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Effects of low dose-rate radiation on the growth of tumour cells

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Title (English)

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Title (Swedish)

Abstract

In targeted radiotherapy, dose-rates are lower than in external radiotherapy. Therefore, it is important to study the effects of low dose-rate radiation on tumour cells. The theory is that cells showing hypersensitivity to low doses also could be unusually sensitive to low dose-rates. Tumour cell lines were irradiated with low dose-rates and acute doses and deviation from normal growth was studied. The colorectal adenocarcinoma cell line HT29, reported to be hypersensitive, showed an indication of inverse dose-rate effect to irradiation with 0.084 Gy/h. Reported to be hyper sensitive is also glioblastoma astrocytoma cell line U118 MG, but it showed no inverse dose-rate effect in this study. At last, the glioblastoma astrocytoma cell line U373 MG showed an inverse dose-rate effect to a dose-rate of 0.12 Gy/h compared to acute doses, even though it was reported not to be hypersensitive.

Keywords

Low dose-rate, hyper radiosensitivity, inverse dose-rate effect, radionuclide therapy, HT29, U118, U373

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Effects of low dose-rate radiation on the growth of tumour cells

Emma Håkansson

Sammanfattning

Vid målsökande radioterapi mot cancer används ett radioaktivt ämne kopplat till något som kan binda till tumörcellen. Doshastigheten kan i detta fall bli låg, och det är därför viktigt att undersöka vilka effekter låga doshastigheter kan ge. Tumörceller har i vissa studier visat sig vara känsligare för riktigt låga doser strålning än för högre, något som kallas hyperkänslighet. Hypotesen som testas här, är om låg doshastighet kan ge hyperkänslighet hos tumörceller på liknande sätt som låga doser givna med hög doshastighet. Att låg doshastighet ger större effekt än hög doshastighet kallas omvänd doshastighetseffekt. Metoden som används är att jämföra avvikelse från normal tillväxt efter bestrålning med låg eller hög doshastighet.

En tumörcellinje från tjocktarmscancer, HT29, har tidigare rapporterats vara hyperkänslig för låga doser givna med hög doshastighet, även kallade akuta låga doser. I denna studie visar de antydan till omvänd effekt vid bestrålning med en doshastighet på 0,084 Gy/h jämfört med akut dos. En tumörcellinje från gliaceller i hjärnan, gliom U118, visade ingen omvänd effekt trots att de har visats vara hyperkänsliga för låga akuta doser. Gliomcellinje U373 har rapporterats inte visa någon hyperkänslighet, men visade i denna studie en omvänd effekt vid bestrålning med 0,12 Gy/h.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet Februari 2002

Contents

Introduction

Today, cancer therapy saves many lives. Still, cancer is not by far controlled, and it is one of our most feared diseases. As an alternative to conventional radiation therapy, targeted treatment is at present a challenge for many research groups. In targeted treatment a radionuclide or a toxic agent is coupled to a targeting agent which will bring the substance to the cancer cell. With this approach, the radionuclide will be brought in vicinity of the malignant cells and also target spread, undiscovered metastases.

When targeted treatment is used, doses and dose-rates are usually difficult to obtain. The dose-rate can be much lower than in external radiation therapy, and therefore it is important to investigate low dose-rate effects carefully.

Radiation Biology

In short, ionising radiation causes formation of free radicals and breakage of chemical bonds. As a result of these reactions, damage is done to DNA and other biologically important molecules. The effects on DNA can, among others, be single and double strand breaks or cross-linkage within a strand and between strands, and it is shown that DNA damage is the most serious effect of radiation and a critical event in cell killing (1). Among DNA damages, double strand breaks are believed to be closest related to cell death. Radiation of cells can also result in chromosomal aberrations. The injury can be lethal, depending on the type of alteration of the chromosomes.

A large part of the DNA damage is repaired by the cell. The repair system can with varying result restore the DNA molecule, sometimes with the loss of genomic information (1). As discussed later, DNA repair is very important for cell survival.

Low Doses

The dose – response relationship is conventionally seen as rather simple; a low dose giving a small effect on living cells. Studies of clonogenic survival show a plateau for less sensitive cells in the low dose region in a plot of survival against dose. However, when looking closer at the results for very low doses, there have been indications that low doses can be more harmful to tumour cells than higher doses (2). This phenomenon is called hyper radiosensitivity (HRS).

Human glioma cells T98G has been shown to be hyper radiosensitive (2, 3). They show a very drastic reduction in survival in the dose region of less than 0.3 Gy (*Figure 1*). Above this point, the cells regain radioresistance, getting increased radioresistance (IRR). After about 1 Gy, the survival curve has a slightly accelerating decrease, as expected.

The phenomena of HRS and IRR have been shown by several groups and for several cell types. The explanation most believed in the literature is that the repair machinery is somehow triggered above a certain dose. Under this triggering point, the repair is not optimal and the cell is therefore hypersensitive. When the dose is increased, repair is turned on, and the survival increases at first before the rate of repair becomes limiting for cell survival (2).

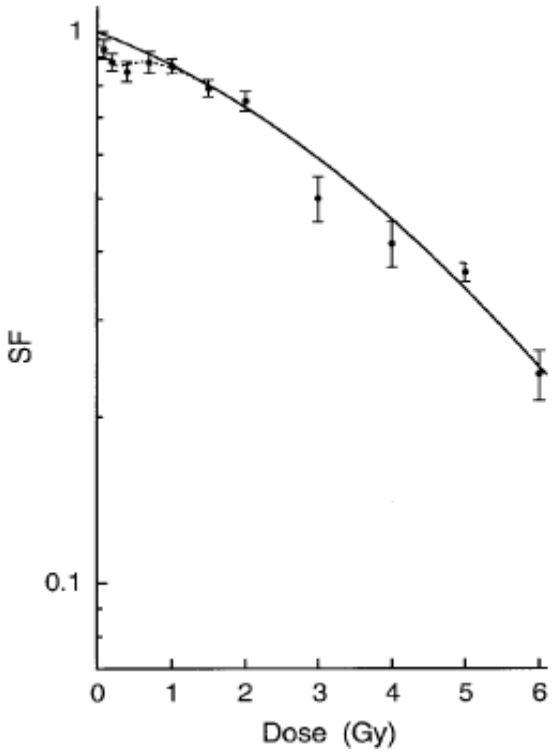

Figure 1: Survival plot of T98G human glioma cell line irradiated with X-rays, exemplifying the shape of a curve with hyper radiosensitivity. (Reprinted from Short et al. (3))

Other hypotheses are those of apoptosis or cell cycle delay. As a response to low doses, apoptosis would be turned on to protect the population from genomic errors, on the expense of single cells. If apoptosis were to be used in a great extent to remove the errors caused by high doses, the cell loss would be too large. Apoptosis would therefore be down regulated to preserve the cell population (2). However, studies have revealed a lack of evidence to support that apoptosis should be the reason for HRS or IRR (2). The apoptosis marker of cleavage of the DNA-PK complex has been used to try to establish the apoptosis theory. On the contrary, this study showed an unchanged level of DNA-PK when comparing cells irradiated with 0.2 or 0.5 Gy. It has not been proved that cell cycle delay can explain the effect either. The cell cycle is delayed in many cell lines when the cells are exposed to radiation, to allow repair to take place. Higher dose gives longer delay and thus better repair, suggested to explain low dose hypersensitivity. No reliable parallel can be drawn, though, to HRS/IRR when looking at the results from many cell lines (2).

Low Dose-rates

The low doses above are all given at high dose-rates, i.e. as acute doses. The effects of low dose-rate radiation are often less severe than high dose-rate radiation on cell level. This phenomenon is referred to as the direct dose-rate effect. Nevertheless, there are also indications of an inverse effect, where low dose-rates can give a worse outcome than higher dose-rates (4).

Results in the Literature

In the literature, there are reports of both direct and inverse effects of dose-rates. Figure 2 shows a schematic overview of results published 1999 to 2001.

A human lymphoblastoid cell line WIL2NS showed a direct dose-rate response in a study made in Japan 1996 (5). Survival decreased with higher dose-rates in survival curves for 0.006, 0.17 and 30 Gy/h irradiated cells. Gamma rays were produced by a ^{60}Co source for irradiation with 30 Gy/h, and a specially designed ^{137}Cs source for the lower dose-rates. In another study, pig vascular smooth muscle cells were irradiated with 90 Gy/h by a ^{60}Co source or with 0.675 Gy/h by adding ^{45}Ca (β-emitter) in a chelated complex to the culture medium (6). Both in survival curves and growth delay studies a higher dose was required of low dose-rate radiation to give the same effects as from the high dose-rate radiation.

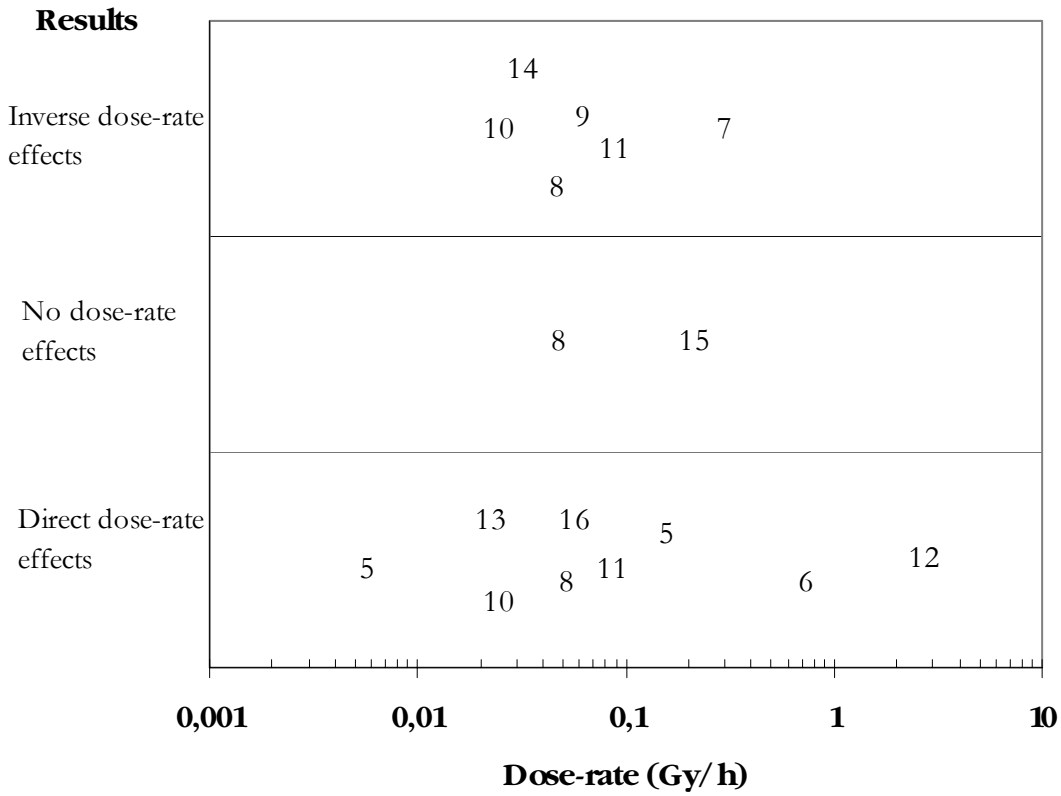

Figure 2: Overview of dose-rate related results published 1999-2001. Reported effects are symbolised on the y axis. Numbers are reference numbers.

An inverse dose-rate effect was shown for human cervical carcinoma cell line NHIK 3025, that has human papilloma virus integrated (7). Cells were irradiated with dose-rates of 0.33 or 0.86 Gy/h, which were achieved by a shielded ^{60}Co irradiator. The survival curve for cells irradiated with the lower dose-rate had a steeper slope than the curve for the higher dose-rate when the accumulated dose was about 7 Gy. Thereby, a greater reduction in cell survival was caused by a dose of more than 7 Gy if given at the lower dose-rate of 0.33 Gy/h. In another study, an inverse dose-rate effect was seen after irradiation with 14 MeV neutrons (8). Human melanoma cell line M4 Beu was irradiated with a dose-rate of 0.048 or 2.4 Gy/h. The survival curves showed an inverse dose-rate effect for doses below 0.3 Gy, where the lower dose-rate gave a significantly larger reduction of cell survival than the higher.

Explanations for dose-rate effects

The longer accumulation time for a certain dose when given at a low dose-rate is the probable cause of the direct dose-rate effect. Since the duration of the irradiation is longer, there is time for repair during the irradiation (6, 7).

There are different theories about the cause of the inverse dose-rate effect. Concerning the cervical carcinoma cells in Furres study (7), the given explanation for the inverse dose-rate effect is an accumulation of cells in the radiosensitive pre-mitotic stage. According to the authors, the population of cells was in a more sensitive stage after 20 h, resulting in an increasing slope in the survival curve. When survival was plotted against dose, there was an increase in the slope of the lower dose-rate curve at a lower dose compared to the higher dose-rate curve.

In a reanalysis of mutational studies (9), the inverse dose-rate effect seen for mutations was explained by induction of repair. By plotting mutations against dose-rate, a minimum was seen between 0.06 and 0.6 Gy/h, where repair was optimally induced. At lower dose-rates, the repair would thus not be fully activated, and the cells would be rather sensitive to radiation.

Project

In targeted radionuclide therapy, the dose-rates can be very low as discussed above. That, beside the desire to know more about the phenomenon of hyper radiosensitivity, is the motivation for this project.

The aim of the study is to investigate if hyper radiosensitivity can be seen when irradiating three different cell lines with low dose-rates, instead of low acute doses. The hypothesis is that the low dose-rate will not trigger optimal repair and therefore maintain the cells in a radiosensitive state. The three cell lines are chosen because of their earlier reported hypersensitivity or lack of hypersensitivity to low acute doses. Colorectal adenocarcinoma HT29 and glioblastoma astrocytoma U118 MG cell lines are reported to be hyper radiosensitive (2). On the contrary, glioblastoma astrocytoma U373 MG is reported not to show HRS (2).

The strategy is to study growth curves, since the problem of repopulation during long exposure times then will be taken into account. In addition, the study of growth curves will to a greater extent mimic the situation of tumour growth in the body, compared to clonogenic survival studies. Growth curves for cells irradiated with low and high dose-rate will be constructed, and the deviation from normal growth for the different dose-rates will be compared.

Materials and Methods

Radiation Chambers

Radiation chambers were manufactured at the local workshop. The walls consist of 15 mm transparent polycarbonate (Macrolon ®), and the exposing area was covered with a 0.5 mm thin transparent polycarbonate foil (*Figure 3*).

Beta emitter ^{32}P (Orthophosphate, Amersham Pharmacia Biotech, the United Kingdom) was dissolved in 400 ml water (2 ml, 740 MBq). The water was boiled shortly before adding the phosphor, in order to minimise air bubbles in the chambers. The three chambers were filled with phosphor solution in equal quantities, which was controlled by measurements with an ion chamber (see *Measurements and Calculations of Dose-rates*).

When the radioactivity in the chambers had declined below the desired dose-rate range, a refilling was made. Since the chambers were equally active, 10 ml liquid was removed from each chamber. These 30 ml were mixed with 2 ml ^{32}P (740 MBq) and 1 ml water, and 11 ml of the new ^{32}P-solution were added to all chambers.

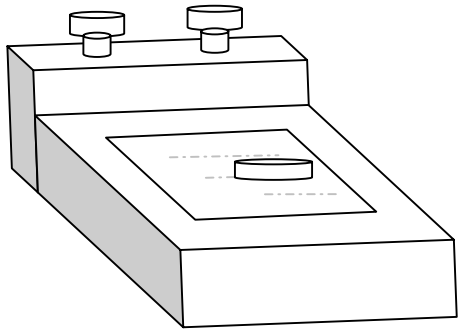

Figure 3: Drawing of radiation chamber with cell dish.

Measurements and Calculations of Dose-rates

Measurements of dose-rates were performed with a thin-walled parallel ion chamber in a cell dish placed on the radiation chambers, to mimic the dose-rate in the cell environment. All three chambers were measured two times, and the measuring-time was found not to affect the result. The chambers were confirmed to radiate with the same dose-rate (*Appendix, Table I*). Measurements were also made with a hand detector (RNI 10/R Intensimeter) and a calibration curve (*Appendix, fig I*) was constructed to facilitate the dose-rate determination.

From these measurements, dose-rates and doses for the different assays were calculated (A*ppendix, Table II-III*). Measurements were made with the hand detector to confirm the dose-rate calculations.

Cell Growth Assays

Cells used were colorectal adenocarcinoma HT29 (American Type Culture Collection, ATCC), glioblastoma astrocytoma U118 MG (ATCC) and glioblastoma astrocytoma U373 MG (Department of pathology, Uppsala University). They were grown in Ham's F-10 medium supplemented with 10% foetal calf serum, 2 mmol/l L-glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin, and 2.5 µg/ml amphotericin B, all components from Biological Industries (Beit Haemek, Israel).

Cell Counting

The medium was removed from the cell dishes and the cells were briefly washed with 0.5 ml trypsin-EDTA (0.25 % trypsin/0.02 % EDTA solution in PBS, Merck Eurolab, Sweden) and then incubated with 0.5 ml trypsin-EDTA (37 °C, 5 % CO_2) until the cells detached. Next, 1.5 ml medium was added to each dish and the cells resuspended to a single cell solution. For counting, 19.5 ml 1xPBS (pH7.4) was added to 0.5 ml cell suspension and a cell counter was used (Coulter Z2, 7-20 µm, Sweden). Two to three dishes were analysed for each time point.

Low Dose-rate Assay

Cells used were seeded at day -3. At day 0, a first counting was made. At the time of counting, four cell dishes were placed on each radiation chamber. The irradiated cells were grown on the chambers in a 5 % carbon dioxide incubator (JPM Med tek AB, Sweden) at 37 °C together with unirradiated control dishes. For counting, one dish per chamber was removed. Counting points were taken every to every other day, depending on cell response and growth.

If cell growth was limiting for the duration of experiment, and thereby also for the accumulation of dose, the cells were trypsinated and split 1:20. They were then allowed to continue to grow. When these cells were counted, correction was made for both dilution and trypsination delay.

High Dose-rate Assay

Cells seeded at day -2 were irradiated at day 0. All dishes, including controls, were transported to the irradiation source (Cs 137 Gammacell 40 Exactor, MDS Nordion, Sweden) in a plastic box with a carbon filter. Cells were exposed to 0, 2.49 or 6.21 Gy, with a dose-rate of 85 Gy/h. A zero point for unirradiated cells was counted immediately after irradiation. All remaining cells were thereafter grown in a 5 % carbon dioxide incubator at 37°C (Galaxy S LabRum Klimat AB, Sweden). Dishes from all three irradiation variants were continuously removed from the incubator and counted the following days depending on cell response and growth.

Results

Deviation from normal growth for each cell line is plotted in figure 4 to 6. In figure 4, results from low and high dose-rate irradiation of colorectal adenocarcinoma HT29 are shown. The acute dose of 6.21 Gy clearly gave the largest decrease in growth. Comparing the low dose-rate irradiation of approximately 0.084 Gy/h to the acute dose of 2.49 Gy, there is an indication of an inverse dose-rate effect, but there are not enough data to draw conclusions. All low dose-rate curves are marked with unfilled symbols at the points where the accumulated dose equals the acute doses of 2.49 and 6.21 Gy (*Figure 4-6*). They were calculated from fitting of growth curves to exponential trend lines (data not shown). The low dose-rate of 0.052 Gy/h gave almost no deviation from normal growth.

The results from assays with glioblastoma astrocytoma U118 MG are shown in figure 5. The acute dose of 6.21 Gy gave the most obvious deviation from normal growth, as for the HT29 cell line in figure 4. The deviation curves for the acute dose of 2.49 Gy and the low dose-rate of 0.037 Gy/h are not clearly separable, and the error bars of the both curves overlap. There is no indication of an inverse dose-rate, though. The U118 cells seem to be quite resistant in all assays performed, regardless of dose-rate.

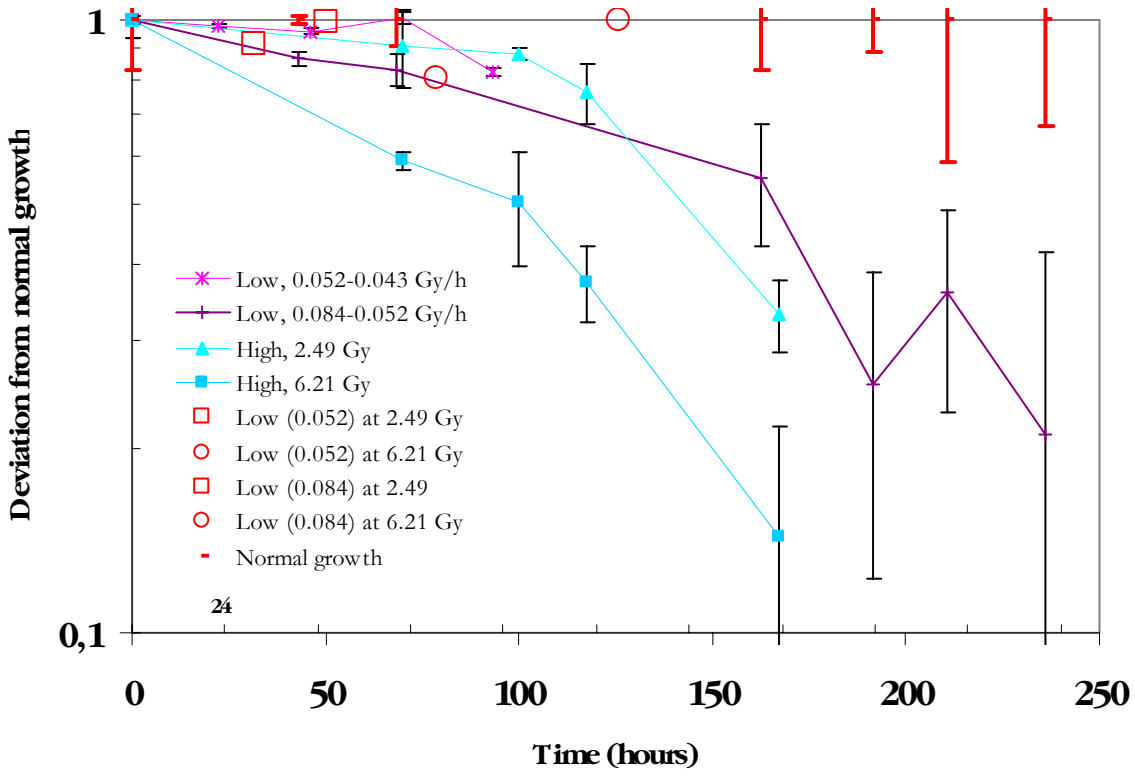

Figure 4: Deviation from normal growth for colorectal adenocarcinoma cell line HT 29 after irradiation with high and low dose-rates. Error bars represent the maximum error (± (max value-min value)/2). All symbols are explained in the figure. Points where the accumulated doses for the low dose-rates are 2.49 and 6.21 Gy are marked with unfilled symbols.

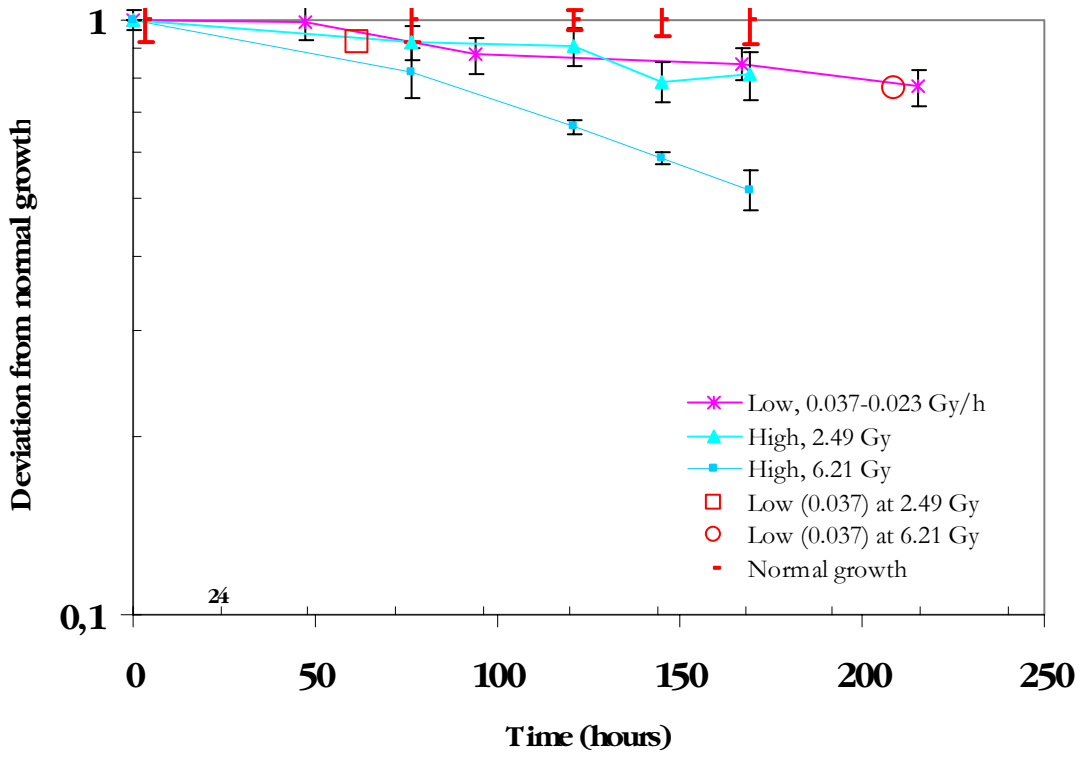

Figure 5: Deviation from normal growth for glioblastoma astrocytoma cell line U118, after irradiation with high and low dose-rates. Error bars represent the maximum error (± (max value-min value)/2). All symbols are explained in the figure. Points where the accumulated doses reach 2.49 and 6.21 Gy for the low dose-rates, are marked with unfilled symbols

The deviation from normal growth for glioblastoma astrocytoma U373 MG is showed in figure 6. The low dose-rate of 0.12 Gy/h gave a surprisingly strong effect for this cell line. The deviation is larger than for the acute doses for the entire time interval, even though the error bars for some points overlap the 6.21 Gy curve. The low dose-rate irradiation of 0.043 Gy/h gave a smaller effect than the 0.12 Gy/h irradiation did, but there is still an indication of an inverse effect compared to both the acute doses. The cells irradiated with acute doses almost recovered and the deviation from normal growth tends to diminish.

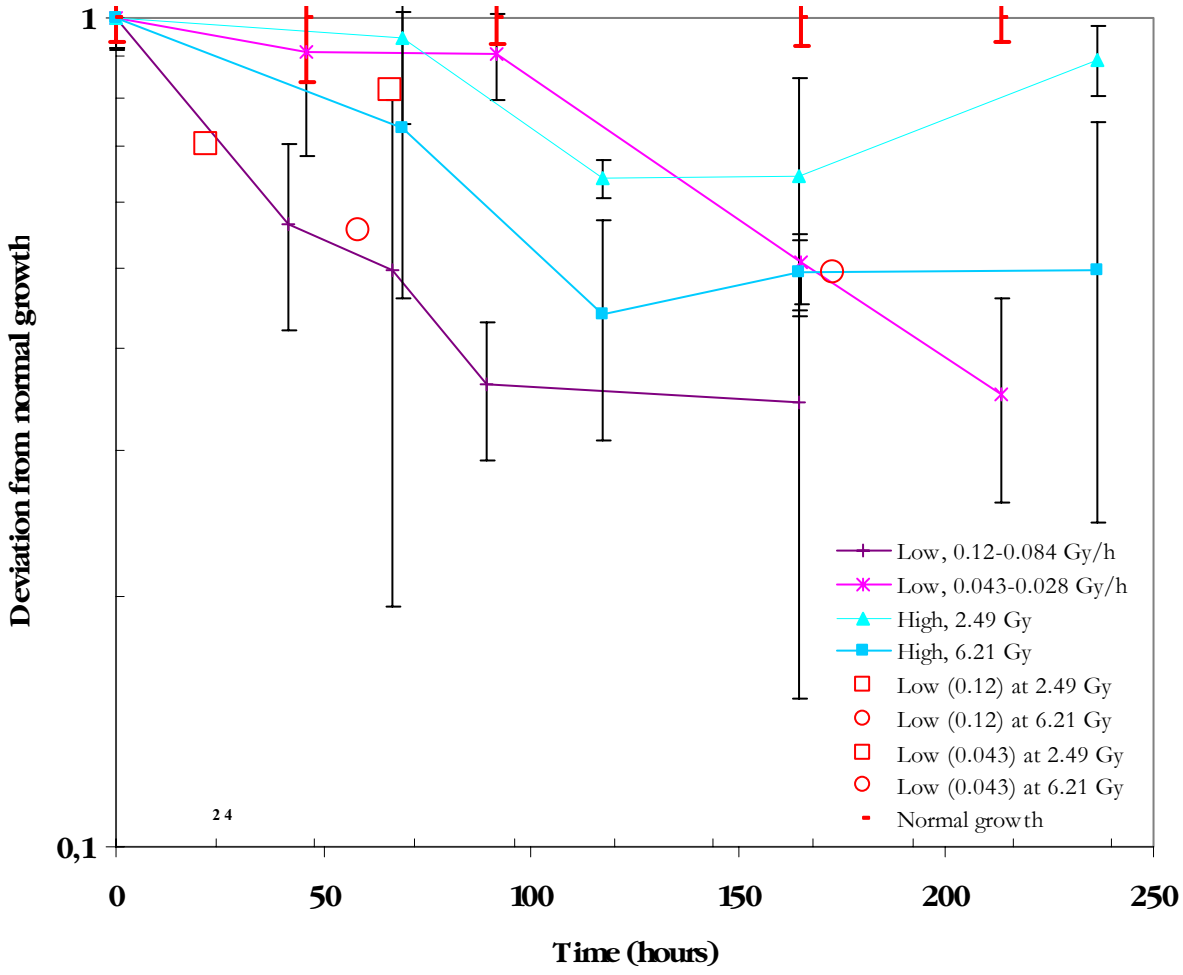

Figure 6: Deviation from normal growth for glioblastoma astrocytoma cell line U373 MG, after irradiation with high and low dose-rates. Error bars represent the maximum error (± (max value-min value)/2). All symbols are explained in the figure. Points where the accumulated doses reach 2.49 and 6.21 Gy for the low dose-rates, are marked with unfilled symbols

Discussion

The choice of cell lines in this study was made with regard to their earlier reported response to low doses. Both cell lines HT29 and U118 are reported to be hyper radiosensitive, as discussed above (2). Cell line U373 MG on the other hand is reported not to show hyper radiosensitivity.

Looking at the results from irradiation of colorectal adenocarcinoma HT29, inverse dose-rate effects cannot be confirmed. There could be an indication of an inverse effect, comparing the acute dose of 2.49 with the dose of 2.49 Gy with a dose-rate of 0.084 Gy/h (*Figure 4*). The deviation from normal growth is slightly larger for the lower dose-rate than for the acute dose-rate curve at this point. However, the curves are within each others error bars at some of the measuring points, and therefore the experiment must be repeated before valid conclusions can be drawn. The lowest dose-rate of 0.052 Gy/h can be concluded not to give hyper radiosensitivity, since the deviation from normal growth is very small compared to the acute doses.

In the case of glioblastoma astrocytoma U118 MG, there is no indication of an inverse dose-rate effect. There is no significant difference between the curves for deviation from normal growth after irradiation with an acute dose of 2.49 Gy and irradiation with a dose-rate of 0.037 Gy/h (*Figure 5*). The U118 MG cell line seems to be more resistant to radiation than the other cell lines in this study.

The results from the third cell line, glioblastoma astrocytoma U373 MG, are very interesting. This cell line, earlier reported not to be hypersensitive (2), has a large deviation from normal growth after irradiation with low dose-rates (*Figure 6*). Especially irradiation with a dose-rate of 0.12 Gy/h gives a strong reduction in number of cells. This dose-rate gives a greater growth retardation than both of the acute doses for the entire time period. Even the very low dose-rate of 0.043 Gy/h shows indications of an inverse dose-rate effect, but not without overlap of the error bars.

The deficiencies of this study are mainly the lack of repeated experiments. To really be able to draw conclusions from these results, all assays need to be duplicated. In addition, if points were taken at the same time of growth and for corresponding accumulated doses given with high and low dose-rates, the curves could more easily be compared. Possible dose-rate effects could thereby be statistically analysed.

In conclusion, the results from irradiation with low dose-rates are somewhat contradictory to what was expected. The most dramatic effect is observed for U373 MG, the cell line chosen for its lack of hypersensitivity, while the two cell lines HT29 and U118, chosen for their hypersensitivity, responds poorly. It is hard to conclude if these results are due to low dose-rate effects or consequences of the method used. The earlier observations of the three cell lines are all made after low acute doses (2). Therefore, it could be that the cells react differently to low dose-rates than to low doses. On the other hand, the method frequently used in the literature is clonogenic survival assays, and there might be differences in the effects detectable with one or the other method. The growth assays used in this study still could be believed to mimic the situation of a tumour growing in the body in a more accurate way.

Future experiments to be made are, evidently, to repeat all assays to get a solid ground for conclusions. Another course to take is to study the effect on the cells, to determine if the cause of diminishing growth is cell death or cell cycle arrest. The latter can be studied by analysis of incorporation of radioactively labelled thymidine.

Finally, it is important to study low dose-rate effects, especially regarding the development of targeted therapy. This study shows that the choice of assay can be important, and that hyper radiosensitivity to low doses not necessarily equals inverse dose-rate effects. It also shows that the choice of dose-rate might be critical for inverse effects.

Acknowledgments

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Appendix

Equations

Calculation of dose-rate: $DR=(DR_0*e^{(-\ln 2 * t / T_{1/2})})$, where $T_{1/2}$ = 343.2 hours

Calculation of accumulated dose: $D_{(at\ time\ t)}=-DR_0*(T_{1/2}/\ln 2)*[\ e^{(-\ln 2 * t / T_{1/2})} - e^{(-\ln 2 * t_{start} / T_{1/2})}]$

Measurements

Table I: Measurements of dose-rates in a cell dish on the radiation chambers. The date of measurement was 2001-11-08. Conversion constant = 61.996 nC/Gy. Background measurements in cell dish gives 0.0028 nC/min

Chamber	**Current** (nC/min)	**Measuring time** (min)	**Average-Background** (nC/min)	**Dose-rate** (Gy/h)
1	0,0262	5		
1	0,0262	32	0,0234	0,0226
2	0,0264	5		
2	0,0258	5	0,0233	0,0225
3	0,0268	5		
3	0,0266	5	0,0239	0,0231
Average			0,0235	0,0228

Calculations

Table II: Calculated dose-rates and doses for the first preparation of radiation chambers.

Date	**Time**	**Ion chamber** (in dish, Gy/h)	**Time from 8/11** (hours)	**Calculated dose-rate** (Gy/h)	**Calculated dose** (Gy)
20011108	~10.00	0,0228	0		
20011022	11.00		-407	0,0519	
20011023	09.30		-384	0,0495	1,17
20011024	10.00		-360	0,0472	2,33
20011025	09.45		-336	0,0449	3,43
20011026	09.20		-312,5	0,0429	4,46
20011029	11.40		-238,5	0,0369	
20011031	11.10		-191	0,0335	1,67
20011102	09.40		-144,5	0,0305	3,16
20011105	10.40		-71,5	0,0263	5,23

20011107	10.55		-23	0,0239	6,45
20011108	03.40		-6,5	0,0231	6,84

Table III: Calculated doses and dose-rates for the radiation chambers after refilling.
***1**)* Cells removed from chambers
***2**)* Cells placed at chambers
0.0731 Gy subtracted from there on following doses.

Date	**Time**	**Calibration curve** (Gy/h)	**Time from 12/11 14.30** (hours)	**Calculated dose-rate** (Gy/h)	**Calculated dose** (Gy)	
20011112	14.30	0,118	0			
20011112	16.15		1,75	0,118	0	
20011114	09.45		43,25	0,108	4,69	
20011115	10.45		68,25	0,103	7,33	
20011116	09.30		91	0,0984	9,62	
20011119	13.00		166,5	0,0844	16,51	
20011119	13.30	0,086				
20011119	15.00		168,5	0,0841	0	
20011121	09.50		211,33	0,0771	3,45	
20011122	11.15		236,75	0,0733	5,36	
20011122	12.05		237,58	0,0732	5,42	***1**
20011122	13.05		238,58	0,0730	5,50	***2**
20011126	09.45		331,25	0,0605	11,7	
20011127	14.35		360,08	0,0571	13,4	
20011128	09.40		379,17	0,0550	14,4	
20011129	11.00		404,50	0,0522	15,8	
20011203	13.45		503,25	0,0428	0	
20011205	11.45		549,25	0,0390	1,88	
20011207	09.20		594,83	0,0356	3,58	
20011210	10.50		668,33	0,0306	6,01	
20011212	11.10		716,67	0,0278	7,42	

Calibration curve

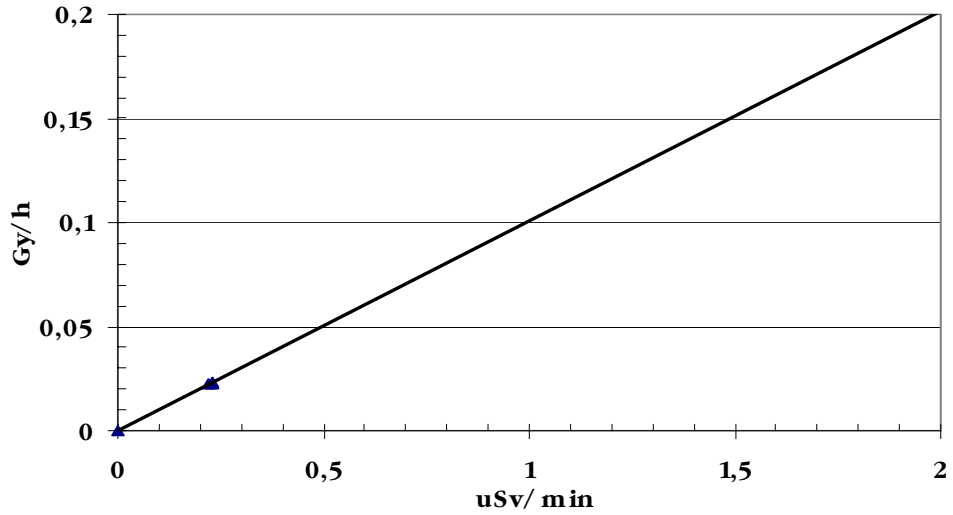

Figure I: Calibration curve for comparison between ion chamber and hand detector measurements on radiation chambers. Ion chamber measurements were performed in a cell dish placed on the radiation chamber. Hand detector measurements were made with the protection lid of transparent polycarbonate (Macrolon ®) between the detector and the chamber surface. Equation for the line is: $y = 0.1008\ x$.

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