In vitro studies on radiation and temozolomide in human glioma

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2011

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Chapter 7

General discussion

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The main aim of the experimental studies described in this thesis was to investigate the potential of 'targeted' therapy to improve glioma therapy by combining radiation with drugs targeting specific molecular processes within the glioma tumour cell. Even trimodal combinations were tested by combining radiotherapy and the alkylating agent temozolomide (TMZ) with the anti-epileptic drug valproic acid (VPA) and the cyclooxygenase-2 (COX-2) inhibitor meloxicam (MLC). Also investigated was the role of the O^6-methylguanine-DNA methyltransferase (MGMT) status in predicting the sensitivity of the tumour cells to TMZ-induced alkylating DNA damage, and the use of genetic profiling to discriminate between second primary and recurrent glioblastoma multiforme (GBM). This chapter discusses some topics relevant to the studies presented in the preceding chapters based on published and unpublished observations.

Established cell lines versus primary cell cultures

Experiments in the field of translational research often use primary cell cultures as their working model. Primary cell culture models are considered to better represent the original tumour in the patient. However, processing of tumour material and establishing primary cell cultures is a complicated process. The processed tumour cells occasionally did not attach to the surface of the culture plates and/or flasks or, if they did attach, cell division was often insufficient (unpublished data). The cell cultures that did undergo cell division often showed a highly variable range in progression of cell growth (population doubling time). Another point in using primary cell cultures was the limited potential of these cells to maintain a regular cell division pattern over a prolonged period of time. Even so, it was not always possible to use many of the growing cell cultures for the clonogenic assay. Plating efficiencies were often low and, in many cases, the colonies were difficult to score.

Another important point in case of using primary cell cultures is the presumed representation of the original tumour. Therefore, genetic alterations that are frequently found in GBMs were analysed on both the original tumour and of the derived primary cell culture. These analyses demonstrated that the majority (~70%) of the primary cell cultures did not represent the genetic profile of the corresponding original tumour cells (unpublished data). These cell cultures did not even show any of the genetic alterations that were found in the original tumour cells. The genetic profiles of the remaining cell cultures (~30%) usually showed a complete match with the original tumours, although about half of these cell cultures could not be used for experiments due to lack of viability (unpublished data). The remaining cell cultures were acceptable for experiments, although most had limitations of their own (as discussed before), including slow growth, low plating efficiency, limited reproducibility or difficult scoring of the colonies (clonogenic assay). Because of the limitations that were faced with early passage primary cell cultures, experiments were performed on primary cells that had been cultured for a longer period of time (so-

called long-term primary cell cultures), thus allowing for reproducibility in maintaining the cells in culture and when testing cell survival by clonogenic assay.

Established cell lines, originally started as primary cell cultures, are well-defined, genetically stable, reproducible and workable models for tumour cells. Contrary to primary cell cultures, which are heterogeneous, established cell lines are homogenous cell cultures, which can be seen as both an advantage and disadvantage. The first studies in this thesis were performed using long-term primary cell cultures, but this *in vitro* model was replaced by established cell lines in the latter studies.

Regarding the genomic representation of primary cell cultures it was shown that the genomic profiles of primary cell cultures from GBM are frequently deviant from parental tumour profiles [8]. In long-term cell cultures even progressive genetic changes had developed. On the other hand, biopsy spheroids were found to be genetically more representative of the parental tumour. Similar results were found when expression profiles of glioma biopsies were compared with the cell lines derived from those tissues [19]. The expression profiles of the tumour cell lines varied greatly from the parental tissues and clustered together rather than with the parental tissues [19]. Both latter studies, and our own unpublished data, advocate use of caution in calling primary cell cultures representative of the parental tumour without proper genetic analysis for verification. Altogether, the use of (genetically manipulated) established cell lines would be preferred to primary cell cultures for research applications, linking specific single genetic alterations to tailored therapy of the patient.

Treatment protocols

A variety of *in vitro* treatment protocols can be used, especially when combining multiple modalities. Radiation can be given as single doses or in several fractions. Drugs can also be given once or repeatedly, for instance as daily exposures. When radiation therapy is combined with one or more drugs, the succession of the treatment modalities can also be important for the acquired effect.

The *in vitro* treatment protocols need more investigation of the fractionated dose schedules (radiation and/or drugs), as this is more in accordance with daily clinical practice. For example, fractionation of 4 x 24h TMZ exposure has a different effect on clonogenic capacity of tumour cells than application of a single 24h exposure [36]. Therefore, testing only single-dose exposures of drugs *in vitro* may not be reliable for extrapolation to the clinical situation. Fractionation of the radiation dose in 2 Gy fractions can also reveal another outcome in combination with a drug that was found to be a radiosensitiser in single-dose survival curves. Thus interpretation of single-dose treatments (radiation and/or drugs) must be assessed with caution, but can be helpful in guiding the direction of research and elucidating the mechanism of action. On the other hand, accomplishing *in vitro* experiments with fractionated doses of radiation or radiation in combination with a specific drug(s) requires extensive exploratory work and expertise, and reproducibility of the executing person.

Modulation of the radiation response is often dependent on the moment a specific drug was administered, e.g. before, during or after the radiation treatment. As, for

example, was seen with three different COX-2 inhibitors that were given before or after irradiation [17]. The only radiosensitising effects were seen when the drugs were given as preirradiation treatment and no effects were seen after postirradiation treatment. Another example with the drug VPA showed the most efficient enhancement of the radiation response when combining both pre-and postirradiation treatment of VPA [3, 7]. Postirradiation treatment of VPA was far more effective than preirradiation treatment, which had hardly (or no) effect at all. This is in contrast with the study presented in this thesis (Chapter 5) in which VPA caused radiation enhancement only after preirradiation treatment, and not postirradiation treatment.

Surviving cells

In this thesis the concept of surviving cells was used [32, 33]. Surviving cells are those that ultimately survive any given treatment after a certain period of time, and include both the effects of the given treatment on cell proliferation as well as the effects on the clonogenic capacity of the cells. The surviving cells are determined by multiplying the total cell number with the fraction of clonogenic cells at the same time point after treatment. For instance, to determine the effects of TMZ on cell proliferation during the fractionated radiation experiments, surviving cells were determined (Chapter 4) [32]. Only treatment of cells with TMZ plus radiation resulted in a decrease in the number of surviving cells, especially after increasing the numbers of fractions, meaning that cell proliferation was more than compensated for by clonogenic cell death.

Another application for the surviving cells is presented in Chapter 6 [33] where surviving cells were determined to examine the recovery of the cell population during a prolonged period of time (>11 days) with the repopulation assay. Determination of the fate of cells for a longer period of time after treatment revealed that the repopulation rates of the ultimately surviving cells were affected far less by treatments than the ultimately non-surviving cells. However, it was also shown that the ultimately non-surviving cells remain present for a prolonged period of time after treatments before dying.

Both of our studies [32, 33] and those of Van Rijn et al. [34] and Van Rijn and Van den Berg [35] demonstrate the additional value of using the concept of surviving cells by contributing valuable extra information to the results obtained from the more commonly used techniques. Without incorporation of both clonogenic cell death and cell proliferation, it is more difficult to determine whether the total amount of tumour cells would grow, remain stable, or decrease after receiving a specific treatment protocol.

Suggestions for future research

Besides the incorporation of the concept of surviving cells and applying fractionation treatment protocols for both radiation and drugs (as mentioned before), other implications and fields of application can be of interest for future research.

The combination treatment protocol of radiation and TMZ can cause enhancement of the radiation response [1, 5, 13, 32, 36]. However, the effects are usually limited or even absent in the cell lines that were tested. The study by Chakravarti et al. [5] demonstrated that TMZ significantly enhanced the radiation response in two primary GBM cell lines with undetectable levels of the MGMT protein, while no radiation enhancement was found in two primary GBM cell lines with high expression levels of the MGMT protein. On the other hand, the study presented in Chapter 4 showed different radiation responses after treatment with TMZ in three long-term primary GBM cell lines lacking expression of the MGMT protein [32]. These two studies show contradictory indications for the role of the MGMT protein in possible radiation enhancement by TMZ. Therefore, further research on the role of the MGMT protein in the response of glioma tumour cells to TMZ plus radiation is warranted.

The role of MGMT as determined by *MGMT* promoter methylation in tumour specimens of patients treated with TMZ alone or combined with radiotherapy is also of interest. Methylation of the *MGMT* promoter proved to be an independent favourable prognostic factor irrespective of treatment [12], although patients with a tumour containing a methylated *MGMT* promoter had a survival benefit when treated with TMZ and radiotherapy compared to patients treated with radiotherapy alone. Furthermore, *MGMT* promoter methylation is also a favourable prognostic clinical factor in association with long-term survival of patients with a GBM [16]. Overall, MGMT appears to be beneficial in the response to radiotherapy and subsequent improved patient survival independent of additional treatment with TMZ. This phenomenon justifies further investigation.

Even though the effect of TMZ on the radiation response is not always interactive, combining TMZ with drugs like VPA and MLC has often shown to significantly increase the cytotoxic response of the tumour cells (Chapters 5 and 6) [33]. Therefore, finding new drugs that can interact both with radiation and TMZ may be worthwhile, especially when the interactions of the drugs are through different pathways.

DNA repair pathways are often reported to be involved in the positive interactions between radiation and TMZ or VPA [3, 5, 7, 13, 25]. DNA repair also appears to be a target for COX-2 inhibitor-induced enhancement of the radiation response [9, 14, 23]. Potential candidates for therapeutic targets may be poly(ADP-ribose) polymerase (PARP) inhibitors. PARP plays an important role in the base excision repair pathway (BER) and is able to detect DNA damage and then recruits cofactors to repair the lesions. The most common DNA adducts (~90%) caused by TMZ are N^7-methylguanine and N^3-methyladenine, which are normally well repaired by the BER pathway [21, 27]. Therefore, although only representing a small amount of the formed adducts (~10%), the O^6-methylguanine adducts are considered to be responsible for the cytotoxic effect of TMZ [2, 31, 37]. Inhibition of the BER pathway by PARP-inhibitors may therefore increase the cytotoxic activity of TMZ by causing additional lethal N^7-methylguanine and N^3-methyladenine adducts to the O^6-methylguanine adducts. PARP inhibitors have shown to enhance the sensitivity to TMZ [6, 10, 18, 28-29] and have also shown promise as radiosensitisers in glioma cell lines and tumour models [11, 22, 26].

In general, there are still many possible pathways and targets that can be explored in the ongoing search for opportunities to improve glioma therapy. Among many options, the following molecular targets can be of interest for further research: WEE1, the DNA repair enzyme MGMT, the PI3K/Akt pathway and the process of angiogenesis [4, 15, 20, 24, 30].

Concluding remarks

Despite considerable efforts to improve our understanding of GBM and their current treatment protocols, median survival of patients with a GBM shows only limited improvement over the last decades. However, increased knowledge about GBM and the underlying mechanisms of these tumours has led to new targets for intervention with new or existing drugs. Exploiting this knowledge in daily clinical practice may hopefully lead to a better therapy protocol and subsequently an increase in median survival for patients with GBM.

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