Anticancer Efficiency of Reovirus in Normoxia and Hypoxia

K. FIGOVÁ, J. HRABĚTA, T. ECKSCHLAGER

Folia Biologica (Praha) 59, 68-75 (2013)

Abstract. Oncolytic viruses infect, replicate in, and lyse tumour cells but spare the normal ones. One of oncolytic viruses is a naturally occurring replication-competent reovirus (RV), which preferentially kills tumour cells with activated Ras signaling pathways. The aim of this study was to survey effects of RV on brain tumour-derived cells in vitro under hypoxic conditions since hypoxia causes resistance to radioand chemotherapy. This study demonstrates that RV replicates preferentially in tumour cells and that the virus is able to overcome cellular adaptation to hypoxia and infect and kill hypoxic tumour cells. RV can both replicate in hypoxic tumour microenvironment and cause the cytopathic effect, subsequently inducing cell death. We found that a large proportion of cells are killed in hypoxia (1% O₂) by caspase-independent mechanisms. Furthermore, we learned that the cell death induced by RV in hypoxic conditions is not caused by autophagy.

Introduction

Despite advances in modern medicine, cancer remains one of the main causes of death in all developed countries. Current efforts to improve cancer therapy are attempting to enhance drug efficacy while maintaining acceptable toxicity levels. In order to succeed in reducing the number of cancer-related deaths, novel therapeutics have been designed to target tumour-specific attributes in order to permit higher doses with fewer side effects. One such example is use of oncolytic viruses (OV).

OV infect, replicate in, and lyse tumour cells but spare the normal ones. Most OV are prepared by genetic engineering for tumour selectivity, although there are a few naturally occurring ones, e.g. reovirus (RV). Replication of OV in tumour cells can increase their effect. However, physical barriers such as necrotic areas, stromal cells, extracellular matrix or basal membrane may limit the spreading of the virus. OV can mediate destruction of tumour cells by more mechanisms than the mere direct lysis caused by viral replication; they may induce antitumour immunity and some viruses express cytotoxic proteins (Ring, 2002). On the other hand, OV induce an anticancer immune response that limits their effect (Smith and Chiocca, 2000). Clinical studies were performed with OV, including reovirus (RV) (Reolysin®, Oncolytics Biotech® Inc., Calgary, Canada) (Eckenschlagr and Figová, 2008).

Reovirus (Respiratory Enteric Orphan virus, RV) is a replication-competent, naturally occurring virus that preferentially kills tumour cells (Coffey et al., 1998). RV replicates in the presence of an active Ras signalling pathway, which is common in cancer cells (Strong et al., 1998). In humans, RV is isolated from the respiratory and gastrointestinal tract, but it is not associated with any disease – orphan virus (Tyler et al., 2001). The RV lytic cycle consists of several steps. It begins with the attachment of virion to the receptor of the host cell, followed thereafter by receptor-mediated endocytosis. Within the endosome, proteolysis of viral outer capsid proteins gives rise to an intermediate subviral particle (ISVP). Receptor binding and disassembly must occur within the same cellular compartment to elicit an apoptotic response. A critical component of the signalling cascade that leads to apoptosis of RV-infected cells is transcription factor NF-κB (O’Donnell et al., 2006). RV also activates c-Jun N-terminal kinase and extracellular signal-related kinase (Clarke et al., 2001), but their involvement in apoptosis induction is not yet understood. The triggered ISVPs then penetrate through the endoso-
mal membrane. Afterwards, transcription of 10 RNA segments mediated by viral dsRNA-dependent RNA polymerase proceeds. Later, synthesis of minus RNA strands occurs and secondary transcription of late viral mRNAs begins. The final composition of the outer capsid yields virus particles (Norman and Lee, 2005). However, viral transcription is not indispensable, as inhibitors of viral RNA synthesis do not diminish the capacity of RV to induce apoptosis (Connolly and Dernody, 2002).

The mechanism of RV tropism in transformed cells is a defective cellular anti-viral response in Ras-pathway-transformed cells. In cells with present reoviral dsRNAs activate protein kinase RNA-activated (PKR), which in turn phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2α). The phosphorylation shuts off any further protein translation and thus inhibits the initiation of translation of viral transcripts. In cells with an activated Ras pathway, the phosphorylation of eIF-2α is inhibited, resulting in viral translation and subsequent entrance into the viral lytic cycle (Strong et al., 1998). RV compels cell cycle arrest at G1 and G2/M, induces apoptosis, and activates MAPK cascades. RV-induced apoptosis involves members of the TNF-related apoptosis-inducing ligand (TRAIL) family and is associated with the activation of both death receptor and mitochondrial-associated caspases (Smakman et al., 2005).

Hypoxic areas are frequent in solid tumours, as a consequence of pathological microcirculation within the tumour and of poor quality of the tumour vessels (Vaupel et al., 1989). Notably, hypoxia-induced resistance is not limited to conventional chemotherapy, but it can also decrease the efficiency of targeted therapies, as documented in imatinib cases of chronic myeloid leukaemia (Giuntoli et al., 2006). Additionally, hypoxia induces genomic instability that leads to progressive transformation of cancer cells into more malignant phenotypes (Huang et al., 2007). The presence of hypoxic regions correlates with more aggressive phenotypes, lower response rates, and shorter survival (Hockel et al., 1999).

The aim of this study was to survey effects of RV on brain tumour-derived cells in vitro in hypoxic conditions.

**Material and Methods**

**Cell lines and virus**

Human glioblastoma cell line U373, medulloblastoma cell line Daoy, non-transformed human fibroblasts from human foreskin – normal human fibroblasts (NHF), and mouse L929 cell line were used for virus cultivation and titration. Cells were cultured in IMDM supplemented with 10% foetal bovine serum, 1% glutamine, streptomycin and penicillin (PAA Laboratories, Pasching, Austria) at 37 °C in 5% CO2 under normoxia (21% O2). Hypoxia (1% O2) was generated in a closed system – hypoxic chamber (Billups-Rothenberg, Inc., Del Mar, CA) with a defined gas mixture containing 1% O2, 5% CO2 and 94% N2.

RV serotype 3 Dearing was purchased from ATCC (LGC Standards Sp. z o.o., Lomianki, Poland). Virus was purified according to the protocol of Smith et al. (1969) with the exception that 2-mercaptoethanol was omitted from the extraction buffer. L929 cells grown in 6-well plates to approximately 80% confluence were infected with RV at an MOI of 20. After 72 h of incubation, the cells and supernatants were subjected to three freeze-thaw cycles and centrifuged to omit cell debris. Viral titre was determined by plaque assays using the L929 cells. The homogenous virus was aliquoted and stored at −80 °C.

A new aliquot was always used for each experiment.

**Immunoblotting**

Cells were washed with PBS, trypsinized and counted. The amount of 2.5 × 10^6 cells containing pellets was washed and stored at −80 °C. Thawed pellets were used for preparation of whole-cell extracts. Protein levels in cell lysates incubated on ice were measured using the DC protein assay (Bio-Rad Laboratories, Hercules, CA) with serum albumin as a standard. Equal amounts of protein were electrophoresed on 11% and 16% (for LC3 protein) polyacrylamide gel. Proteins were transferred by wet electroblotting to a nitrocellulose membrane (Bio-Rad Laboratories). Non-specific binding was blocked in 5% non-fat milk in PBS with 0.1% Tween-20 or TBS with 0.1% Tween-20 and 1% BSA for LC3. The membranes were then exposed to rabbit polyclonal antibody to HIF-1α (Upstate Biotechnology, Lake Placid, NY) diluted 1 : 3000, the mouse monoclonal antibody to β-actin (Abcam, Cambridge, MA) at a dilution of 1 : 3000, or the polyclonal anti-reovirus antibody that was prepared for us by immunization of rabbits (Seva-Imuno, Prague, Czech Republic) at a dilution of 1 : 200,000. The appropriate HRP-conjugated secondary anti-mouse or anti-rabbit antibody (Bio-Rad) was used at a dilution of 1 : 2000. Antibody binding was visualized by enhanced chemiluminescence following manufacturer’s instructions.

**Flow cytometry analysis**

**Cell cycle analysis**

Cycle distribution of cells exposed to ambient or hypoxic conditions was evaluated. U373 and Daoy cells were either infected with RV at an MOI of 10 or left uninfected and exposed to either ambient oxygen concentration or 1% oxygen for 12, 24, 48, 72 and 96 h. Cells were washed with PBS, trypsinized, and the washed cell pellet was resuspended in PBS containing 10^6 cells/ml. Cells were permeabilized and stained by propidium iodide using the DNA Prep Reagent kit (Coulter Immunology, Hialeah, FL) according to the manufacturer’s protocol; the samples were measured with a FACSCalibur cytometer (Beckton Dickinson, San Jose, CA), and the data were analysed by software ModFit LT (Verity Software House Inc., Topsham, ME).
Apoptosis quantification

Cells cultivated in the presence or absence of RV in ambient or hypoxic conditions were harvested at various time points. Apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit according to manufacturer’s instructions (Biovision, Mountain View, CA). Cells were washed in PBS and resuspended in a binding buffer, incubated with annexin V and propidium iodide for 10 min at room temperature and then analysed using flow cytometry (FACSCalibur). Data obtained from flow cytometry were evaluated using the technique described by Bossy-Wetzel and Green (2000).

RV antigen-producing cells

RV+ cells were detected by indirect immunofluorescence measured by flow cytometry after permeabilization of the cells using a Fix & Perm kit (An der Grub, Kaumberg, Austria) according to the manufacturer’s protocol. As the primary antibody, we used MAB994 monoclonal antibody that reacts with RV type 3 σ1 haemagglutinin (Millipore, Billerica, MA); as the secondary antibody we used the FITC-Conjugated Goat Anti-mouse Immunoglobulin Polyclonal Antibody (BD). As a negative control we used non-infected cells cultivated under the same conditions.

Cell viability MTT test

After the desired period of time to permit the cells to grow in different concentrations of RV in a microtitre plate, the MTT solution (Sigma-Aldrich, Prague, Czech Republic) (2 mg/ml PBS) was added, the plates were incubated for 4 h and cells lysed in 50% N,N-dimethyl formamide (Sigma-Aldrich) containing 20 % of SDS (LifeTechnologies, Prague, Czech Republic), pH 4.5. The absorbance at 570 nm was measured for each well by the multi-well ELISA reader Versamax (Molecular Devices, Sunnyvale, CA). The mean absorbance of medium controls was the background and was subtracted. The absorbance of control cells was taken as 100 % viability and the values of treated cells were calculated as a percentage of the control cells. Each value is the mean of eight wells with a standard deviation. The IC50 values were calculated from the linear regression of the dose-log response curves by SOFTmaxPro (Molecular Devices).

Caspase-3 assay

Using Biovision Caspase-3/CPP32 Colorimetric Assay Kit (BioVision, Milpitas, CA) the level of caspase-3 was detected according to manufacturer’s instructions. Cells were lysed in a cell lysis buffer and placed in a microplate together with the caspase-3 substrate. After incubation for 2 h at 37 °C, the absorbance was measured, the background reading from cell lysates and buffers was subtracted, and fold-increase in caspase-3 activity was determined by comparing these results with the level of the uninduced control.

Statistical analysis

All experiments were performed at least in triplicates. All numerical data were presented as mean ± standard deviation and were analysed statistically using Student’s t-test. P values of less than 0.05 were considered significant. Software SPSS version 10.1 was used for statistical calculations.

Results

In vitro reovirus infection

To determine the susceptibility of human glioblastoma and medulloblastoma cell lines to RV compared to its effect on NHF, we infected these cells with RV at an MOI of 10. As shown in Fig. 1B, no morphological changes were detected in the NHF, even at 96 h after infection. In contrast, both tumour cell lines infected with RV exhibited meaningful cytopathic effects, i.e., rounding and clumping of cells. At 96 h post infection, nearly 85 % of both cancer cell lines examined were destroyed, as examined by optical microscopy (Fig. 1 D, F).

Hypoxia induces expression of HIF-1α

The HIF-1α expression was evaluated to verify whether the hypoxic phenotype was established in cell lines subjected to hypoxia. In all cancer cell lines used in this study, HIF-1α protein expression was increased in hypoxic conditions (1% O2) compared to ambient conditions (21 % O2). The results indicate that hypoxic conditions used in all experiments incited physiological response and did not reduce cell viability. It also suggests that in cell line Daoy used in this experiment, RV decreased the degradation of HIF-1α, and the effect of RV and of hypoxia was cumulative, see Fig. 2. Similar results were also observed in cell line U373 (data not shown).

Hypoxia does not reduce reoviral oncolysis in hypoxic cells

The aim of these experiments was to determine whether hypoxia affects the replication of wild-type RV and thus reduces its oncolysis. We evaluated the progression of RV infection using immunofluorescence microscopy and flow cytometry to compare the numbers of efficiently infected (producing RV proteins) cancer cells under ambient and hypoxic (1% O2) conditions. There was no significant difference between hypoxic and ambient samples infected with RV during the entire experiment, and the number of positively stained cells augmented with time (data not shown). We then determined by immunoblotting whether there was a difference in viral protein accumulation in normoxic and hypoxic cells. The levels of λ, μ and δ viral proteins appeared to be similar in normoxic and hypoxic infected cells (Fig. 3 A).

The cytopathic effects of RV under ambient or hypoxic conditions were compared, also using MTT test.
The percentage of surviving cells at various time points (12, 24, 48, 72, 96 h) after RV infection did not differ significantly between hypoxic and ambient samples, Fig. 3 B, C.

The mode of tumour cell death following RV infection differs in hypoxic and normoxic conditions

After we learned that RV is capable of killing cells in hypoxic as well as in normoxic conditions, we measured the proportion of apoptotic cells in these samples. Using cytometric detection of annexin V and propidium iodide binding we detected that significantly more infected cells died of apoptosis in normoxia than in hypoxia. To test our findings, prior to RV infection we treated cells with pan-caspase inhibitor ZVAD-fmk, which irreversibly binds to the catalytic site of caspase proteases and inhibits induction of apoptosis, to block apoptosis (Slee...
We proved in previous experiments that the concentrations of ZVAD-fmk we used were harmless for the cells (data not shown). At various time points post RV infection, we measured apoptosis using flow cytometry. As a positive control we used cells incubated with CDDP (10 μM), which is a well-known inducer of apoptosis via sequential activation of caspases. We found that the levels of induced caspase-3 did not differ significantly in hypoxia and normoxia. After adding RV to the cells, there was an increased activation of caspase-3 in normoxic but not in hypoxic conditions, see Fig. 5.

**Caspase-3 assay**

We performed a caspase-3 assay to confirm the apoptosis by a second independent method because some viral infections can induce annexin V binding even when apoptosis is not induced. As a positive control we used cells incubated with staurosporine, a non-selective protein kinase inhibitor that blocks many kinases through the prevention of ATP binding to them and thus initiates apoptosis (Chae et al., 2000). We found that the viability was the same in hypoxia and normoxia during the entire experiment, the levels of apoptosis significantly differed (Fig 4 E, F). There was a higher rate of early apoptosis (annexin V+ propidium iodide- cells) after RV infection in normoxia compared to corresponding hypoxic samples. However, after adding ZVAD-fmk to samples prior to RV, the level of apoptosis changed dramatically. ZVAD-fmk blocked apoptosis in normoxic samples completely, while in hypoxic samples it did not. There still remained some level of apoptosis in hypoxia which seemed to be caspase-independent.

**Is autophagy the type of death reovirus induces in hypoxia?**

We found no significant difference in normoxic and hypoxic samples nor in the samples infected with RV compared to non-infected ones, see Fig 6; similar results were observed in U373 cells. This means that the low level of autophagy induced by RV after 48 h in hypoxia was similar to that in the normoxic non-infected sample, while cells incubated with chloroquine showed a massively increased level of converted LC 3-II. Autophagy is, therefore, not the type of death that RV induces in hypoxia.

**Discussion**

We have proved that RV replicates preferentially in tumour cells. This is due to the fact that many cancer cells have a defect in the Ras transcriptional pathway, which makes them susceptible to RV (Smakman et al., 2005; Marcato et al., 2007). Normal cells are able to suppress viral infection even after RV has entered the cell, as we have demonstrated on NHF as a model of non-cancer cells. The differences between cancer and normal cells create a wide potential of therapeutic uses of RV as anticancer agent.

Since RV naturally colonizes gastrointestinal and respiratory tracts, it is exposed to a higher level of oxygen...
pressure. However, the conditions within a tumour mass differ with oxygen concentrations 10 mm Hg or lower (Hockel and Vaupel, 2001). It has been reported that this decline in oxygen concentration negatively affects ade-

Fig. 4. Apoptosis following RV infection in hypoxic and normoxic conditions. A – Graph of Daoy and B – U373 cell viability (percentage of annexin V- and propidium iodide-negative cells) 48 h post infection; C – Daoy; D – U373, early apoptosis (percentage of annexin V-positive and propidium iodide-negative cells) 48 h post infection; E, F – Prior to reovirus infection cells were treated with ZVAD-fmk inhibitor and staurosporine-treated cells were used as positive control. Percentage of surviving U373 cells – E; and percentage of early apoptotic Daoy cells – F.

Fig. 5. Caspase-3 assay. Cells were treated with RV (MOI = 10) and placed in hypoxia and normoxia. After 48 h post infection cells were harvested to perform caspase-3 assay. Cells treated with cisplatin (CDDP) (10 μM) were used as positive control.

Fig. 6. Detection of autophagy. Cells were treated with reovirus (MOI = 10) and placed in normoxia or hypoxia, incubation of cells with chloroquine (CHQ) was used as positive control. After 48 h cells were harvested and the expression of LC 3-II as an indicator of autophagy was examined by immunoblotting using anti-LC 3 antibody.

noviral (Pipiya et al., 2005) and VSV replication (Hwang et al., 2006). We have shown that RV is able to overcome cellular adaptation to hypoxia and infect and kill tumour cells. It can not only replicate in a hypoxic tumour microenvironment, but it can also induce the cytopathic effect and subsequently induce cell death. A large proportion of cells in hypoxia are killed by caspase-independent mechanisms. This might be important in cells
with caspase-8, were detected in several cancers (Olsson and Zhivotovsky, 2011). Furthermore, we found out that cell death induced by RV in hypoxic conditions is not caused by autophagy.

Hypoxia is regarded as a negative prognostic factor for malignant tumours because it causes resistance to radio- and chemotherapy (Shannon et al., 2003; Um et al., 2004). Therefore, agents are searched that are efficient both in normoxia and hypoxia. Hypoxia induces resistance by both HIF-1-dependent and -independent mechanisms (Rohwer and Cramer, 2011). HIF-1α is a transcription factor which mediates adaptive responses to changes in tissue oxygenation and is degraded in the presence of oxygen, while in hypoxic conditions it is stabilized (Metzen and Ratcliffe, 2004; Semenza, 2010). The actual contribution of different transcription factors to hypoxia-induced apoptosis resistance depends on several factors, e.g. cell type, severity and length of hypoxia, type of pro-apoptotic stimuli. We have shown an important role of HIF-1α in neuroblastoma cell lines in experiments with HIF-1α inhibition. The role of HIF-1α as an anti- or pro-apoptotic transcription factor is still discussed (Piret et al., 2002; Rohwer and Cramer, 2011) and constitutive expression of HIF-1α has been shown to restrict RV replication (Cho et al., 2010). We did not detect a decrease of HIF-1α caused by RV in brain tumour-derived cell lines as it was described in different carcinoma cells (Cho et al., 2010). Contrary to that, we found increased expression of HIF after RV infection in normoxia. A possible explanation of this contradiction may be the different biology of the tested cells or use of oxygen concentration – Cho et al. (2010) used 2% of O₂ whereas we used 1% concentration.

Preclinical studies have shown that RV proliferates only in tumour cells with activated genes of the Ras family or its pathway, which could be found in 60–80% of human malignancies. Oncolytics Biotech Inc. is currently guiding clinical studies with RV – Reolysin®. Completed studies, which included more than a hundred patients, demonstrated that intratumoral and intravenous application of Reolysin® is being well tolerated by patients (Morris et al., 2012). Also tested was a combination of Reolysin® with chemotherapy and/or radiotherapy (summarized by Figova et al., 2006). All of these studies have demonstrated the potential of RV as an anticancer agent. We have shown an additional advantage of the oncolytic RV, namely its ability to replicate in a hypoxic tumour microenvironment and kill even cancer cells with caspase deficiency. We have shown that it is not autophagy that RV evolves in hypoxia.

References


