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

Summary, Conclusions and Future Directions
Radiosensitivity of tumor cells monitored by chromosome aberrations

In chapters 2 and 3 we studied whether measurement of chromosomal aberrations with the FISH technique could predict the outcome of radiation treatment. The results in both chapters show that the quantitative correlation between cell lethality in vitro and the incidence of chromosome aberrations is highly significant. We also investigated whether this good correlation was preserved when radiation of the cells was combined with radiosensitizing agents. In chapter 2 we show that for the halogenated pyrimidine, BrdU, the good prediction of the outcome of treatment is maintained. From the experiments with the cytostatic drug gemcitabine (chapter 3) it appeared that a good prediction of the outcome was not that clear and requires knowledge beforehand whether gemcitabine had been present or not. These results show that the use of the FISH technique to stain chromosomes and count aberrations as a predictive assay for the effects of radiation treatment of cancer has a great promise, but, would this technique be useful as a predictive assay in the clinic, the limitations of this technique, when treatment is combined with chemotherapy have to be carefully considered.

The studies described in this thesis were designed as a first step in evaluating the capacity of this technique under circumstances that possibly could be encountered in the clinic, i.e., radiation treatment combined with an additional modality like a cytostatic drug or a sensitizer. All the studies described in this thesis were performed on metaphase preparations of cultured cells in vitro, and therefore the chromosome spreads were of good quality. However, when metaphase preparations would be made from cells out of human tumor biopsy specimens, this would imply that cells had to be forced to pass through at least one cell cycle in culture. This in vitro culturing of fresh tumor tissue in practice would automatically result in a selection of cells, as only a fraction of cells for which by chance the in vitro culture conditions are good will start a cell division cycle. As a result of this the cells available for the FISH analysis will only represent a fraction of the tumor cells, and therefore results with these cells may not be representative for the entire tumor. This problem could be solved if the FISH technique could be applied directly on cells from fresh tumor material, preferably on all or most cells, independent of cell cycle phase. For this reason it is a prerequisite that the technique has to be developed further. The premature chromosome condensation technique (PCC) might offer the solution. Notably chemically induced PCC obtained with an inhibitor of phosphatase 1 and 2A (calyculin A) has much potential. Currently our laboratory is investigating the possibilities of application of the PCC technique directly on fresh tumor material. The PCC technique may have some disadvantage as cells in S-phase cells can not be picked up.
The advantage of the FISH technique above other methods used to determine the radiosensitivity of tumor cells, like the clonogenic assay or the growth delay assay, is the shorter time necessary to perform the analysis. Ideally FISH analysis could be ready within days in stead of weeks. Whole chromosome painting and subsequent color junction analysis are relatively easy to perform and standard assays and kits are available. Caution should be taken when radiation treatment is combined with chemotherapy, like we have shown in chapter 3 for gemcitabine.

In a recently published article Bezrookove et al. (2003) demonstrate that a novel approach to the PCC technique combined with multi-color 'cobra'-FISH can generate good quality chromosome preparations both from normal and established cell lines and most importantly from cells isolated from fresh biopsies. As these authors also showed that the yield of PCC's from the biopsies was high enough to yield a representative sample this will allow for a better and quicker analysis and holds promise for the further development of the FISH technique as a predictive assay. In addition the use of multi color FISH would give additional information of the chromosomal rearrangements in tumors, which will eventually leading to a better understanding of the different steps in tumor development.

**Radiosensitization by gemcitabine**

The exact mechanism of radiosensitization of gemcitabine has not been elucidated yet. Recently clinical studies have shown that gemcitabine can be combined with radiation treatment without major toxicity (Blackstock et al. 2001), which warrants further study into the potential of this chemotherapeutic agent to sensitize effects of radiation. In Chapter 4 we show that the cell cycle dependency of the effects of gemcitabine, plays an important role in its ability to radiosensitize cells in culture. Confluent glioma cultures containing relatively large numbers of G1-phase cells and large glioma cell spheroids can not be sensitized to effects of radiation by gemcitabine pretreatment. This observation could have implications for clinical use. If the radiosensitizing potential of gemcitabine is of advantage in clinic, correct scheduling of application of the agent and radiotherapy is important. Moreover, it might be more advantageous to use gemcitabine in conditions where many tumor cells are dividing, possibly after so-called “debulking” surgery when it is known that an increase in proliferation takes place.

Currently gemcitabine is used in the treatment of NSLCC and pancreatic carcinoma. As the agent would be used more frequently, an interest arises on information whether resistance to
gemcitabine also renders tumor cell resistant to other cytostatic drugs. In chapter 5 we determined in a gemcitabine resistant lung carcinoma cell line whether cross resistance for several other drugs used in lung cancer treatment exists. We show that in our cell line resistance to gemcitabine was due to a deficiency in deoxycytidine kinase (dCK), the enzyme involved in the rate limiting step of gemcitabine phosphorylation. This dCK deficiency resulted in increased resistance to gemcitabine and ara-C. We did not observe cross-resistance to other cytostatic drugs involved in the treatment of lung cancer. Furthermore the sensitivity to ionizing radiation was not altered in two different dFdC-resistant cell lines. We also demonstrated that in gemcitabine resistant cell lines still sensitization of the effects of radiation took place.

Analysis of survival curves after irradiation with and without gemcitabine, using the linear quadratic model indicates that the effect of gemcitabine is mainly on the alpha parameter (see table introduction and chapter 6). Low dose rate irradiation is characterized by the fact that sublethal and potentially lethal damage is repaired during radiation. As a consequence the beta-parameter (of the LQ model) is reduced so that only the alpha parameter remains. In chapter 6 we determined to what extent gemcitabine was able to sensitize effects of pulsed low dose rate irradiation. We observed that sensitization indeed occurs under these conditions, but that the enhancement factor is slightly lower than after HDR irradiation.

These 3 studies confirm that gemcitabine is a promising drug. If resistance to the drug by dCK deficiency will occur this will not hamper treatment of patient with other cytostatic drugs except other deoxycytidine analogues as e.g. Ara-C. Gemcitabine can also be combined with low dose rate irradiation in the form of brachytherapy which has advantage of highly localized dose deposition. The timing of gemcitabine treatment in relation to the cell cycle is important for obtaining the maximum radiosensitizing potential of gemcitabine.

In this thesis 2 reports are included wherein the effect of gemcitabine on induction of chromosome aberrations by irradiation (monitored as color junction induction, chapter 3 and chapter 6) is studied. In chapter 3 we show that incubation of lung carcinoma cell lines with 10 nM gemcitabine for 24 hours combined with HDR irradiation resulted in a reduction of the amount of color junctions compared to radiation alone. In chapter 6 we show that in the same experimental setup no effect on color junction induction is observed after pulsed low dose rate irradiation. After HDR irradiation no clear effect on chromosome fragment induction could be observed while after low dose rate for chromosome 18 a decrease in fragments was scored while chromosome 2 yielded more fragments. These results differ markedly from earlier
published results by Rosier et al. (2003), and are discussed in chapter 6. Effects of gemcitabine on induction of chromosome aberrations still needs to be investigated further.

**Pulsed low dose rate irradiation**

In Chapters 7 and 8 we have studied effects of pulsed low dose rate irradiation. In chapter 7 we determined the levels of thymidine kinase and deoxycytidine kinase after p-LDR irradiation. As described for HDR irradiation (Boothman et al., 1994, Wei et al., 1999), after p-LDR irradiation a rise in the activity of the TK and dCK is observed. This radiation induced rise in enzyme activity might possibly be exploited in clinical practice. Several agents are dependent on these enzymes for their activation. These include the pyrimidine analogs like bromodeoxyuridine, which are dependent on TK for their activation and the anti-cancer agent gemcitabine which is activated by deoxycytidine kinase. Based on the results presented in chapter 7, it would be advantageous to apply these drugs after radiotherapy instead of before, thereby potentially leading to an increase of their effect.

The focus of chapter 8 was on the role of different DNA-repair mechanisms in the so-called dose rate effect. Functional proteins involved in NHEJ, the AT-like mutation and XRCC2 are essential for the dose rate effect whereas intact single strand break repair appears to play a minor role in the dose rate effect. Another important observation of this study is that the HR mutant irs-ISF is much more sensitive to ionizing radiation in exponentially growing phase than in confluent phase. This finding again confirms that in the various cell cycle phases different mechanisms of DNA repair dominate.
Summarizing

All studies presented in this thesis were designed to investigate aspects of either sensitivity to irradiation or sensitization of the effects of irradiation, this with evidently the ultimate aim of improving cancer treatment. We have shown that the FISH technique holds promise as a predictive assay, notably with the new developments in the technique that lead to an improved yield in PCC’s in biopsy material as described by Bezrookove et al.(2003) The results presented in this thesis on the sensitizing properties of gemcitabine in combination with radiation will contribute to a better understanding of the working mechanism of this drug. Our results on the dose rate effect have given valuable insights into the role of the different DNA repair mechanisms after low dose rate radiation.
References


Rosier JF, Michaux L, Ameye G, Cedervall B, Libouton JM, Octave-Prignot M et al. 2003 The radioenhancement of two human head and neck squamous cell carcinomas by 2'-2' difluorodeoxycytidine (gemcitabine; dFdC) is mediated by an increase in radiation-induced residual chromosome aberrations but not residual DNA DSBs. Mutat Res 527:15-26.