

# Improving the Radiosensitivity of Brain Tumors by Gene-directed Enzyme Prodrug Therapy

**Ph.D. thesis**

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## **1. Introduction**

Radiotherapy continues to remain one of the most effective treatment modalities of malignant tumors. However there are significant variations among various tumor types regarding the effectiveness of radiation therapy due to differences in tumor radioresistance. Malignant gliomas are among tumors considered relatively radioresistant. Despite the aggressive treatment modalities, usually they have a bad prognosis. The conventional therapies for glioblastoma multiforme are surgical removal of the bulk tumor mass, combined with radio- and chemotherapy. However the median survival time is only 9 months. Therefore there is an urgent need for new therapeutic approaches and for tumor models to elaborate these new treatment modalities.

Currently, several animal brain tumor models are available; however, none of them can mimic exactly human high-grade gliomas. Tumor models vary in their immunogenicity, growth patterns and invasiveness. G1261 is a frequently used murine brain tumor model; nevertheless, no detailed description of the model is available.

In the first part of this work the main biological characteristics of the G1261 cell line were investigated. In the second part the efficacy of radiosensitizing gene-therapy was studied in G1261 cells and in two other well-characterized tumor models: the rat C6 and human U373.

Radiosensitizing gene therapy is a form of gene-directed-enzyme-prodrug therapy (GDEPT). The principle of GDEPT is to use non-toxic prodrugs as therapeutic agents. These prodrugs require intracellular metabolic activation to exert their toxic effect. The gene encoding the enzyme responsible for the prodrug activation is introduced into tumor cells using various vectors. Thus the activation of the drug becomes more effective in the tumor and systemic toxicity might be reduced. Prodrugs with radiosensitizing properties are used in the radiosensitizing gene-therapy approach. We have used adenoviruses as vectors in our study. Adenoviruses are capable to carry large fragments of DNA and they don't cause insertional mutagenesis because they are non-integrating vectors. Adenoviruses transduce a wide range of dividing and non-dividing

cells. Their main benefit is that they are only mildly pathogenic in humans: they can cause diseases of the upper respiratory tract.

In this work an adenovirus vector encoding the human deoxycytidine-kinase (dCK) gene was introduced into glioma cell lines. Our aim was to increase the cytotoxic and radiosensitising effect of gemcitabine (2',2'-difluoro-2'-deoxycytidine) by the means of this gene-therapy approach. gemcitabine is a prodrug that becomes activated by intracellular phosphorylation. The rate-limiting step in this process is the phosphorylation into its monophosphat form by the dCK enzyme. gemcitabine has a wide range of antitumor activity and has been shown to be a potent radiosensitizing agent both in laboratory and clinical studies.

## **2. Aim of the study**

The first part of the present study was designed to investigate important biological characteristics of the G1261 murine brain tumor model. We had the following goals:

- To study the growth characteristics of the G1261 tumor model.
- To determine the radiation sensitivity of this cell line
- To define sensitivity of the G1261 cells toward adenoviral vectors and to determine the persistence of protein expression after adenoviral gene-transfer in the G1261 cell line
- To establish certain immunological characteristics of the cell line
- To study the *in vivo* immunogenicity of this cell line

In order to study the enhancement of toxic and radiosensitizing effects of gemcitabine by the means of gene therapy, we had the following main objectives in the second part of this work:

- To construct an adenoviral vector encoding the human dCK gene (Ad-Hu-dCK)
- To establish the transduction efficiency of the vector
- To determine dCK enzyme activity of the wild-type and dCK overexpressing glioma cell lines
- To determine gemcitabine sensitivity of the three glioma cell lines
- To study the effect of increased dCK activity on gemcitabine toxicity and radiosensitization *in vitro* and *in vivo*

### 3. Methods

Murine G1261, rat C6 and human U373 glioma cell lines were used.

Radiosensitivity of G1261 cells was assessed by colony-forming assay.

Adenovirus infection of the glioma cells was made 24 hours after plating. Cells were incubated with the virus for 1 hour in serum-free culture medium then fetal calf serum was added to 10%.

Transduction efficiency was tested using a LacZ encoding adenovirus vector (AdexCALacZ): cells were transduced with this vector at different multiplicities of infection (MOI) followed by quantification of LacZ positive cells.

Expression of the major histocompatibility antigens and co-stimulatory molecules in G1261 cells and alterations in their expression, as a result of various treatments, was investigated by reverse transcription-polymerase chain reactions (RT-PCR). Total cellular RNA was isolated from wild type G1261 cells, from cells transduced with different cytokine encoding (IL-2, IL-4, IL-12, GM-CSF, IFN- $\gamma$ ) adenovirus vectors, and from irradiated cells. The RNA was isolated 48 hours after virus-infection or 24 hours after irradiation. RNA was reverse transcribed and amplified by PCR using MHC I, MHC II, B7-1 and B7-2 specific primers. Products were analyzed by 5% polyacrylamide gel-electrophoresis. Semi-quantitative measurement of mRNA expression was done with the „GDS8000 Complete Gel Documentation and Analysis System”. RNA expressions were normalized on  $\beta$ -actin expression.

In order to investigate the immunogenicity of *in vivo* growing G1261 cells, mice were transplanted intracranially with G1261 cells. Mice were vaccinated with irradiated G1261 cells (20 Gy Co<sup>60</sup>- $\gamma$ -radiation) at different time points before or after tumor transplantation. The survival of the animals was followed up to 100 days.

The dCK encoding adenovirus vector (Ad-Hu-dCK) was generated by homologous recombination between the dCK containing shuttle plasmid and pBHGfrtDE1,3FLP genomic plasmid in HEK293 cells, using CaCl<sub>2</sub> co-precipitation. dCK overproduction of cells transduced with the vector was tested by biochemical assays.

Cells were infected at different MOI with Ad-Hu-dCK. The toxicity of Ad-Hu-dCK on different cell lines and the dCK activity of the cells were assessed.

Sensitivity of glioma cell lines to gemcitabine treatment was also determined: one day after plating, cells were treated with gemcitabine at increasing concentrations and the ratio of surviving cells was determined 3 days later.

To determine the combined effects of Ad-Hu-dCK transduction, gemcitabine treatment and irradiation, cells were transduced with Ad-Hu-dCK one day after plating. On day 3, cells were treated with gemcitabine. Twenty-four hours later, cells were irradiated with 4Gy. On day 7 cell survival was determined. Surviving fraction was calculated and the radio- and chemo-sensitizing effects were studied.

The effects of combined therapy were studied under *in vivo* conditions, too. Intracranial tumors were transplanted on Wistar rats (C6 cells) and C57Bl/6 mice (Gl261 cells) using wild-type or dCK over-expressing glioma cells. Virus-infection was performed *in vitro*, 24 hours prior to tumor transplantation. Three days after tumor transplantation, animals were treated intra-peritoneally with gemcitabine, 24 hours after gemcitabine treatment the head of animals was irradiated with 4 Gy X-rays. Survival of animals was followed.

Statistical analysis was performed using GraphPad Prism 5 software.

## 4. Results

### 4.1. Characterization of the Gl261 cell line

- Gl261 cells grew rapidly under *in vitro* conditions with a population doubling time of 20 hours. Cells did not show contact inhibition. Cell densities of  $2,5 \times 10^5/\text{cm}^2$  were reached without substantially affecting cell viability. Radiation sensitivity of Gl261 cells was investigated using increasing doses of  $\text{Co}^{60}$ - $\gamma$ -radiation. At 2 Gy, survival was approximately 50%, at 10 Gy it was about 0,0009% and at 20 Gy no surviving cells were seen.

- *In vivo* transplanted Gl261 cells can form both subcutaneous and intracranial tumors in the syngeneic host, C57BL/6 mouse. Intracranial tumors had a rapid growth rate with slightly invasive growth pattern. Neither the intracranial nor the subcutaneous tumors gave metastases. Lymphocyte infiltration was hardly detectable in intracranial tumors. Local irradiation of the tumor with 4 Gy x-rays substantially slowed down tumor progression, but no animals were cured by this treatment.

- G1261 cells could be efficiently transduced with adenoviral vectors. At 10 MOI, approximately 70% of the cells carried the vector, whereas at 100 MOI all cells carried the vector. We also demonstrated that after transduction with Ad-Hu-dCK, the expression of the dCK increased proportionally with the applied MOI.

- Since expression of the genes introduced by the adenoviral vector is transient we determined the kinetics of protein expression in infected G1261 cells. We demonstrated on two proteins with completely different functions (dCK and IL-2), that protein expression peaked 2 days after virus transduction, then began to decrease progressively, but was more than double the basal activity even 7 days after transduction. Irradiation did not influence significantly the protein level during this interval.

- We tested the expression of MHC I, MHC II, B7-1, B7-2 molecules in G1261 cells. This was done both on wild type G1261 cells and on cells genetically modified to secrete certain cytokines. We showed that wild-type cells had a well detectable basal MHCI level, which was definitely higher than the MHCI level in healthy brain tissue used as control. The only cytokine that modified MHCI expression was IFN $\gamma$ . MHCI levels increased in parallel with the amount of IFN $\gamma$  produced by transduced G1261 cells. No detectable MHCII expression was observed in wild-type G1261 cells, but IFN $\gamma$  production could induce MHCII expression. A low amount of B7-1 and B7-2 RNA could be detected in wild-type cells, but cytokine production did not change this expression. Irradiation with 20 Gy did not modify MHC I, MHC II, B7-1 or B7-2 expression levels neither in wild-type nor in cytokine-producing cells.

- We studied the *in vivo* immunogenicity of the G1261 tumor. G1261-bearing C57Bl/6 mice were vaccinated or pre-vaccinated with a single subcutaneous injection of irradiated (20 Gy) G1261 cells. When subcutaneous vaccination was carried out 7 or 3 days before intracranial tumor transplantation, approximately 87% or 33% of the animals remained tumor free. However, if subcutaneous vaccination was done on the day of the intracranial tumor transplantation or 3 days later, no significant effect was observed compared to non-vaccinated controls. The vaccination protocol was efficient only if  $1 \times 10^6$  or more G1261 cells were used for vaccination. These data suggest that G1261 cells are moderately immunogenic. When surviving animals were re-challenged 9 months later with intracranial transplantation of G1261 cells, no tumors were formed, showing that the immunity raised by G1261 vaccination was long lasting. If this

vaccination protocol was applied on C57Bl/6 mice challenged by intracranial transplantation of B16 melanoma cells, G1261 pre-vaccination failed to result in an increase in median survival of mice, showing that immune activation was raised specifically against G1261 cells.

#### **4.2. Radiosensitization of brain tumors by combination of chemotherapy and radiotherapy**

- Total and specific dCK enzyme activity was measured in three glioma cell lines. Substantial differences among cell lines were found. Both total and specific dCK activity was the highest in the G1261 cell line, and the biggest difference in the specific compared to total dCK activity was observed in C6 cell line.

- The *in vitro* gemcitabine sensitivity of the three cell lines was compared: G1261 cells were the most sensitive and U373 cells the most resistant. The IC50 values were 11, 39 and 282 nM for the G1261, C6 and U373 cells, respectively.

- Transduction efficiency of the adenovirus was investigated next. The highest transduction efficiency was detected in G1261 cells. Transduction efficiency in U373 cells was slightly lower and the lowest transduction efficiency was found in C6 cells.

- dCK overexpression following Ad-Hu-dCK transduction was cytotoxic for the G1261 cells (21% survival at 100 MOI) and U373 cells (52% survival at 100 MOI). The cytotoxic effect was moderate in C6 cells (82% survival at 300 MOI).

- Enzyme activities increased in parallel with the applied MOI in each cell line, independent from the different basal activities, 24 hours after virus infection. This effect was persistent; the enzyme activity was two-fold higher than the basal activity on the 7th day. The highest relative increase in the dCK level was detected in the cells, where enzyme activity at 300 MOI was about 140-fold higher than the basal dCK activity.

- We investigated the combined effects of dCK overproduction, gemcitabine treatment and irradiation *in vitro*. In these experiments, the different sensitivity of the cell lines toward gemcitabine treatment and adenoviral transduction, and the differences in transduction efficiencies were taken into consideration. Overexpression of dCK enzyme alone had no radiosensitizing effect in any of the cell lines. gemcitabine treatment had a mild, but significant radiosensitizing effect only on the G1261 cell line. Overexpression of the dCK enzyme enhanced gemcitabine toxicity in each investigated

cell line, but the enhancement rate varied. In the G1261 cells dCK overexpression led to 1.3-fold increase in gemcitabine toxicity. Stronger effect was observed in C6 and U373 cells (2- and 3.4-fold enhancement, respectively). Similarly, dCK over-expression improved the radiosensitizing effect of gemcitabine too, but the rate of radiosensitization was again different in the studied cell lines: it was 1.25 fold in G1261, 2.3 fold in C6 and 3.6 in U373 cells.

- Overexpression of the dCK gene had no effect on the *in vivo* tumor growth, neither in the G1261 nor in the C6 model. gemcitabine treatment alone had minor effect on tumor growth in both models and dCK over-expression could not improve this effect. In both animal models, local irradiation alone slightly prolonged the life of the animals, and dCK over-expression increased the anti-tumor effect of local tumor irradiation (about 25 and 28% survival rate in the G1261 and C6 models, respectively). In the murine model, the combination of gemcitabine treatment and tumor irradiation led to about 20% survival rate and this was slightly improved in the dCK/gemcitabine/radiation-treated group (25% survival). However, the lifespan of these animals increased from 30% to 43%. In the rat C6 model, the combined treatment with gemcitabine and tumor irradiation in the absence of dCK overexpression resulted in 16% survival. Significantly improved ( $P=0.0162$ ) survival rate (nearly 70%) was detected when gemcitabine treatment and irradiation was combined with dCK overexpression.

## 5. Conclusions

The G1261 murine glioma model harbors the main characteristics of most glioma models: it has an invasive but non-metastatic growth pattern, a high tumor take rate but survival time is dependent on the initially transplanted tumor cell number. Contrary to most animal glioma models, which often down regulate MHC1 expression, G1261 has an elevated basal MHC1 expression and also expresses low levels of B7-1 and B7-2 RNA, which might be responsible for the immunogenicity of the cell line. The G1261 brain tumor model can be used efficiently to investigate the antitumor effects of several therapeutic modalities. G1261 cells can be efficiently transduced with adenoviral

vectors. The moderate immunogenicity of the cells should be considered when evaluating the experimental data.

Toxic and radiosensitizing effect of gemcitabine can be efficiently improved by the over-expression of the dCK enzyme in the tumor cells by the GDEPT approach, but this effect is strongly cell type specific.

### **List of publications:**

#### **In the topic of the thesis:**

1. **Tünde Szatmári** , Katalin Lumniczky, Szilvia Désaknai, StéphaneTrajcevski, Egon J. Hídvégi, Hirofumi Hamada, Géza Sáfrány (2006): Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy. *Cancer Science* 2006; 97: no 6. 546-553
2. **Tünde Szatmári**, Gergely Huszty, Szilvia Désaknai, Tatjana Spasokoukotskaja, Mária Sasvári-Székely, Mária Staub, Olga Ésik, Géza Sáfrány and Katalin Lumniczky (2007): Adenoviral vector transduction of the human deoxycytidine kinase gene enhances the cytotoxic and radiosensitizing effect of gemcitabine on experimental gliomas. *Cancer Gene Therapy* 2008, 15, 154-164

#### **Other publications:**

1. I. Klementis, K. Lumniczky, E. Kis, **T. Szatmári**, S. Antal, G. Sáfrány (2004): The transgenerational mutagenic and carcinogenic effect of ionizing radiation. *Central European Journal of Occupational and Environmental Medicine*; 2004, 10(3):235-245
2. E. Kis, **T. Szatmári**, M. Keszei, R. Farkas, O. Ésik, K. Lumniczky, A. Falus and G. Sáfrány (2006): Microarray analysis of radiation response genes in primary human fibroblasts. *Int. J. Rad. Oncol., Biol., Phys.*, Vol. 66, No5, pp. 1506-1514, 2006.