The prognosis of malignant brain tumors remains extremely bad in spite of moderate improvements of conventional treatments. A promising alternative approach is the use of oncolytic viruses. Strategies to improve viral toxicity include the combination of oncolytic viruses with standard therapies. Parovirus H-1 (H-1PV) is an oncolytic virus with proven toxicity in glioma cells. Recently it has been demonstrated that the combination of ionizing radiation (IR) with H-1PV showed promising results. Previously irradiated glioma cells remained fully permissive for H-1PV induced cytotoxicity supporting the use of H-1PV for recurrent gliomas, which typically arise from irradiated cell clones. When glioma cells were infected with H-1PV shortly (24 h) after IR, cell killing improved and only the combination of both treatments lead to complete long-term tumor cell killing. The latter finding raises the question whether IR in combination with H-1PV exerts an additional therapeutic effect on highly resistant glioma stem cells. A likely translation into current clinical treatment protocols is to use stereotactic radiation of non-resectable recurrent gliomas followed by intratumoral injection of H-1PV to harvest the synergistic effects of combination treatment.

The treatment of malignant brain tumors remains an unsolved problem. The most important improvement upon standard therapies in the last decade was to combine radiation therapy concomitantly with the chemotherapeutic drug temozolomide. This strategy has raised the medium survival of patients with the first diagnosis of glioblastoma from 13 to 16 months indicating that also for glioma treatment combinatory approaches are promising. However, as even the improved survival times indicate, there is a clear demand for more efficient treatments. Next to improving standard approaches in various settings, the investigation of alternative treatment modalities is another important step towards better therapeutic outcomes. One novel approach is to use the anti-tumor activity of oncolytic viruses to specifically destroy malignant cells without harming normal tissue. For malignant brain tumors, a growing number of viruses have been shown to possess selective oncolytic activity in glioma cells and to be largely innocuous for the normal brain tissue. But despite the variety of tested viruses the ‘magic bullet virus’ has not been discovered yet-and indeed, might never be. Therefore, the strategy to combine the unique therapeutic properties of oncolytic viruses with standard therapies is one current focus of oncolytic virus research.

One promising candidate virus for glioma therapy is Parovirus H-1 (H-1PV). H-1PV belongs to the parovirus family and its natural host is the rat. As the name suggests, the viral particle is small (20–25 nm in diameter), encapsulating a single stranded DNA genome of 5,100 nucleotides. Humans are most likely not naturally infected by H-1PV and no human pathology caused by H-1PV has been observed.
reported. We were able to demonstrate that glioma cells are highly susceptible to H-1PV induced cell killing and viral replication is supported in these cells. Further analysis of the death mechanisms induced by H-1PV demonstrated a relocation of lysosomal proteins, in particular cathepsin, to the cytoplasm leading to an autophagy-like cell death. As a consequence, even glioma cells that were resistant to apoptotic cell death, a frequent problem in the treatment of malignant gliomas, remained fully susceptible to the virus. In animal models therapeutic H-1PV infection of rats bearing large intracranial gliomas resulted in significantly improved survival. In some animals virotherapy resulted in complete remission of tumors even when the animals were symptomatic from tumor growth. In these experiments tumor regression could be achieved after intratumoral infection but also by intravenous injection of H-1PV. To our knowledge, the highly efficient regression of intracranial gliomas by both routes of administration has not previously been demonstrated for other viruses before, possibly opening additional therapeutic strategies.

In the current work we focused on the interaction of H-1PV and radiation therapy (IR). As IR is the standard of care for all primary and selected cases of recurrent malignant gliomas, a possible therapeutic use of H-1PV could be influenced by IR mainly in two ways: (1) infection of recurrent gliomas after previous radiation therapy could be different from highly susceptible primary glioma cells and (2) the combination of both therapies could be more efficient than either therapy alone.

**Previously Irradiated Glioma Cells Remain a Target for H-1PV**

In order to address the influence of previous radiation therapy on H-1PV induced cytotoxicity two different experiments were performed: (1) we infected cells from a recurrent glioblastoma culture at low passages from patient material (NCH-307 cells, courtesy of Dr. C. Herold-Mende, Division of Neurosurgical Research, Heidelberg, Germany). This recurrent glioma had developed after previous radiation therapy as part of the standard treatment protocol following the first tumor resection. (2) In addition to the in vivo irradiated NCH-307 cells, we infected two glioblastoma short-term cultures (NCH-82; NCH-89) and one gliosarcoma culture (NCH-37) after experimental IR. The exposure of the culture flasks to a dose of 10 Gy led to markedly reduced cell growth but did not eradicate all tumor cells. Nine days after IR when cells had resumed to proliferate from surviving cell clones, albeit at a much slower rate compared with control cells, H-1PV infection was performed at a low multiplicity of infection (MOI) of five plaque forming units (pfu) per cell. Cell viability assays five days after H-1PV infection showed efficient cell killing in all cell cultures (NCH-307, NCH-37, NCH-82, NCH-89) indicating intact susceptibility to H-1PV after previous IR (Fig. 1).

At a first glance the persisting susceptibility of high-grade glioma cells after previous IR seems to be less spectacular than the possible improvement of viral toxicity by combining both treatments. However, the intact susceptibility of high-grade glioma cells is in general an important finding prior to designing clinical trials with oncolytic viruses and, surprisingly, this question had not been addressed for any other oncolytic virus before. Different from metastatic cancers that frequently arise in organs distant from the original tumor location, recurrent gliomas form in 70 to 80% of the cases adjacent to, or within 2 to 3 cm of the original tumor site. As the radiation field in high-grade glioma therapy usually extends a few centimeters away from the bulk tumor mass defined by the area of contrast enhancement, the knowledge of the response of recurrent gliomas that arise from irradiated cell clones is relevant for the majority of potential patients, at least in initial clinical trials. The reason is that typically phase I or phase II clinical glioma trials with oncolytic viruses are restricted to patients with recurrent tumors. Thus, by demonstrating the intact susceptibility of malignant gliomas to H-1PV induced oncolysis either after experimental sublethal IR of glioma cultures or even after previous therapeutic IR in vivo, recurrent gliomas remain a suitable target for an initial use of this virus in patients after failed standard therapy.

**Combination of IR Followed by H-1PV Infection Increases Cytotoxicity**

In a next step the possible therapeutic effect of combined IR and H-1PV infection was tested. To this end cell viability of short-term glioma cultures (NCH-37, NCH-89 and NCH-89) was measured after virus infection was performed either before or after IR at different doses (5 Gy, 10 Gy and 20 Gy). In brief, IR enhanced virus induced cell killing in all primary glioma cultures when administered 24 hours before, but not 24 hours after infection. This effect was most pronounced in cultures of NCH-37 cells, which were the most resistant to either IR or viral infection alone. After combination treatment cell killing of NCH-37 cells was elevated to levels similar to the much more
susceptible NCH-82 cells to approximately 80% on day 5 after infection. Also in the more susceptible NCH-82 and NCH-89 cells combination therapy was superior to either therapy alone.

Viral replication could be demonstrated in all cell cultures on the translation and transcription level. The improved cell killing after combination treatment was paralleled by an increase of cells positive for the cytotoxic viral non-structural NS-1 protein as determined by flow cytometry, indicating a synergistic effect. This was again most pronounced in NCH-37 cells supporting the finding of a highly improved efficiency of combination treatment in these relatively resistant cells. Furthermore, virus replication after previous IR remained intact in all glioma cells yielding a 100-fold increase of fully infectious progeny particles from the supernatant of infected cells 3 days after H-1PV infection. As H-1PV replication is S-phase dependent, we examined the cell cycle of irradiated cells prior to infection. In all cell cultures an increase in the percentage of cells in S-phase between +67% to +114% could be observed, a mechanism that is likely to contribute to improved virus replication and toxicity.

Another important finding was that only the combination of IR and virus infection was able to completely eradicate all glioma cells from the culture dishes. Single treatment at the same dose was unable to achieve this goal and after 3 weeks cells began to grow out from remaining clones in all tested cell lines (Fig. 2).

This result deserves further investigation in the future. A number of studies have shown that in cultures of permanent tumor cell lines including gliomas a varying proportion of cells express markers of tumor stem cells. When cell cultures were exposed to cytotoxic treatments including IR it could be shown that cells positive for the expression of stem cell markers were the most resistant subpopulation. In one recent study even IR doses up to 30 Gy were not able to completely eradicate glioma cell cultures but rather to select for CD-133+ (one stem cell marker for glioma stem cells) positive populations. Moreover, when this treatment was repeated after cells had regrown, the ratio of CD-133+ cells increased as a result of IR induced selection. After IR alone, we observed a similar growth pattern in our glioma cell cultures which were different from the cells tested by Kang et al. After a high IR dose of 20 Gy subpopulations of cells survived in all our glioma cultures and started to proliferate after a lag period of 7 days. Similar findings could be observed after cytotoxic H-1 treatment, which did not completely eradicat all glioma cells. It would therefore be of significant interest if the surviving cell populations after either treatment alone express a higher ratio of stem cell markers and if the combination treatment is efficient to also eliminate this resistant subgroup of cells.

**Clinical Considerations**

As the promising data on H-1PV induced cytotoxicity in gliomas support a clinical use of this virus, the question arises whether also the combination of IR and H-1PV infection could possibly be included in current therapeutic strategies. As radiation methods for glioblastoma patients vary depending on the stage of the disease, a possible combinatory approach has to take this aspect into consideration. Furthermore, the initial application of a novel oncolytic virus will most likely be conducted in patients with recurrent tumors. Therefore, in the following the possible combination of IR with H-1PV will only be considered for recurrent gliomas.

While radiation therapy is part of the first line treatment for all primary glioblastomas, the strategy to re-irradiate recurrent glioblastomas has only become more common in the past years. A total dose of 60 Gy that is typically used for primary tumors was considered to be the maximum total tolerable dose and IR was only rarely applied for recurrent tumors. Recently it could be demonstrated that also for patients with recurrent tumors options for IR exist. A main reason is that with more sophisticated radiation techniques it became possible to reduce severe side effects while still applying a therapeutic dose to patients that had already received a full course of IR. Estimating possible options for viro-radiotherapy is complicated by the fact that the choice of the suitable radiation technique for recurrent gliomas has to be made individually for
each patient depending on factors such as tumor size, previous radiation field, clinical status and others. For smaller recurrent tumors it is possible to use stereotactic radiosurgery (SRS) in which a high local dose is applied to the entire tumor in a single treatment. Larger recurrent gliomas are treated with fractionated stereotactic radiotherapy (FSRT) with smaller doses delivered over a longer period of time or hyperfractionated stereotactic radiotherapy (H-FSRT), which also uses repeated dosing but in less fractions than FSRT resulting in a higher single dose.

The results of combination treatment in cell culture experiments presented in this article support a strategy where a relatively high dose of IR should be followed by H-1PV infection after 24 h. Obviously, the most direct transfer of this approach offering the best chances for cytotoxic effects and efficient viral spreading. When considering the timing of a combination of SRS and H-1PV it would be favorable to apply the treatment relatively early after the recurrence of a localized tumor when gliomas are still smaller and before the size of the glioma excludes the use of SRS or makes efficient delivery of H-1PV more difficult.

In the case of recurrent glioblastomas that can be resected, IR is only applied if tumor remnants remain (after incomplete removal) or in case of another regrowth. The type of IR will again depend on tumor volume, as for non-resectable recurrent tumors. Since gliomas patients after a second complete tumor resection will be monitored by MRI in short intervals (typically 3 months) an early detection of the next tumor recurrence is possible and radio-virotherapy could also in this case be applied to more suitable smaller lesions. A question that arises from promising recent animal data is the use of H-1PV not only by local administration but also by intravenous injection.8 One goal of a planned initial clinical trial, which does not include IR, is to assess whether also in human glioma patients H-1PV is able to cross the blood-brain barrier. Without this basic information the consideration of a possible intravenous virus application in combination with IR or with IR plus local H-1PV seems premature and, consequently, this option is labeled with a question mark in Figure 3.

In conclusion, previous IR did not reduce H-1PV induced toxicity in glioma cells, while the combination of IR followed by H-1PV infection after 24 h improved oncolytic activity. In patients, this effect could be used in particular for the treatment of non-resectable recurrent gliomas. Whether the positive long-term effects of the combination of IR and H-1PV result from increased toxicity for highly resistant glioma stem cells remains to be shown.

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