In vitro studies on radiation effects and radiosensitization: HDR vs LDR and cytotoxic agents
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Chapter Two:

Chromosome aberrations detected by FISH and correlation with cell survival after irradiation at various dose-rates and after bromodeoxyuridine radiosensitization.

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Abstract:

**Aim:** To determine whether measurement of chromosome aberrations by fluorescence *in situ* hybridization (FISH) predicts cell survival after irradiation at different dose rates and after radiosensitization by bromodeoxyuridine (BrdU) in a lung carcinoma cell line

**Materials:** The human lung carcinoma cell line SW1573 was irradiated at high dose rate (HDR: 0.8 Gy/min) or at pulsed – low dose rate (p-LDR: average dose rate of 1Gy/h) with or without radiosensitization by bromodeoxyuridine (BrdU). Cell survival was determined with the clonogenic assay. Chromosome aberrations (color junctions) were measured by whole chromosome FISH of chromosome 2 and 18 and were scored according to the PAINT method.

**Results:** Clear radiosensitization by BrdU was observed both after HDR and p-LDR irradiation. Chromosome 18 was more radiosensitive than chromosome 2. There was a good correlation between induction of color junctions and cell survival both after HDR and p-LDR irradiation and after radiosensitization by BrdU.

**Conclusion:** Determination of chromosome aberrations by FISH can predict cell survival after different dose rates and after radiosensitization by BrdU.
Chapter 2

Introduction

Much recent research has focused on the development of an assay that could predict the outcome of radiation treatment so that patient treatment could be tailored individually. In several studies intrinsic radiosensitivity of the tumor cells as determined by clonogenic cell survival or growth assay has correlated well with treatment outcome (West et al., 1997, Girinsky et al., 1994). However, this technique is time-consuming and not fast enough to individualize treatments.

A good correlation between chromosome aberrations determined by whole chromosome fluorescence in situ hybridization (FISH) and cell kill has been shown for human tumor cells and human fibroblasts (Coco-Martin et al., 1994, 1996, 1999, Franken et al., 1999a, Russel et al., 1995). This technique is much faster than the average clonogenic assay and thus could be of clinical use.

At low dose rate (LDR) the increase in treatment time allows repair processes to take place during the irradiation leading to higher cell survival than after high dose rate (HDR) irradiation. In the clinic next to conventional HDR irradiation, LDR irradiation in the form of brachytherapy is used in the treatment of several malignancies. Owing to the very localized placement of the sources, there is optimal sparing of normal tissues.

Halogenated pyrimidines (HP) are known to sensitize cells to ionizing radiation in vitro (Iliakis et al., 1989). They are thymidine analogues that need to be incorporated into the DNA to be effective. The level of radiosensitization by HP correlates with the degree of thymidine replacement (Philips et al., 1989, Lawrence et al., 1990). Incorporation of HP into DNA increases the amount of DNA double strand breaks (dsb’s) induced by radiation (Ling et al., 1990, Wang and Iliakis, 1992, Iliakis et al., 1992, Lawrence et al., 1995), reduces clonogenic cell survival and increases the amount of chromosome aberrations after ionizing radiation (Wilt et al., 1994, Franken et al., 1999a,b).

To evaluate the use of FISH as a predictive assay, we have investigated whether chromosome aberration induction measured with FISH correlates with cell survival after irradiation at different dose rates and with or without radiosensitization by the halogenated pyrimidine bromodeoxyuridine (BrdU) in a lung carcinoma cell line.
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Materials and Methods

Cell culture

The human squamous lung carcinoma cell line SW-1573 was grown in Leibowitz-15 medium (L15; GIBCO-BRL life technologies, Breda, The Netherlands) supplemented with 10 % fetal bovine serum and 2 mM glutamine at 37°C with no CO₂. The doubling time of the SW-1573 cells in exponential growth is 22-24 h (Haveman et al., 1995).

For experiments, the cells were plated in 30 mm or 60 mm Petri dishes (p-LDR irradiation) or 75 mm² tissue culture flask (HDR irradiation) (Costar Europe LTD, Badhoevedorp, the Netherlands). For sensitizing experiments cells were incubated with medium containing 4 μM bromo-deoxyuridine (BrdU; Sigma, St. Louis, USA) for 48 hours before irradiation. Before the start of irradiation medium containing BrdU was removed, cells were washed two times with PBS and fresh medium was added. Cells were irradiated in late exponential growth/early plateau phase (50-65 % of the cells in G1-phase, 30-40 % in S-phase, 7-15 % in G2-phase as determined by BrdU incorporation). The percentage of thymidine replacement at the time of irradiation was 19.6 ± 0.8 %.

Irradiation

HDR irradiation was performed with X-rays from ¹³⁷Cs sources at a dose rate of 0.8 Gy/min. Two ¹³⁷Cs sources, one above and one below the culture flask were positioned at a distance of 22.5 cm. Lead flattening filters were used to ensure a homogeneous dose through out the culture flask.

Pulsed LDR (p-LDR) irradiations were performed with a Siemens Stabiliplan 2 X-ray machine (Siemens, Germany). Pulse dose was 0.1 Gy at 9.16 cGy/min and the resting period between the pulses was 4 min 52 s resulting in a mean dose rate of 1 Gy/h. The distance between focus and culture dish was 1.60 m and a 1 mm Cu- filter was used. During irradiation cells were kept at 37°C in a water bath. A maximum of six 100 mm culture dishes, fifteen 60 mm culture dishes or thirty-six 35 mm culture dishes could be irradiated simultaneously with 95 % dose homogeneity. Dosimetry was performed with a BF-vat detector and a Farmer electrometer once a month.
**Clonogenic Assay**

Cell survival was determined by clonogenic assay. Twenty-four hours after HDR irradiation or directly after p-LDR irradiation cells were trypsinized and replated in appropriate dilutions in six-well culture plates (Costar). Eight days later the colonies were fixed and stained in 6% glutaraldehyde with 0.05% crystal violet. Colonies of ≥ 50 cells were scored as originating from a single clonogenic cell. The plating efficiency of SW-1573 cells was 80-100%.

Surviving fractions \( \frac{S(D)}{S(0)} \) after dose D, corrected for toxicity of BrdU alone, were calculated and survival curves analyzed using Graphpad Prism statistical software (Graphpad Software Inc, San Diego, USA) or BMDP (Los Angeles, USA). The HDR data were fitted by multiple regression to the LQ formula:

\[
\frac{S(D)}{S(0)} = \exp - (\alpha D + \beta D^2)
\]

The LDR data were fitted by a pure exponential model:

\[
\frac{S(D)}{S(0)} = \exp - (\alpha D)
\]

**Metaphase slide preparation**

SW1573 cells were plated in 100 mm culture dishes (p-LDR irradiation) or 75 cm² tissue flasks (HDR irradiation). For sensitization experiments cells were cultured for 48 hours in the presence of 4 μM BrdU before irradiation. Twenty-four hours after HDR irradiation and directly after p-LDR irradiation, the cells were transferred to 172 cm² tissue culture flasks. Twenty four hours later, cells were incubated for 2 hours with colcemid (0.1 μg/ml, Sigma) and mitotic cells were shaken off. Mitotic cells were treated with hypotonic HCl for 10 min at 37°C and subsequently washed and fixed in methanol/acetic acid (3:1). Finally the cells were dropped onto moist slides.

**Fluorescence in situ hybridization**

Chromosome 2 and chromosome 18 were selected to study the induction of color junctions. In these chromosomes no spontaneous exchanges were observed. Directly labelled whole chromosome-specific probes (chromosome 2-Cy3 and chromosome 18-FITC) were obtained from Cambio (Cambridge, UK). Metaphase double staining was performed with the method described by Pinkel et al. (1986) and Natarajan et al. (1992). Enhancement of the FITC signal was achieved with a commercially available amplification kit (Cambio). Metaphase slides were counterstained with DAPI (2.5 μg ml) in PBS and embedded in anti-fade solution (Vecta Shield, Vector Laboratories, Burlingame, CA, USA).
Scoring of aberrations

Slides were examined using a fluorescence microscope (Ortholux: Leica, Weltzar, Germany) with a green light (552 nm) emission filter (615 nm) to detect Cy3 or a (495 nm) emission filter to detect FITC (519 nm). DAPI-stained metaphases were detected with a UV (372 nm) emission filter (456 nm). A total of 300 - 600 metaphases from three different experiments were scored for each dose and each chromosome. Aberrations were scored according to the PAINT method (Tucker et al., 1995). All aberrations involving a painted chromosome and an unpainted chromosome were scored as color junctions and are reported here.

Three copies of chromosome 2 and two copies of chromosome 18 are present in SW-1573 cells. In total SW-1573 cells contain between 60 and 67 chromosomes.

Dose-effect curves for induction of color junctions by radiation were analyzed using Graphpad Prism statistical software. The data were fitted to a pure linear model:

\[ F(D) = \alpha D \]

To determine the relative DNA content of chromosome 2 and chromosome 18, the length of all the chromosomes from ten photographs of well-spread metaphases was measured. For this purpose, DAPI-stained metaphases and metaphases after FISH were photographed with a CCD camera (Hi-Sis slowscan cooled, Lambert Instruments, Leutingewolde, The Netherlands) and the ratio of stained chromosome and total genome was calculated.
Chapter 2

Results

Cell survival after HDR and p-LDR irradiation

Survival curves for control and BrdU treated cells after HDR and p-LDR irradiation are shown in figure 1 and 2. The survival curve after p-LDR irradiation was adequately described by a pure exponential model with only the linear component (figure 2a and table 1). However analysis of the initial part of the survival curve, i.e. 0-4 Gy resulted in a different value of the α term when compared to the α value obtained from the entire survival curve i.e. 0-20 Gy (figure 2b and table 1). For the BrdU-sensitized curve this was not observed.

<table>
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<tr>
<th>HDR irradiation:</th>
<th>LQ: *</th>
<th>Control</th>
<th>BrdU</th>
<th>Enhancement Factor</th>
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</thead>
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<tr>
<td>Cell survival</td>
<td>α</td>
<td>0.12 ± 0.02</td>
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<td></td>
<td>β</td>
<td>0.04 ± 0.006</td>
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<td>1.5 ± 0.4</td>
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<td>Chromosome 18</td>
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<td>0.042 ± 0.002</td>
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<td>Total genome relative to 2</td>
<td>α</td>
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<td>1.22 ± 0.04</td>
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<tr>
<td>Total genome relative to 18</td>
<td>α</td>
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<td>1.68 ± 0.08</td>
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<th>BrdU</th>
<th>Enhancement Factor</th>
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<td>Cell survival 0-4 Gy</td>
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<td>Total genome relative to 18</td>
<td>α</td>
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<td>1.32 ± 0.1</td>
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</table>

*α (Gy⁻¹) and β (Gy⁻²)

Table 1. LQ parameters describing cell survival and induction of colour junctions after HDR irradiation and p-LDR irradiation with and without BrdUrd-sensitzation:

In the radiation dose range in which cell survival and chromosome aberrations are compared (0-6 Gy) no changes in cell cycle distribution were observed, after 6 Gy p-LDR irradiation the S-phase was 30-40 %.
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Figure 1: Cell survival of plateau-phase SW-1573 cells after HDR irradiation with or without 4 μM BrdUrd. Each point represents the mean value of at least 3 different experiments ±SEM. HDR data are fitted with a linear-quadratic model.

Pulsed-LDR irradiation in combination with 4 μM BrdU resulted in an increase in clonogenic cell killing by a factor of 1.5. After HDR irradiation the α value was increased by 2.2 and the β value was increased by 1.5 (table 1). Incubation with 4 μM BrdU did not result in growth delay or in a decrease of the plating efficiency.
Figure 2 a,b: Cell survival of plateau phase SW-1573 cells after p-LDR irradiation with or without 4 μM BrdUrd. Each point represents the mean value of at least 3 different experiments ±SEM. Pulsed-LDR data are fitted with a pure exponential model. b: Enlargement of the initial part of the p-LDR cell survival curve.

Chromosome aberrations

In figure 3 and 4 the dose-effect relationship for color junctions for chromosome 2 and 18 with and without 4 μM BrdU are presented for HDR and p-LDR irradiation respectively. In table 1 the LQ parameters for color junctions are shown. Curves were analyzed with a pure
exponential model with only the linear component because this resulted in the best correlations ($R^2 = 0.92 - 0.99$). For p-LDR irradiation LQ parameters were calculated based on the 0-4 Gy region. It is striking that, whereas cell survival after p-LDR and HDR irradiation at low doses was not very different, the efficiency of induction of color junctions differed by a factor of 3 for chromosome 2 and a factor of 2 for chromosome 18 (chromosome 2: $p<0.005$, chromosome 18: $p=0.03$). HDR was more effective than p-LDR irradiation.

**Figure 3:** Yield of colour junctions per cell after HDR in chromosome 2 and chromosome 18 with and without incorporation of 4 μM BrdUrd. Each point represents the mean value of at least two different experiments ±SEM. Data are fitted with a pure exponential model.
After HDR irradiation we could not score color junctions above 4 Gy because of insufficient yield of metaphases. This is in contrast to the situation after p-LDR irradiation (figure 4). A striking increase in the number of color junctions is observed in the p-LDR curve without BrdU, above 4 Gy notably in chromosome 2. At these doses, radiation-induced abnormalities such as dicentrics (data not shown) were observed. These were not seen after lower doses of p-LDR or after HDR. Even though a large percentage of the cells are in S-phase no S-phase specific chromosome aberrations were observed.

*Figure 4:* Yield of colour junctions per cell after p-LDR irradiation in chromosome 2 and chromosome 18 with and without incorporation of 4 μM BrdUrd. Each point represents the mean value of at least two different experiments ±SEM. Data are fitted with a pure exponential model.
The increase in color junctions in the p-LDR curve without BrdU could not be attributed to the appearance of dicentrics. A clear enhancement of dicentrics by BrdU can be seen in both chromosomes (figure 5). Because no centromeric probe was used the detection of dicentrics might be underestimated in this study.

**Figure 5:** Induction of dicentrics after p-LDR irradiation in chromosome 2 and chromosome 18 with and without BrdU. Each point represents the mean value of at least two different experiments ±SEM.

Based on their physical length chromosome 2 and 18 represent $7.8 \pm 0.6\%$ and $2.5 \pm 0.07\%$ of the entire genome. Extrapolation of the values of LQ parameters for the entire genome resulted in $\alpha$ values for chromosome 2 and chromosome 18 of $0.79 \pm 0.03$ and $1.16 \pm 0.08$ respectively for HDR irradiation and $\alpha$ values of $0.27 \pm 0.01$ and $0.56 \pm 0.03$ respectively for p-LDR irradiation. This indicates a relative overrepresentation of chromosome 18 for color junction induction.

The induction of color junctions in chromosome 2 and 18 after HDR irradiation combined with BrdU was increased with a factor of $1.5 \pm 0.1$ in both chromosomes. After p-LDR
irradiation color junction induction in chromosome 2 and chromosome 18 was increased with a factor of 2.8 ± 0.2 and 2.4 ± 0.2 respectively.

**Figure 6:** Correlation between cell survival and color junctions in chromosome 2 and 18 after HDR irradiation

**Correlation between in vitro cell survival and induction of chromosome aberrations**

In figure 6 and 7 the correlation between cell survival and induction of chromosome aberrations is shown after HDR and p-LDR irradiation with or with our BrdU. For all conditions a correlation coefficient >0.85 was obtained (0.85-0.98).
Chromosome aberrations detected by FISH and correlation with cell survival

Figure 7: Correlation between cell survival and colour junctions in chromosome 2 and 18 after p-LDR irradiation.

![Graph showing correlation between cell survival and colour junctions in chromosome 2 and 18 after p-LDR irradiation.](image)
BrdU is a known radiosensitizer at HDR but little is known about its radiosensitizing capacities after p-LDR. The enhancement factors obtained for cell survival in this study are in agreement with which enhancement factors found in previous studies using human cervical carcinoma cells (Tishler and Geard 1991, 1992).

The $\alpha$ value of the entire p-LDR curve (0-20 Gy) is slightly higher than the $\alpha$ value of the overall curve. Since our p-LDR irradiation consists of repeating pulses of 0.1 Gy followed by a rest period, it can be speculated that the lower $\alpha$ value in the initial part of the curve may be due to an adaptive response (Raaphorst et al., 2000). The first fraction may induce certain repair processes so that cell survival after the second fraction is slightly higher than expected. This has been reported for several cell lines which were primed to a small conditioning dose of radiation, followed by a second larger radiation dose (Raaphorst et al., 2000, Raaphorst and Boyden, 1999, Marples and Joiner 1995). The $\alpha$ value for the BrdU sensitized survival curve did not change, indicating that this adaptive response is absent in the presence of BrdU.

The induction of color junctions after HDR is considerably enhanced compared to p-LDR. The efficiency of color junction induction differs by a factor of 3 for chromosome 2 and by a factor of 2 for chromosome 18. However, cell survival after HDR or p-LDR irradiation does not differ much, indicating that the difference in induction of chromosome aberrations is not translated into a difference in survival. Color junctions are scored in the first mitosis after irradiation, while cell survival is determined after 6 cell divisions. Coco-Martín et al. (1994, 1996) showed that the number of aberrations in surviving cells is much lower than in the first mitosis after irradiation. We speculate that during HDR irradiation more aberrations are formed and scored but that most of them do survive to form colonies. On the other hand after p-LDR irradiation fewer aberrations occur that could be compatible with long term survival.

In the present study, both HDR and p-LDR irradiation of chromosome 18 induced more color junctions than expected when compared to chromosome 2. This suggests that chromosome 18 has a different radiosensitivity which contrasts with the assumption that radiation damage is random throughout the genome. A previous study in our laboratory showed no difference in radiosensitivity between chromosome 2 and chromosome X (Franken et al., 1999a). In literature, there is no clear consensus with respect to difference in radiosensitivity of chromosomes. Several authors have found that the induction of chromosome aberrations is proportional to the length or DNA content of the chromosomes (Tucker et al., 1993, Matsuoka
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et al., 1994, Johnson et al., 1998), while other authors have observed aberrations yields that are not relative to the length or DNA content (Wilt et al., 1994, Dominguez et al., 1996, Boei et al., 1997, Barguinero et al., 1998, Xiao and Natarajan 1999). Differences in gene density, chromatin organization and heterogeneity of repair processes have been suggested to explain this difference (Natarajan et al., 1996, Surrallés et al., 1998).

Even though there is a difference in radiosensitivity between chromosome 2 and chromosome 18, the enhancement by BrdU for the induction of color junctions was the same for both chromosomes. When the BrdU enhancement factors for chromosome aberration induction after p-LDR and HDR irradiation are compared, it is striking that the enhancement factor after p-LDR irradiation is higher than after HDR irradiation. However, when the BrdU enhancement factors for cell survival are compared the opposite is observed. It is speculated that the DNA damage induced by combining p-LDR irradiation with BrdU is less complex than when HDR irradiation is combined with BrdU, thus resulting in more viable color junctions. After HDR irradiation combined with BrdU the damage may be more complex resulting in more cell death.

Even though there is no significant difference between the survival after HDR and p-LDR in the low dose rate area and a difference in color junction induction was observed, the correlation between cell survival and color junctions is still high both for HDR irradiation and p-LDR irradiation. The present study tried to validate the predictive value of scoring chromosome aberrations with the FISH technique. Both after HDR and p-LDR irradiation and after radiosensitization with BrdU the correlation between cell survival and chromosome aberration determined by FISH was very good. This indicates that the technique could be used to predict the outcome of radiation treatment even after different irradiation conditions.

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