in normal tissue as compared to tumour tissue. As the dose-rate is lowered and the irradiation time protracted, a point is reached at which all sublethal damage is repaired.

Lowering the dose-rate further may invoke an inverse dose-rate effect. There appear to be different mechanisms that induce a higher cell kill as the dose-rate is lowered. In human cervix cancer HeLa cells (*43*) and NHIK 3025 cells (*44*) but not in human breast cancer T-47D cells (*45*) an inverse dose-rate effect was observed, which was demonstrated to be due to accumulation of the cells in the radiosensitive G_2-phase when the dose-rate was lowered from ~0.9 to ~0.3 Gy/h. T-47D cells did also accumulate in G_2 during low dose irradiation, but the lack of inverse dose-rate effect was ascribed to the observation of a population of arrested G2 cells with pRB protein bound in the nucleus, which might protect cells against radiation-induced cell death in G_2 (*45*).

On the other hand, the inverse dose-rate effect observed in human prostate carcinoma PC3, and human glioma T98G, and A7 cell lines, but not in HRS-negative human astrocytoma U373MG cells, was suggested to reflect HRS and be due to the lack of induction of repair processes occurring in response to exposures at the lowest dose-rate in HRS-competent cell lines (*46*). In support of this explanation, it was found that the

enhanced cell kill in DU-145 cells irradiated at the very low dose-rate (LDR) of 9.4 cGy/h equivalent to ~4-5 DSBs/h compared to at the high dose rate (HDR) of 45 Gy/h could be attributed to inefficient activation of the damage sensor ATM and its down-stream target H2AX; the LDR effects could be abrogated by preactivation of ATM or simulated in HDR-irradiated cells by inhibiting ATM function (*26*).

The effect on HRS of LDR-priming with dose-rates comparable to the LDR used in this thesis has previously been examined in one study. In this a pre-irradiation with total doses of 2 or 5 Gy ^{60}Co γ-rays using various LDRs from 5 to 60 cGy/h did not abolish HRS in asynchronous T98G cells when the response to an immediate subsequent HDR X-ray exposure was measured, but HRS was reduced after the higher dose LDR pre-exposure compared with the lower dose (*47*). In paper I, we found that not only did a LDR priming exposure (0.3 Gy at 0.3 Gy/h) remove HRS in T-47D cells, but it did not return within the 14 weeks studied. In paper II, this time course was extended to 14 months and later experiments, done more than 2 years after the LDR priming, indicate that this seems to be a permanent elimination of HRS.

1.2.3 Responses mediated by medium transfer (bystander effects)

Radiation-induced bystander effects are biologic responses in unirradiated cells that have received signals from irradiated cells either via cell-to-cell gap junctions or by secretion of soluble factors. Experiments involving medium transfer from cells irradiated with low LET-radiation to unirradiated cells have demonstrated a highly significant reduction in plating efficiency of both normal and malignant recipient cells (*48*). This bystander effect suggested that irradiated cells secreted a molecule into the medium that was capable of killing cells when the medium was transferred onto unirradiated cells. The precise nature of factors that mediate the bystander effect is unknown, but reactive oxygen and nitrogen species as well as various cytokines have been implicated (*49*).

While it has been observed that repair-deficient cells produce cytotoxic “bystander” effects after γ- irradiation which reduce the plating efficiency in recipient cells (*50-52*), medium harvested from repair-competent cells γ-irradiated with a small dose seems to be capable of inducing an adaptive response to subsequent irradiation in unirradiated cells (*53-57*). There also appears to be an inverse relationship between the ability to produce cytotoxic signals and the presence of HRS/IRR in cell lines (*58*).

Consistently, we have not found any cytotoxic effect of medium transfer from irradiated T-47D cells. On the contrary, we observed an increase in plating efficiency in

cells receiving medium transferred from cells irradiated with 0.3 Gy both at LDR and HDR. We also found that medium transferred from T-47D cells irradiated with 0.3 Gy at the LDR could remove HRS transiently in recipient unirradiated T-47D cells. However, medium transferred from HDR-primed cells did not have this effect (paper II).

1.2.4 Hypoxia

In cancer treatment, it is a major problem that tumour cells are to some extent protected against radiation damage because they are hypoxic. As a solid tumour expands, the ability of the existing vasculature to supply growth factors, nutrients, and oxygen is surpassed. Tumour hypoxia can stimulate angiogenesis but the newly formed vasculature is malformed and poorly organized resulting in regions with poor delivery of oxygen as well as intravenously administered chemotherapeutics (*59*). Tumour cells that are deprived of oxygen are resistant to radiation (and some chemotherapeutic drugs) because oxygen is required to prevent the return of short lived radiation induced free radicals to their original un-reactive state. As the oxygen concentration increases, the cells become gradually more sensitive to radiation and reach a sensitivity about 3 times as high as during anoxia (*60*). However, prolonged oxygen depletion has been shown, in addition to the radiochemical effect, to exert biological effects which can influence the radiation response. Hall et al. showed already in 1966 that cells irradiated under chronic extreme hypoxia were more radiosensitive than cells irradiated under acutely hypoxic conditions, and consistently, that cells reoxygenated following prolonged hypoxia were more radiosensitive than cells not pre-treated with hypoxia (*61*). Although this was shown not to be the case with Chinese hamster V79 cells (*62*), it has been confirmed for another human cervix cancer cell line NHIK3025 (*63, 64*). Koritzinsky et al. found that the intrinsic radiosensitization induced by prolonged hypoxia (<4 ppm O_2 for 20 h) lasted for 7 hours following reoxygenation and could be attributed to biological gene-expression-induced changes, not the altered cell cycle kinetics that arise when the cells resume cell cycle progression after the hypoxia induced arrest (*63*).

Chronic moderate hypoxia does not induce cell cycle arrest. Depending on the cell line, cells can adapt to a certain level of hypoxia and grow seemingly indefinitely. T-47D cells were cultured in a hypoxia box with 4% O_2 in the gas-phase, which between reculturings gave a peri cellular oxygen concentration reaching below 0.1% while the oxygen concentration did not drop below 3.5% for cell grown in ambient air (*65*). After

being cultured for 7 weeks with 4 % O_2 in the box, the radiosensitivity was the same when irradiated with acute doses of ^{137}Cs as for cells cultivated in ambient air.

In paper IV, the effect of moderate chronic hypoxia on HRS was investigated. This is particularly of interest in radiotherapy of solid tumours in connection with exploiting HRS in ultrafractionation. It was demonstrated that T-47D cells grown for 3-6 weeks in 4% O_2 had lost HRS but they regained it after reoxygenation. In contrast to our previous findings (*65*), the cells that had been cultured at 4% O_2 appeared to be slightly more resistant to high acute doses than cell cultivated in ambient air.

1.3 Clinical implications of HRS

The presence of HRS may have implications for in cancer radiotherapy in which the aim is to control the eradication of tumour tissue while minimizing the damage to normal tissue. The introduction of intensity modulated radiation therapy (IMRT) in cancer treatment results in irradiation of a larger proportion of normal tissue but at lower doses when compared to conventional treatment. In some situations, one could fear that HRS will tend to increase the effect of low doses in normal tissue and thus negate the benefits of using IMRT, in particular in tissues with a pronounced volume effect (*66*). Since the HRS is related to the fraction of cells in G_2-phase, it may be of more consequence for early-responding proliferating tissues, such as skin, than for slowly proliferating normal tissues with a small fraction of cells in G_2. In support, evidence of HRS has been demonstrated in studies with human skin using basal cell density (*67*) or skin erythema (*68, 69*) as endpoint as well as with mouse skin (*70*).

On the other hand, it may be possible to exploit HRS in tumour cell killing. However, to compensate for cell proliferation, a decrease in dose-fraction size must be accompanied with a decrease in time between fractions which induce a risk of a priming effect of the previous fraction reducing the efficacy of the treatment. This may be part of the explanation why *in vivo* experiments have had diverging outcome. For example were data from experiments with ultrafractionation of 0.4 Gy per fraction, 3 fractions per day in murine DDL1 lymphoma (*71*) or in human A7 glioblastoma xenografts (*72*) not showing HRS.

A combined chemo-radio therapy approach has been demonstrated to improve the therapeutic potential of ultrafractionation. A protocol using a taxane (paclitaxel), which synchronize cells in G_2-phase (*73*), in combination with carboplatin and low dose

fractionated radiation was extremely well tolerated by the patients and showed a synergistic effect in patients with squamous cell cancer of the head and neck (*74*).

Another approach to exploit HRS in tumour tissue is to inhibit the induction of IRR. Several biotechnology companies are currently developing improved inhibitors of ATM, DNA-PK, Chk1, Chk2, and PARP-1 (*12*).

Both for exploitation of HRS in tumour tissue and for protection against HRS in normal tissue, knowledge of the transition from HRS to IRR response is requisite. In papers I-IV, a number of ways to eliminate HRS from T-47D cells either transiently or presumably permanently were demonstrated. The HRS/IRR transition appeared to be affected by a factor that LDR primed cells induced in the medium if serum was present during LDR priming. These findings prompted further investigations in pursue of a mechanistic understanding of the phenomena.

2. Experimental models and techniques

2.1 Cell line

The T-47D cell line was established from the pleural effusion of a 54 year old patient with breast carcinoma in 1974 (*75*). The cells exhibit epithelial morphology and form monolayers in culture. T-47D cells are hemizygous for the p53 gene and contain a single point mutation in the remaining allele at codon 194 (*76*).

A subculture of T-47D cells was primed august 2005 with 0.3 Gy at 0.3 Gy/h and cultured as a stock culture since then, interrupted by two periods of approximately two weeks during which the cells were stored in liquid nitrogen. These cells are denoted T-47D-P.

2.2 Clonogenic survival assay

The presence and degree of HRS was measured using the clonogenic survival assay technique of Puck and Marcus (*77*). In short, a certain number of single cells were plated, irradiated and subsequently incubated until each single cell had had time to grow into a colony. At this time, the flasks were fixated in ethanol and dyed with methylene-blue for manual colony counting. The surviving fractions were calculated as the plating efficiency of each dose divided by that of the control flasks.

In order to improve the precision of the colony formation assay, which is necessary to detect the rather small deviations in surviving fractions in the HRS/IRR region, different methods have been used. The most common is to identify single cells by use of a cell sorter (*78, 79*). However, in the present experiments with T-47D cells, making dilutions after counting the cells in a Bürker-chamber provided significant and reproducible data. For all doses up to 2 Gy, the cells were seeded from the same dilution as the controls with 200 cells/ml and particular care was taken to maintain the homogeneity of the dilution by repeated pumping with a 2 ml pipette.

As described in paper II, trypsinization and plating affected the degree of HRS up to 8 h after handling the cells. In order to avoid any artefacts from trypsinization, a time of 16-20 h was chosen between plating and (challenge-) irradiation even though this implied a higher multiplicity (mean number of cells per colony-forming unit) at the time of irradiation because the cells had had time to progress through cell cycle. The surviving

fraction of irradiated cells (when values were recorded below 1) was therefore corrected for increased mean multiplicity per colony-forming unit according to a formula published by Gillespie et al. (*80*). In light of recent publications on bystander effects, the relevance of Gillespie's formula based on the target-hit theory could be questioned for low doses, but we have chosen to apply it because T-47D cells did not show any cytotoxic bystander effects by medium transfer from irradiated cells (paper II) and it has been demonstrated that bystander effects dependent on cellular interaction and functioning gap junctions are induced only by radiation with high linear energy transfer (LET), but not by low LET radiation (*49*).

In the experiments in paper I, where the challenge doses were given 6 and 24 h after the priming dose, respectively, the cells were plated after the priming dose. When it was discovered, as described in paper II, that trypsinization reduced HRS for at least 8 h, the experiments with 6 h between priming dose and challenge dose were repeated with the cells plated for colony formation the day before the priming dose was given. The same elimination of HRS was found by this assay (data not shown) excluding that the results found in paper I were an artefact caused by trypsinization.

2.3 Irradiation

2.3.1 ^{60}Co γ-rays

The ^{60}Co facilities were situated at the Radium Hospital. For the experiments in papers I and II , the flasks with cells attached were γ-irradiated from below with a Molbatron 80 (T. E. M. Instruments, Crawley, UK). The irradiation field was 25 × 25 cm^2 and the source-to-flask distance was adjusted to yield the dose-rate of 40 Gy/h used for acute irradiation. For doses implying a radiation time of more than two minutes, the flasks were placed in sealed plastic bags and submerged in an open water bath (42×35 cm^2, height 20 cm). The water bath maintained a temperature of 37˚C by use of a temperature-controlled heater (Techne Templette TE-8D, Princeton, NJ, USA) that also kept the water in circulation.

The low dose-rate of 0.3 Gy/h was obtained by shielding the source by a 10 cm thick block of Roos metal (Sn 25%, Pb 25%, Bi 50%, melting point 96˚C, specific weight 9.85 g/cm^3).

The Mobaltron 80 was replaced by a Theratron 780-C (MDS Nordion , Ontario, Canada). At this facility, the cell flasks were placed on a hollow perspex plate. The Perspex plate was heated to maintain 37˚C in the medium of the flasks by circulating water from a water bath (Grant Instruments, Cambridge, England). The irradiation field was 40 × 40 cm^2 and the source-to-flask distance was 84.5 cm giving a dose-rate of ~ 40 Gy/h. The survival curve for T-47D cells used in paper III and IV was drawn from data pooled from experiments done with both ^{60}Co -sources.

The low dose-rate of 0.3 Gy/h was obtained by shielding the source by a 11.4 cm thick block of Roos metal. The irradiation field was 40 × 40 cm^2 and the source-to-flask distance was 66 cm. The temperature of 37˚C was maintained as for the Mobaltron 80, by submerging the flasks in sealed plastic bags in the waterbath.

Dose measurements were done by Torbjørn Furre using thermoluminescence dosimetry.

2.3.2 220 kV X-rays

Cells were irradiated from above by a Pantak HF225 X-ray tube through a 0.5 mm copper filter. The flasks were placed in a steel chamber with inner diameter of 25.5 cm and lid thickness of 1 mm and the steel chamber was placed on a heat plate maintaining 37˚C in the medium of the flasks. The heat plate consisted of an aluminum plate with circulating water in tubes from a water bath (Ecoline, Lauda, Germany).

Dose measurements were was done by Eirik Malinin using alanine dosimetry.

3. Summary of the publications

3.1 Paper I

Title: Recovery of low-dose hyper-radiosensitivity after a small priming dose depends on priming dose-rate

Paper I investigates the effect of a small priming dose on the pronounced low-dose hyper-radiosensitivity of T-47D cells. Since IRR sets in as a result of enough DNA damage to exceed the threshold for ATM activation of the early G_2-phase checkpoint, 0.3 Gy was chosen as priming dose from the survival curve of the T-47D cells where the transition from HRS to IRR response occurs at approximately this dose. Consistent with this assumption, HRS was removed by the priming dose at 6 h and returned within 24 h.

Giving the same dose at a low dose-rate of 0.3 Gy/h should reduce the damage density and provide enough time for the cells to repair damage concurrently and it could therefore be expected that priming at the low dose-rate would not influence HRS. Surprisingly, instead we found that not only did the LDR priming dose remove HRS, but HRS did not return for the 14 weeks examined in this paper.

Flow cytometry was used to obtain DNA histograms to illustrate the effect of the priming dose on cell cycle distribution and no effect was found by the priming dose at either dose-rate. However, the trypsinization and plating caused an increase in the number of cells in G_1-phase and a corresponding decrease in the number of cells in G_1- or S-phase. Cells selected and sorted out while in G_1-phase were then irradiated and it was found that these cells did not exhibit HRS and were more sensitive to larger doses than asynchronously growing cells. In the experiments with priming doses, the cells were plated for colony formation a certain time after the priming dose and the challenge doses were given 6 h or 24 h after the trypsinization and plating. A trend was observed in which the cells given the challenge dose 6 h after trypsinization were more sensitive to larger doses than those irradiated after 24 h. It was speculated that this could be due to the sensitivity of the larger fraction of cells in G_1-phase observed in the DNA histograms.

3.2 Paper II

Title: The elimination of low-dose hyper-radiosensitivity by transfer of irradiated cell conditioned medium depends on dose-rate

In paper II it was reported that trypsinization reduced HRS in T-47D cells for at least 8 h and for this reason a time of 16-20 h after plating was chosen for challenge irradiation. At the time this paper was written, we had cultured two different LDR primed T-47D cell cultures for 9 and 14 months, respectively, neither of which showed any signs of HRS.

The different impact of the HDR and LDR priming was further investigated in experiments where medium was harvested from the irradiated (0.3 Gy) cells and transferred to unirradiated T-47D cells. The recipient cells were exposed to the donor medium for 24 h before being plated for colony formation in fresh medium and subsequently challenge irradiated 16-20 h later. The medium from HDR irradiated cells had no impact on HRS in the recipient cells while medium from LDR primed cells not only removed HRS but induced a higher surviving fraction in the low dose irradiated cells than in the controls. Even when medium was harvested from the cell cultures that had been LDR primed 9 or 14 months earlier it still removed HRS in recipient cells.

When the donor medium was diluted with fresh medium, the effect was reduced but even a 1% dilution still had a slight effect and saturation appeared to occur between 5% and 20%.

The recipient cells regained HRS within 2 weeks. Thus, it was only the LDR primed cells in which the absence of HRS was persistent. Apparently, LDR priming but not HDR priming induces the T-47D cells to produce or release a factor into the medium. This factor in turn removes HRS in cells exposed to it possibly by manipulating the early G_2-arrest either so it is activated by the smaller amount of ATM or by dissociating the activation of the G_2-arrest from the ATM dependence.

When the plating efficiency of cells receiving medium transfer from primed cells was measured, there was a significant increase in survival both in cells that received medium from LDR and HDR primed cells compared to controls that received fresh medium. Thus, there was no cytotoxic bystander effect in T-47D cells and the observed protective effect of the medium transfer was dose-rate independent.

3.3 Paper III

Title: Mechanisms of the elimination of low dose hyper-radiosensitivity in T-47D cells by low dose-rate priming

T-47D-P cells, a LDR primed HRS-deficient T-47D cell culture, which by this time had been cultured for more than two years after priming, were observed to show a trend of decreasing mitotic ratio with increasing radiation dose measured using the mitotic marker histone H3 phosphorylation indicating induction of the early G_2-checkpoint in response to doses in the HRS-range for unprimed T-47D cells. In contrast, the mitotic ratio of the unprimed HRS-positive T-47D cells was unaltered compared to unirradiated cells at doses between 0.1 and 0.3 Gy, i.e. these cells failed to show a low-dose, early-G_2 phase check point. Thus, the elimination of HRS in T-47D-P cells can be correlated to induction of the early G_2-checkpoint in response to doses normally in the HRS range. The persistent alteration of the T-47D-P cells was also expressed in an increased resistance towards cisplatin.

The effect of the LDR priming was not impeded by the presence of protein synthesis inhibitor 4,6-benzylidene-D-glucose (BG) during LDR priming and the following 6 h.

The ability of medium harvested from LDR primed cells to remove HRS in recipient cells was found to depend on the presence of serum during the LDR priming. However, to our surprise, the cells did not need to be present during the LDR priming as long as the medium had previously been exposed to cells. Thus, LDR priming of cell conditioned medium seemed to induce the factor which removes HRS in cells exposed to it while unprimed or HDR primed cell conditioned medium as well as LDR primed fresh medium had no effect on HRS in recipient cells. For the LDR primed cell conditioned medium to affect HRS of the recipient cells, serum needed to be present during cell conditioning.

A model was developed on the basis of the data in papers I, II, and III in attempt to explain the mechanisms induced by the LDR priming in cooperation with a serum constituent.

3.4 Paper IV

Title: Low dose hyper-radiosensitivity in T-47D cells is removed by chronic moderate hypoxia but returns after reoxygenation

Paper IV presents data on how chronic moderate hypoxia affects the radiosensitivity of T-47D cells, in particular the response to doses in the HRS-range. To irradiate the cells while still hypoxic or after 5 min of reoxygenation, a 220 kVp x-ray machine was used instead of the ^{60}Co source used otherwise. When control experiments were done with T-47D cells grown in ambient air, it turned out that the higher LET of the 220 kVp x-rays as compared to ^{60}Co γ-rays resulted in a shallower HRS-"dip", i.e. an earlier transition from HRS to IRR response.

X-ray irradiation of T-47D cells that had grown for 3-6 weeks in a hypoxia box operated with 4% O_2 in the gas phase disclosed a significantly altered response to low doses. The hypoxic cells were HRS-deficient and the surviving fractions in the low dose range appeared to lie above the LQ-curve (not significant). In order to exclude that the radioresistance was due to the low amount of oxygen present during irradiation the experiment was repeated with a flushing of the cell flasks 5 min before irradiation with ambient air with 5% CO_2. This resulted in slightly lower surviving fractions in the low dose range, but they still were above the LQ-curve.

When cells that had been cultured in the hypoxia box operated at 4% O_2 for 4 weeks were reoxygenated in ambient air for 48 h, the survival was best described by the LQ-curve, but after 2 weeks in ambient air, HRS was reestablished. The time scale for HRS to be reestablished was concordant with the findings in paper II for cells receiving medium transfer from LDR primed cells but different from the cells receiving the direct HDR (HRS back within 24 h) or LDR irradiation (persistent elimination of HRS). When medium was harvested from the cells that had been hypoxic for 4 weeks, the recipient cells also lost HRS. Thus, it is tempting to speculate in a connection between the effect of LDR priming on cell conditioned medium (paper II) and that of chronic moderate hypoxia in activating a factor in the medium.

4. Discussion

There appear to be general consensus on the model associating HRS/IRR to the early G_2-phase checkpoint as proposed by Marples et al. (2003) and it is corroborated by the findings in this thesis. In paper I, the effects of a HDR priming dose of 0.3 Gy on HRS in T-47D cells was investigated. The dose size of 0.3 Gy was chosen from the survival curve of the T-47D cells since the transition from HRS to IRR response seems to occur at approximately this dose. Xu et al. (*20*) found that HeLa cells arrested in the early G_2-phase checkpoint were released 12 h after irradiation at which time they began to reenter mitosis. Consistently, we found no HRS in the HDR-primed cells in response to a challenge exposure given 6 h after the priming dose but a full HRS response to a challenge dose given 24 h after the priming dose. A full time scale for the effect of the HDR priming was examined in a master thesis by Christiansen (*81*) in which the duration of the effect was found to be approximately 12 h. Thus, the observed effects of the 0.3 Gy HDR priming dose are consistent with an induction of the early G_2-phase checkpoint.

Prolonging the exposure time by giving the priming dose of 0.3 Gy at 0.3 Gy/h, one would expect that the low density of ionizing events would not be able to raise the levels of phosphorylated ATM sufficiently to activate the early G_2-phase checkpoint and that the LDR primed cells would retain their HRS response. This, however, was not the case. Instead HRS seemed to be persistently lost (we have so far tested for almost three years) after the LDR priming dose. Experiments measuring the mitotic ratio using the mitotic marker histone H3 phosphorylation (paper III) of the unprimed and the LDR primed (more than two years previously) T-47D cells indicated an early G_2-arrest for the LDR-primed cells in response to all doses between 0.1 and 1 Gy while the unprimed T-47D cells unaffectedly continued to enter mitosis after challenge doses between 0.1 and 0.3 Gy. The two responses corresponded to those observed by Krueger et al. (*11*) for HRS-deficient and HRS-proficient cell lines, respectively. This confirmed our notion that the early G_2-checkpoint somehow is activated even by a dose of 0.1 Gy in the LDR primed cells. We were not able to establish the exact threshold dose because the shutter mechanism of the available ^{60}Co source was too slow to give doses below 0.1 Gy without changing the dose-rate.

In paper II, experiments with medium transferred from cells primed with 0.3 Gy to unirradiated cells confirmed our suspicions that the mechanisms induced by the LDR priming dose were of a different nature than those induced by the HDR priming. Cells receiving medium from HDR irradiated cells retained their HRS response while cells receiving medium form LDR primed cells lost HRS transiently. The data indicate that LDR primed cells release a factor into the medium which eliminates HRS in recipient cells. The recipient cells regained HRS within two weeks in contrast to the cells given the LDR priming, which not only remained without HRS but persistently released the putative factor into the medium.

Dilution of the medium from LDR primed cells led to a reduction of the HRS-eliminating effect in the recipient cells (paper II). A full effect was obtained at 20% dilution suggesting a mechanism of saturation concordant with that of growth factors. This inspired an experiment in which the cells were deprived of serum in the medium during the LDR priming. The medium was then harvested, filtered, supplied with serum, and transferred to unirradiated cells. The recipient cells remained HRS-proficient (paper III) indicating that a constituent of the serum which the T-47D cells can not produce themselves was requisite for the effect of the LDR priming.

Experiments with irradiation of the medium alone lead to a surprising result (paper III). Transfer of irradiated fresh medium did not influence HRS in the recipient cells, but medium that had previously been harvested from unirradiated cells and subsequently, after filtration, exposed to 0.3 Gy at LDR (but not HDR) was able to eliminate HRS in recipient cells. However, if serum was not present during the cell conditioning, the recipient cells retained HRS even if serum was added before LDR irradiation of the medium.

Exposure to chronic moderate hypoxia (4% O_2) also eliminated HRS in T-47D cells and induced a factor which, by medium transfer, eliminated HRS in recipient cells (paper IV). In the cells that had been grown for 4 weeks at 4% O_2 in the gas phase, HRS returned within 2 weeks of reoxygenation. Thus, chronic hypoxia appeared to induce effects similar to those induced in cells receiving medium transferred from LDR primed cells or LDR primed CCM.

The experiments with cisplatin and bleomycin described in paper III were also performed with cells that were incubated with 3% O_2 in the gas-phase in the 24 h between plating for colony formation (200 cells/flask) and during the 24 h exposure to the cytostatic (unpublished data, figure 2). Exposure to 3% O_2 in the gas-phase induced a significant resistance towards bleomycin (figure 2, panel A) both in wild-type T-47D cells and in T-

47D cells in which a LDR (0.3 Gy/h) priming dose of 0.3 Gy given more than two years earlier had eliminated HRS (denoted T-47D-P cells) ($P<0.01$ for all dose points in both cell cultures (null hypothesis was coincidence of data points from three individual experiments with either hypoxic cells or cells grown in ambient air)).

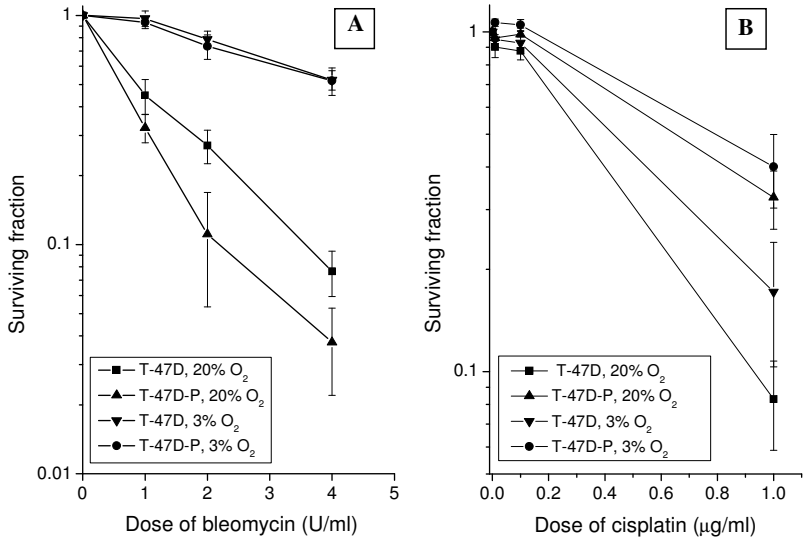

Figure 2: The sensitivity towards bleomycin (panel A) and cisplatin (panel B) was measured on T-47D cells and T-47D-P cells (i.e. T-47D cells LDR primed two years previously) in either 20 or 3 % O_2

Exposure to 3% O_2 also appeared to protect both the T-47D cells and the T-47D-P cells against 1 µg/ml cisplatin though the increases in surviving fraction were not significant.

As described in paper III, the LDR priming also protected the cells against exposure to 1 µg/ml cisplatin and the T-47D-P cells showed a significantly higher clonogenic survival than the unprimed T-47D cells (figure 2, panel B) whereas the cells exposed to bleomycin showed a slightly (non-significant) lower survival in T-47D-P compared to T-47D cells (figure 2, panel A). This difference in responses are probably related to the observations that cisplatin pretreatment (1µM) has been shown to eliminate HRS in CHO cells (*9*) whereas bleomycin pretreatment did not (*82*). Interestingly, the protective effect in response to cisplatin treatment of moderate hypoxia and a previous LDR priming was

additive. Calculating the surviving fraction from un-treated T-47D cells by adding the effect of each pre-treatment (LDR priming in T-47D-P cells and 3% O_2) gave 0.41 ± 0.10 compared to the measured surviving fraction of T-47D-P cells in 3% O_2 of 0.40 ± 0.10.

In view of the concordance of the time scale for regaining HRS after exposure to medium from LDR primed cells or to chronic moderate hypoxia, it is tempting to hypothesize that it is the same factor that is present in the medium after the two different treatments. The additive protective effect of moderate hypoxia and LDR priming towards cisplatin could be taken to indicate otherwise, but the exposure to 3% O_2 in the gas phase was for only 24 h in flasks containing only 200 cells. Since T-47D cells grown at the high density normal for stock cultures in 20% O_2 may experience oxygen concentration close to 3% between reseedings (*65*) and still retain HRS, the mechanisms that eliminate HRS are most likely induced at much lower oxygen concentrations than was obtained by an atmospheric concentration of 3% O_2 with such low cell density (200 cells/flask). The mechanisms related to the observed protection by moderate hypoxia against the cytotoxic effects of cisplatin are therefore more likely to be similar to those protecting the cells against exposure to bleomycin and different from those involved in HRS elimination.

It has been suggested that mitochondria may play a crucial role in low dose/low dose-rate induced adaptive responses. Pandey et al. (*83*) irradiated density-inhibited normal fibroblasts with ^{137}Cs γ-rays using either 4 Gy at 198 Gy/h or 0.1 Gy at 0.2 cGy/h. Protein (matrix precursor protein frataxin) import was decreased in mitochondria isolated from high dose/high dose-rate irradiated cells whereas it was enhanced in mitochondria isolated from low dose/low dose-rate irradiated cells. Also the membrane potential $\Delta\Psi$ was decreased in response to the high dose/high dose-rate and increased in response to the low dose/low dose-rate. However, it is not evident how mitochondria can be correlated to the data concerning LDR priming of cell conditioned medium without cells present or the requirement for serum present during LDR priming.

In paper III, a model was presented which is illustrated in figure 3 (redrawn from paper III). Experiments with serum-deprived medium demonstrated the involvement of a serum constituent, which in the model is called **C**. Because of the short time of serum deprivation required for inhibition of the effect of LDR priming, it is not likely that **C** crosses the cell membrane to exert its effect. An attachment to a receptor on cell membrane

can on the other hand be quickly reversed. It is even possible that continuous fresh recruitment of **C** is required.

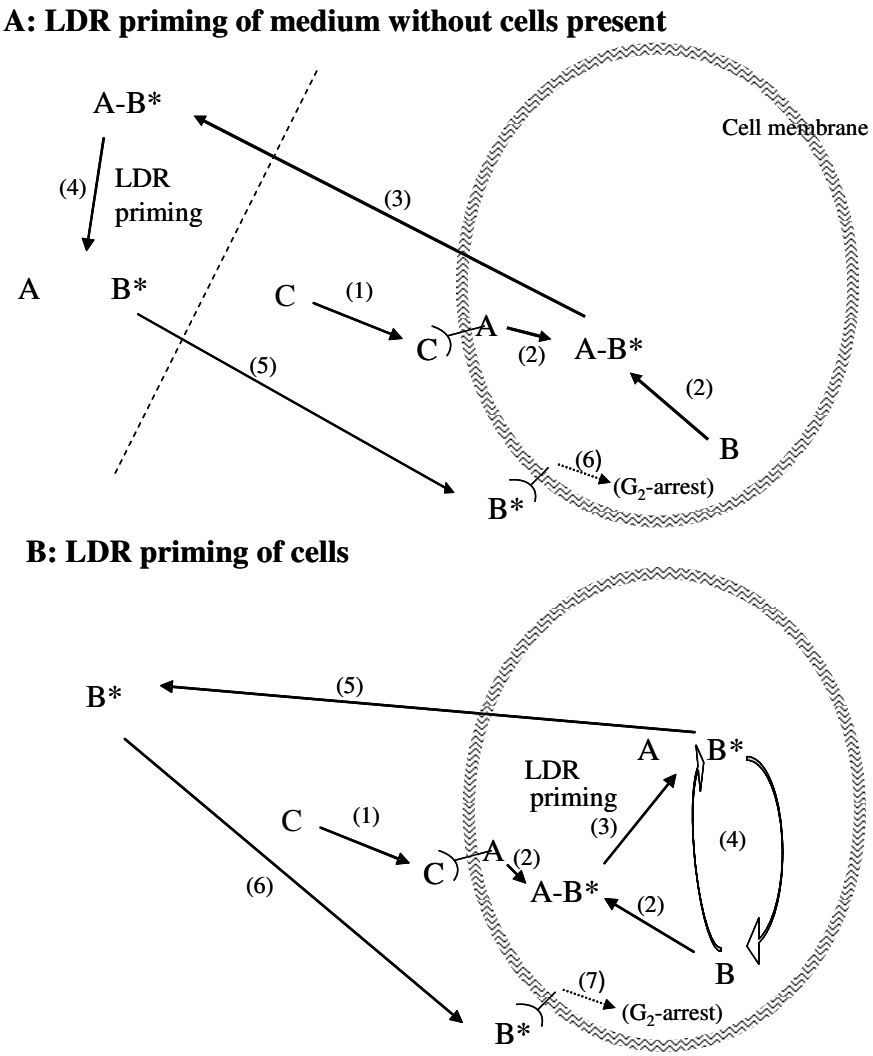

Figure 3: Illustration of the model presented in paper III: *Panel A: LDR priming without cells present.* A serum constituent (C) attaches to receptor on the plasma membrane (1) resulting in release of a substance A which interact intracellularly with B, a substance produced in the cell, forming a complex in which B is modified to B* (2). The A-B*

complex is then secreted into the medium (3). LDR irradiation of the medium releases B* from the complex (4) and if the medium is transferred to cells, B^* will bind to receptors on the recipient cells (5) inducing the non-HRS response (6).. The process (4) can take place without cells present indicated by the dashed line.
Panel B: LDR priming on cells. A serum constituent (C) attaches to receptor on the plasma membrane (1) resulting in release of a substance A which interact intracellularly with B, a substance produced in the cell, forming a complex in which B is modified to B* (2). LDR irradiation of the cell releases B* from the complex (3) before secretion. The presence of B* intracellularly induce an autoreaction in which all Bs are modified to B* without needing to be linked to A (4). The B*s are secreted (5) and bind to receptors on available cells (6) inducing the non-HRS response (7).

The next factor introduced in the model is an endogenously supplied substance **A** which in an inactive state is attached to the cell membrane from where it is released by **C**'s reaction with the receptor. When released, it can form a complex with a second endogenously supplied substance **B**.

In the process of forming the complex, it is assumed that **B** is modified to **B***. One could imagine the modification to consist of phosphorylation or methylation, but since the modification appears to be irreversible, a conformational change is perhaps more plausible. In unirradiated cells, the **A**-**B*** is inactive and freely secreted into the medium. The action of LDR irradiation is assumed to target the **A-B*** bond dissociating **B*** from **A** and in the free state **B*** will attach to cell membrane receptors and open for an increased sensitivity for early G_2-arrest. When unirradiated cells received medium transfer form LDR primed cells, the effect of the transferred medium depended on the dilution with a full effect at 20% dilution (paper II). The saturation effect is consistent with receptor mediated effects.

The action of LDR irradiation on the **A-B*** complex can take place both in the medium and in the cell. However, only when it takes place inside the cell, is the elimination of HRS permanent. This could be explained if the presence of **B*** intracellularly induce the modification of **B** to **B***, before **B** forms a complex with **A**, in a continuous chain of auto-reactions. Another possibility could be that **B*** acts on the **A-B*** complex in the same way as LDR irradiation by a mechanism that requires the presence of certain enzymes and therefore does not take place in the medium. However, protein synthesis inhibition did not affect the elimination of HRS by LDR irradiation (paper III) which makes **B** a more likely target for the actions of **B***.

When **B*** is attached to a receptor on the cell membrane, the **B***-receptor complex is thought to activate a sensor that responds to signals induced by HDR challenge doses as

low as and perhaps even lower than 0.1 Gy by induction of the early G_2-checkpoint through a pathway which either evades or changes the threshold dose for the ATM-control. We have initiated microarray analysis of mRNA regulation, which hopefully will elucidate the pathways that are specifically activated in the LDR primed cells.

The LDR of 0.3 Gy/h was obtained by shielding with 10 cm Roos metal. However, Monte Carlo analysis showed no significant LET difference for the LDR and HDR arrays and only small deviations were found in the secondary electron spectra which therefore can not account for the different effects induced by the two dose-rates.

In paper II, a theory proposed by Vilenchik and Knudson (*84-86*) was discussed in which it was suggested that at that particular dose-rate that induces DSBs at a rate in the order of the induction of endogenous DSBs, the damage was more easily recognized by the cells which resulted in optimal error-free DNA repair; this explanation was used to account for the inverse dose-rate effect. However, the results in papers III and IV suggest that the action of the LDR irradiation can also take place outside the cell and point away from DNA damage as the inducer of the observed effects.

In a cell with diameter of 15 μm, a dose of 0.3 Gy at 0.3 Gy/h deposits approximately 3.3 x 10^6 eV per hour. The energy is not deposited uniformly, but is located along the tracks of secondary electrons. In the case of X- or γ-rays, 95% of the energy deposition events are so-called "spurs", which have a diameter of about 4 nm, contains up to 100 eV, and involves, on average, three ion pairs ($OH^{\cdot}$ and e_{aq}) (*42*). Thus, the 0.3 Gy results in ~33000 spurs per cell or ~99000 $OH^{\cdot}$ radicals per cell.

Since background radiation does not eliminate HRS, a certain amount of ionizations are required, and since HDR irradiation also does not eliminate HRS permanently, it seems that time is a necessary factor. One can only speculate on the actions of LDR irradiation on the putative **A- B*** complex but since time is an important factor, a slow reaction with a transition state, which depends on a second ionization or radical interaction for the reaction to proceed rather than to be reversed, could be imagined.

In paper IV, it was demonstrated that cells, which were grown for 3-6 weeks in a hypoxia box with 4% O_2 in the gas phase at a density that resulted in a much lower O_2 concentration at the cell surface, lost HRS. Medium from the hypoxic cells removed HRS in recipient cells and HRS returned within 2 weeks after reoxygenation, a time scale similar to that of unirradiated cells receiving medium transferred from LDR primed cells.

Thus, there were indications that the same factor was induced in the medium of the hypoxic cells as after LDR irradiation. A suggestion of a mechanism could be that the hypoxic T-47D cells that are adapted to atmospheric 4% O_2 continuously release or secrete reactive species with the same effect on the putative **A-B*** complex as LDR irradiation. When the cells are reoxygenated, the flow of reactive species stops and at that time, the cells correlate to cells that has been transiently exposed to medium from LDR primed cells containing the putative **B*** factor.

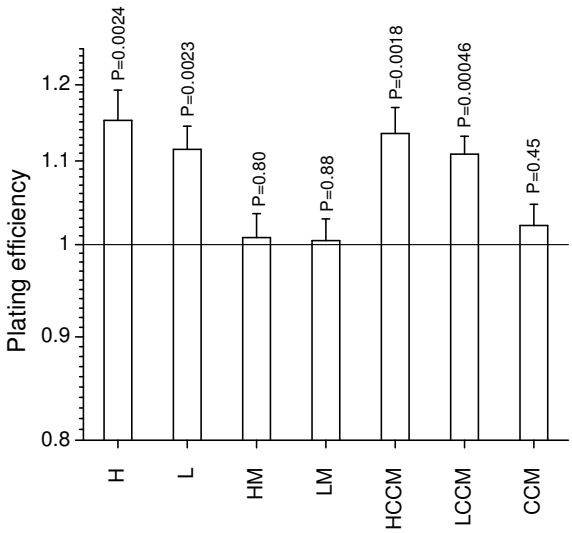

Figure 4: Plating efficiencies of unirradiated cells that had received either 1: Medium harvested from irradiated cells (H: at HDR, L: at LDR) or 2: Fresh medium irradiated without cells present (HM: at HDR, LM: at LDR) or 3: Medium harvested from unirradiated cells; the medium was either unirradiated (CCM) or irradiated without cells present (HCCM: at HDR, LCCM: at LDR). The plating efficiency was calculated as surviving fraction relative to that of cells receiving fresh unirradiated medium. Error bars indicate standard error. P-values are from a two-tailed student's t-test.

In paper II, figure 8 shows the plating efficiencies of the control flasks of the data in figure 4 showing the effect of medium transfer from either LDR or HDR primed cells on the radiosensitivity of the recipient cells. Similar measurements were done of the plating efficiencies of the control flasks in figure 5 in paper III which had received transfer of medium irradiated without cells present. The plating efficiencies, measured relative to that

of cells receiving fresh, unirradiated medium, are shown in the present figure 4, which include data from both papers II and III as well as unpublished data. The cells pre-treated with conditioned medium that had been irradiated without cells present (HCCM and LCCM) show enhanced plating efficiency relative to that of cells receiving fresh unirradiated medium. The increase is similar to that induced by medium irradiated while on cells (H and L). This effect did not depend on dose-rate. Cell conditioning by itself (on unirradiated cells) (CCM) or irradiation of fresh medium (LM and HM) did not have any significant impact on the ability of the medium to influence colony formation.

In cells exposed to the HDR irradiated medium, an increase in plating efficiency was observed but there was no effect on the response of the exposed cells to small doses. When the medium was irradiated at LDR, both an increase in plating efficiency in cells exposed to the irradiated medium was observed as well as an early-G_2 phase arrest in response to doses normally below the IRR threshold dose. Thus, the effects on plating efficiency and HRS/IRR appear to be through independent mechanisms.

The LDR primed cells as well as the cells receiving medium transfer from LDR primed cells, transfer of LDR irradiated cell conditioned medium or exposed to chronic moderate hypoxia all responded to the lowest doses with surviving fractions above 1. The increased plating efficiencies of the controls shown in figure 4 can not explain this phenomenon. On the contrary, if the surviving fractions are adjusted accordingly, the distance from the data points to the LQ-curve is increased. The surviving fractions above 1 imply that the plating efficiencies of the irradiated flasks were higher than in the unirradiated but also pre-treated controls. A reason for this could be either that the irradiated cells form more than one colony or that the colonies of the control flasks merge or loosen. This explanation was examined by time-lapse photography of the LDR primed as well as unprimed cells with or without a challenge dose of 0.3 Gy by Fenne in her master thesis (*87*). The collected data did not confirm that the observed surviving fractions above 1 could be attributed to colony splitting, migration or merging. On the contrary, though these phenomena did appear they seemed to mask the effect instead. A second hypothesis, in which the control population is assumed to contain a subpopulation in G_0-phase that can be induced to re-enter cell cycle by a small challenge dose, is currently being tested. However, in the development of the model illustrated in figure 3 a more likely explanation evolved. If B* leads to increased ability to activate the early G_2-checkpoint even by the smallest challenge doses used, the G_2-delay may improve the

ability of T-47D cells to repair damage that has accumulated under normal growth and thereby restore clonogenic ability of a few cells that would otherwise have lost it.

The time-lapse films by Fenne never showed any signs of apoptosis in T-47D cells HDR irradiated (X-rays) with 0.3 Gy. Tan et al. (*88*) also found that T-47D cells failed to demonstrate typical DNA fragmentation associated with apoptosis in response to exposure to cytotoxic Epipremnum pinnatum (L.) Engl. Hexane extracts and the expression levels of caspase-3 mRNA were not significantly regulated. However, they found strong indications of Type II non-apoptotic programmed cell death (autophagy) which also could be consistent with our film observations. Thus, it is possible that not only apoptosis could be associated with HRS as suggested by Enns et al. and Krueger et al. (*29, 30*) but that Type II non-apoptotic programmed cell death could be an alternative in cell lines with defects in the apoptotic induction pathway. This is an area for future investigations.

5. Conclusions

The results of the four papers included in this thesis can be summarized in the following main conclusions:

- Exposure of T-47D cells to LDR ^{60}Co γ-irradiation (0.3 Gy/h) during 1 hour changes the phenotype making the early G_2-phase checkpoint accessible for activation by doses in the HRS-range of wild type T-47D cells. Inhibition of protein synthesis does not impede the actions of LDR priming
- The LDR induced phenotype secretes a factor inducing transitory elimination of HRS in cells exposed to it. The effect depends on the presence of serum in the medium during LDR irradiation. Dilution of the transferred medium showed a saturation effect
- The factor is also secreted in the unirradiated progeny of LDR primed cells
- The factor secreted from the LDR primed cells can also be induced by LDR irradiation in medium without cells present. This requires that the medium has preciously been in cell contact and that serum was present during cell contact
- None of the observed effects of a LDR priming irradiation was observed when the same priming dose was given at HDR
- Chronic moderate hypoxia eliminated HRS in T-47D cells, but HRS returned after reoxygenated within the same time frame as the return of HRS in cells exposed to medium from LDR primed cells, and medium from the hypoxic cells appear to contain the same factor that removes HRS as medium from LDR primed cells.

6. Future directions

As mentioned in the discussion, we have initiated microarray analysis of mRNA from LDR primed T-47D cell compared to unirradiated cells as well as HDR irradiated cells as well as investigations of the mechanisms causing that the plating efficiencies of irradiated cells can exceed those of the controls.

In this thesis, the experiments were designed to elucidate the mechanisms behind the surprising observation of the persistent elimination of the HRS response in T-47D cells. In addition to pursuing the questions that have emerged in the discussion of this research, starting with identification of the serum constituent (denoted **C** in the model), which should be possible through experiments in which certain fractions of the serum are removed, it is evident that the investigations should be extended to include more cell lines.

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