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Genetic determinants of cell sensitization to parvovirus H-1-induced activation of non-apoptotic death pathways

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Alea iacta est.

(Julius Caesar at Rubicon River
January 10, 49 BC)

Summary

Gliomas are the most common brain cancers, characterized by an exceptionally wide cellular heterogeneity and extreme migratory features. The structural development of these neoplasms renders surgical removal of the tumoral mass almost prohibitive and inefficient. Moreover, these tumors are often resistant to chemotherapy treatments as a result of the onset of survival mechanisms occurring during astrocytes malignant transformation and counteracting the induction of apoptotic cell death. An alternative therapeutic approach relies on the use of autonomous parvoviruses. These small, non enveloped, single-stranded DNA viruses are endowed with the capacity to kill malignant cells while being non-cytopathic towards healthy tissues.

In particular, recent analyses on low passage cultures of human gliomas have demonstrated that the rodent parvovirus H-1 (H-1PV) induces death in cells resistant to conventional anticancer compounds. Among these, NCH82 cells have been chosen in this study to investigate the mechanisms of parvovirus H-1-induced glioma cell death. It has been observed that H-1PV triggers the formation of autophagic vesicles that are eventually involved in the cytosolic activation of lysosomal cathepsins B and L. The virus promotes efficient killing even in glioma cells overexpressing Bcl-2, an oncogene interfering with both apoptosis and autophagy induction. Besides, H-1PV-induced cathepsin B cytosolic activity is favoured by the down-regulation of cystatin B and C, two cathepsins inhibitors, and modulates caspase 3 induction. Glioma cells are protected from the viral lytic effect by autophagy inhibition, cathepsin B or L inactivation or cystatin B overexpression. Finally, cathepsin B *in vivo* activation upon parvovirus H-1 infection is associated with the regression of rat glioma cells intracranially implanted into recipient animals.

To set the basis for an extensive future study on the identification of the key genetic alterations that render tumor cells permissive to H-1PV, a preliminary analysis has been conducted on rat embryo fibroblasts (REFs). Different immortalized and transformed phenotypes have been induced in these cells by overexpressing *c-myc*, *SV40 large T antigen* and activated *Ha-ras* oncogenes, or by inactivating the anti-oncogene *p53*. Programmed cell death activation has been further analyzed in this model system to correlate the genetic determinants of H-1PV sensitiveness with specific molecular events leading to virus-induced cell killing. This work demonstrates that *c-myc* overexpression is sufficient to render REFs permissive to H-1PV-mediated cytolysis. While the virus accomplishes cytosuppression of *ras*/p53dn*-transformed REFs by activating classical apoptosis, it triggers in all the other transfectants a non-apoptotic death pathway characterized by the cytosolic accumulation of autophagic vesicles and active cathepsin B.

These observations indicate parvovirus H-1 as a potential novel therapeutic tool for cancer treatment through its ability to efficiently hijack both autophagic/cathepsins and apoptotic pathways, thus jeopardizing tumor cells survival.

Zusammenfassung

Gliome sind die häufigsten Hirntumoren. Sie weisen eine sehr hohe zelluläre Heterogenität auf und sind sehr stark migratorisch. Die strukturelle Entwicklung dieser Neoplasmen macht die chirurgische Entfernung des kompletten Tumorgewebes fast unmöglich. Desweiteren sind diese Tumoren oft resistent gegenüber Chemotherapien. Dies ist auf Mechanismen zurückzuführen, die das Übereleben der Zellen gewährleisten und die sich während der Entartung von Astrozyten zu bösartigen Tumorzellen entwickeln. Diese Mechanismen unterdrücken den apoptotischen Zelltod. Einen alternativen Therapieansatz stellt der Einsatz von autonomen Parvoviren dar. Diese kleinen, hüllenlosen, einzelsträngigen DNS Viren können maligne Zellen töten ohne dabei gesundes Gewebe zu gefährden.

Vor allem neuere Untersuchungen an Kurzzeitkulturen menschlicher Gliome zeigen, dass das Nager Parvovirus H-1 (H-1PV) Zelltod in Zellen induziert, die resistent gegenüber konventionellen Therapien sind. Die NCH82 Zellen wurden in der vorliegenden Arbeit ausgewählt, um die Mechanismen des parvovirus-induzierten Zelltods von Gliomzellen zu untersuchen. Es wurde beobachtet, dass H-1PV die Bildung von autophagischen Vesikeln auslöst. Diese Vesikel sind dann an der zytosolischen Aktivierung der lysosomalen Cathepsine B und L beteiligt. Das Virus führt selbst in Gliomzellen, die Bcl-2, ein antiapoptotisches Protein, das auch Autophagie verhindern kann, überexprimieren, zu einer hohen Zelltodrate. Außerdem wird die H-1PV induzierte zytosolische Cathepsin B Aktivität durch die Herunterregulierung von Cystatin B und C, zwei Cathepsin Inhibitoren, begünstigt und moduliert die Caspase 3 Induktion. Die Inhibition der Autophagie, Cathepsin B oder L Inhibition sowie Cystatin B Überexpression schützen Gliomzellen vor der Virus-bedingten Lyse. Die *in vivo* Aktivierung von Cathepsin B nach Parvovirus H-1 Infektion führt zu einer Regression von intracranial implantierten Gliomzellen der Ratte.

Um die Basis für intensive Untersuchungen zu schaffen hinsichtlich der Identifikation der wichtigsten genetischen Veränderungen, die dazu führen, dass Tumorzellen permissiv für H-1PV sind, wurde eine vorläufige Analyse an Fibroblasten von Rattenembryos (REFs) durchgeführt. Verschiedene immortalisierte und transformierte Phänotypen wurden in diesen Zellen induziert, indem die Onkogene *c-myc*, *SV40*, das *large T antigen* und aktiviertes *Ha-ras* überexprimiert wurden beziehungsweise das Tumorsuppressorgen *p53* inaktiviert wurde. Die Aktivierung des programmierten Zelltods wurde in diesem Model weiterhin untersucht, um die genetischen Determinanten der H-1PV Sensitivität mit molekularen Ereignissen, die zu Virus-induziertem Zelltod führen, zu korrelieren. Diese Studie zeigt, das die Überexpression von *c-myc* ausreicht, um REFs permissiv gegenüber H-1PV vermittelter Zelllyse zu machen. Während das Virus in *ras/p53dn*-transformierten REFs klassische Apoptose induziert, löst es in all den anderen transformierten Zellen einen nicht apoptotischen Weg des Zelltods aus, der sich durch zytosolische Akkumulation autophagischer Vesikel und aktives Cathepsin B auszeichnet.

Diese Beobachtungen offenbaren das Parvovirus H-1 als ein potenziell neues Werkzeug in der Krebstherapie, da es sowohl autophagische/Cathepsin-abhängige als auch apoptotische Wege des Zelltods in Tumorzellen induziert.

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ii List of Abbreviations

CDK	cyclin-dependent kinase
cds	gene coding sequence
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
$\Delta\Psi m$	mitochondrial membrane potential
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERK1/2	extracellular signal-regulated kinases
G6PD	glucose-6-phosphate dehydrogenase
GFP	green fluorescent protein
H-1PV	parvovirus H-1
HSV-1	herpes simplex virus type 1
largeT	SV40 *largeT* antigen
LB	Luria-Bertani broth
LC3	microtubule-associated protein 1 light chain 3
LMP	lysosomal membrane permeabilization
MAPK	Mitogen Activated Protein Kinase
MVM	minute virus of mice
myc	human *c-myc* oncogene
MMP	mitochondrial membrane permeabilization
mTOR	mammalian target of rapamycin
NDV	Newcastle disease virus
NS1/2	parvoviral non structural proteins
p53dn	dominant negative mutant form of human *p53* tumorsuppressor
PCD	programmed cell death
PDGFR	platelet-derived growth factor receptor
pfu	plaque forming units
PI3K	phosphotidylinositol-3 kinase
PKR	RNA-dependent protein kinase
PLA_2	phospholipase A_2

PTEN	phosphatase and tensin homology
ras*	activated human *Ha-ras* oncogene
REFs	rat embryo fibroblasts
(m)RNA	(messenger) Ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
RTK	receptor tyrosine kinase
SV40	Simian vacuolating virus 40
TNF	tumor necrosis factor
VP1/2/3	parvoviral structural proteins
VSV	vesicular stomatitis virus
VV	vaccinia virus

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1 Introduction

1.1. Gliomas

During vertebrate embryogenesis, stem cells that originate in the dorsal lip of the neural tube undergo epithelial-to-mesenchymal transition and migrate outwards to give rise to all cells of the peripheral and central nervous systems, including neurons, astrocytes and oligodendrocytes. When the developmental process is complete, neurons cease to proliferate and only a small compartment of stem cells remains, while glial cells retain the ability to propagate throughout life (Teng and Labosky, 2006). The most common adult neurological tumors are termed gliomas as they arise from glial cells, which are all non-neuronal cells of the nervous system and provide neurons with structural support and insulation, nutrients and oxygen supply, protection from pathogens. Gliomas encompass neoplasms composed predominantly of oligodendrocytes (oligodendrogliomas), astrocytes (astrocytomas), mixtures of various glial cells (i.e. oligoastrocytomas) and ependymal cells (ependymomas) (Kleihues and Cavenee, 2000). Neurological tumours include also cancers derived from non-glial cells of the central nervous system (CNS), like medulloblastoma, and from cells of the peripheral nervous system (PNS), the most common being neurofibroma and Schwannoma (Fig. 1). Neurological tumours have been classified on the basis of morphological and immunohistochemical criteria that identify their predominant cell type(s) (Zhu and Parada, 2002). This classification could result obsolete in the perspective of more recent findings demonstrating that brain tumours might be originating from brain-cancer stem cells arisen from normal $CD133^+$ stem or early progenitor cells (Singh et al., 2004).

Gliomas are short lasting cancers characterized by an exceptionally wide cellular heterogeneity and extreme migratory features. Surgical removal of the tumoral mass is almost prohibitive and inefficient, since these neoplasms infiltrate diffusely along the structural interfaces of the brain such as the corpus callosum, meninges, and the ventricular lining (Tysnes and

Mahesparan, 2001). Invasion of the surrounding tissue occurs via passive flowing within the cerebrospinal fluid or by active cell migration mediated by cell surface receptors (Pedersen et al., 1993; Thorsen and Tysnes, 1997). Moreover, the accumulation of multiple genetic alterations renders brain tumor cells resistant to death-inducing agents and in particular to chemotherapy treatments (Tysnes and Mahesparan, 2001). The prognosis for diagnosed gliomas is generally poor, even though it can differ according to clinical factors such as the age of the patient, the grade and the position of the tumour (Cooper, 2004). In England and Wales these neoplasms account for 1.6% of all cancers, being respectively the twelfth and fifteenth most frequent tumors in males and females, with survival rates around 12–13% for males, 15% for females (Cooper, 2004). In the United States, about 17.000 new malignant brain cancer cases are diagnosed each year, and more than 13.000 deaths are attributed to these cancers (American Cancer Society Surveillance Research, 2002). Worldwide, the median survival for primary glioblastomas patients is less than one year (Maher et al., 2001) while 20% are the long-term survivors, referred to as brain tumor cases with a life expectancy of more than 5 years (Wrensch et al., 2002).

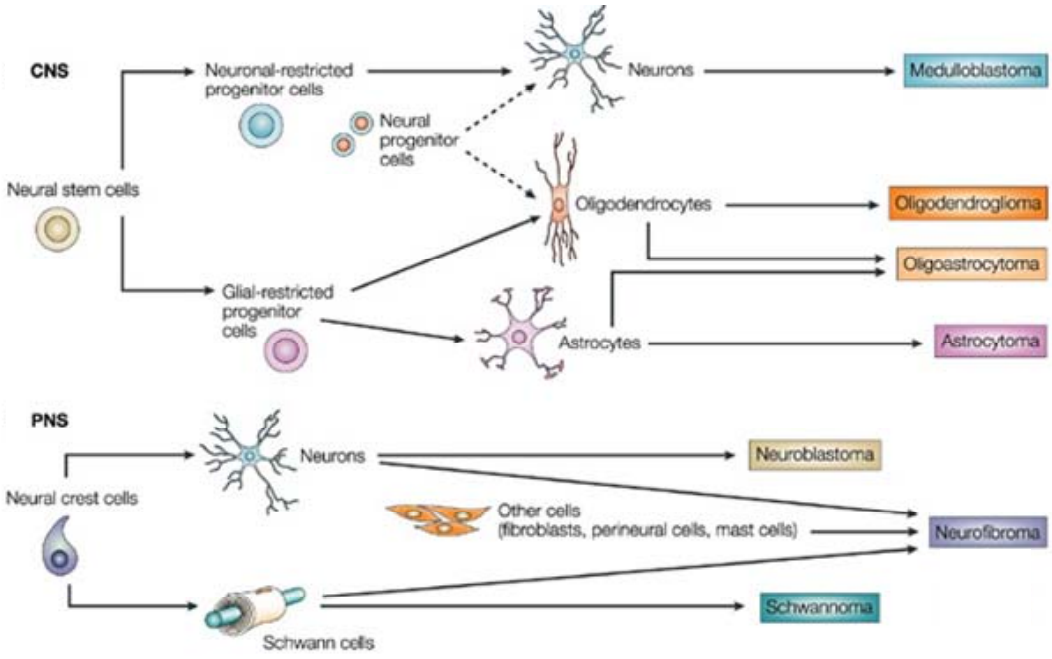


Figure 1. *Development of neuroectodermal cells and genesis of neurological tumors.* Adapted from Zhu and Parada (2002) The molecular and genetic basis of neurological tumours.

Altogether, gliomas seem to have withstood all attempts for curative treatment, and alternative therapeutic approaches are therefore eagerly sought.

1.2. Genetic alterations in gliomas

Brain cancer can progress *de novo* from glial cells to primary glioblastomas or from astrocytic tumors towards a more malignant phenotype and finally to secondary glioblastomas. The World Health Organization (WHO) grading system classifies gliomas into grades from I to IV by scoring their malignancy degree on the basis of histopathological identification (Fig. 2). Astrocytic brain tumours (WHO grade I) are generally benign neoplasms and might be in many cases circumscribed, whereas low-grade astrocytomas (WHO grade II) show diffuse infiltration capacities and tend to progress over a period of 5–10 years towards a more malignant phenotype. Astrocytomas account for more than 60% of all diagnosed brain tumours. Anaplastic astrocytomas (WHO grade III) have more pronounced invasive features and acquire migratory capacity, finally turning into secondary glioblastomas (WHO grade IV) that share histopathological and biological characteristics with primary glioblastomas (WHO grade IV). Primary and secondary gliomas, generically referred as glioblastoma multiforme (GBM), are considered the most malignant form of infiltrating brain cancers, and ranked among the most aggressive human tumors (Holland, 2001; Kleihues and Cavenee, 2000; Maher et al., 2001; Zhu and Parada, 2002).

Gliomagenesis results from the accumulation of multiple genetic alterations which cooperate in promoting uncontrolled proliferation, expanded migration, apoptosis evasion and a striking resistance to conventional drug treatments. Despite the extremely broad glial cell heterogeneity, it is possible to divide the mutations occurring with the highest frequency into five groups: inactivation of p53-dependent pathways, enhancement of RTK-activated pathways, interference with pRb-mediated cell cycle regulation, loss of PTEN locus, and promotion of the interactions between integrins and the extracellular matrix (Fig. 2).

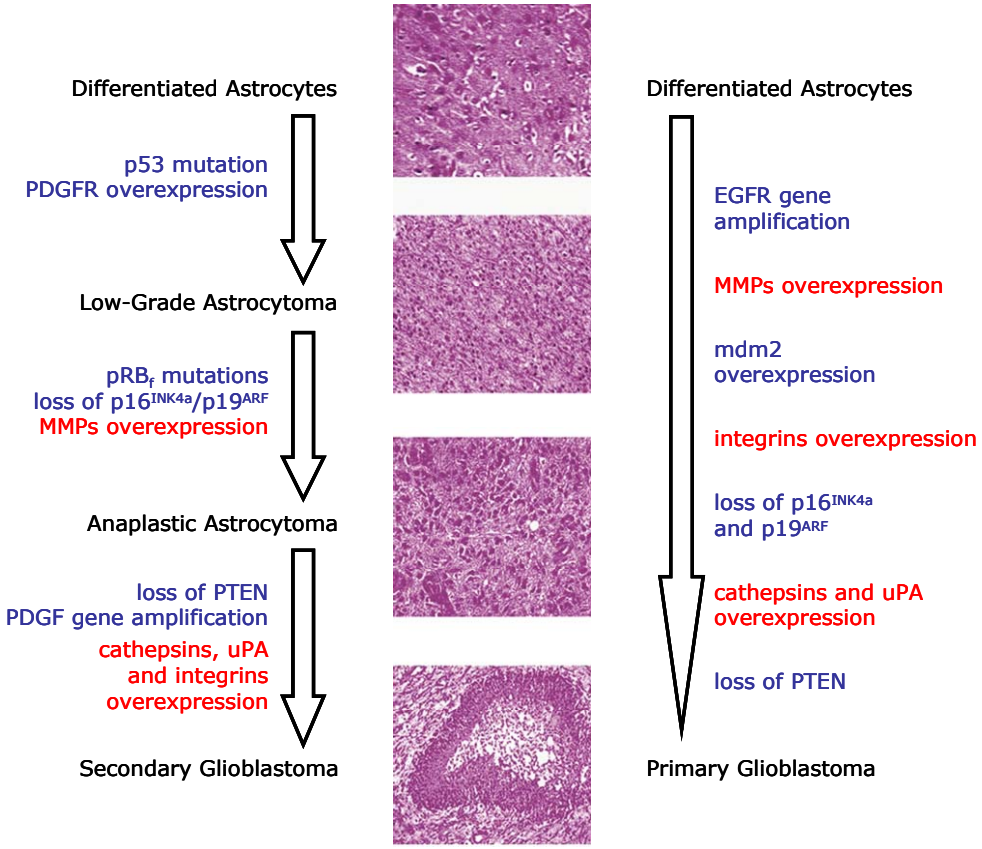


Figure 2. *Multiple genetic alterations during gliomagenesis.* Histological sections from Tysnes and Mahesparan (2001) Biological mechanisms of glioma invasion and potential therapeutic targets. Alterations proven to be associated with glioma evolution and progression are marked in blue. For abbreviations refer to text.

1.2.1. Inhibition of p53-dependent signalling

p53 has multiple functions (Vogelstein et al., 2000), including cell cycle control in response to DNA damage (Kastan et al., 1991), DNA repair (Smith et al., 1995), and induction of apoptosis (Lowe et al., 1993). p53 is directly inactivated in low-grade gliomas, anaplastic astrocytomas, and secondary glioblastomas by allelic loss of chromosome 17p or missense mutations targeting crucial residues for DNA binding (Maher et al., 2001). Moreover, loss of p53 leads to glial cell immortalization (Mathon et al., 2001). In mammals, p53 modulates cell response to DNA damage by blocking cell cycle progression through p21-dependent inactivation of G1 phase cyclin/cyclin-dependent kinases (CDK) complexes (Waldman et al., 1995). p53 also acts as a sequence-specific DNA binding protein which

activates the transcription of target genes, such as the pro-apoptotic protein Bax (Miyashita et al., 1994). Nevertheless, mutations in p53 downstream targets such as p21 and Bax have not been documented in gliomas (Maher et al., 2001). Frequently detected in primary glioblastomas is the gene amplification or overexpression of Mdm2 (Biernat et al., 1997a), which inhibits p53 transcriptional activity by direct binding and promotes p53 ubiquitination (Kubbutat et al., 1997). In addition, 40% of primary glioblastomas carry deletions of the INK4 locus (Zhu and Parada, 2002), whose alterative splicing product $p19^{ARF}$ (Sherr, 2001) stabilizes p53 by blocking Mdm2 (Pomerantz et al., 1998). Remarkably, p53 direct mutation and indirect inactivation seem to be mutually exclusive (Fulci et al., 2000). Furthermore, p53 mutations occur in the early phases of glioma development, in contrast to quite all the other human cancers having p53 mutations associated with the advanced stages (Zhu and Parada, 2002). This might reflect the need for astrocytoma cells to evade apoptosis in order to migrate and survive in adverse microenvironments, and could partially explain why gliomas, while being extremely invasive, are not metastatic (Tysnes and Mahesparan, 2001).

1.2.2. Activation of RTKs-mediated pathways

Receptor tyrosine kinases (RTKs) are transmembrane proteins with intrinsic tyrosine kinase activity, which control multiple downstream signalling pathways. Upon binding of diffusible growth factors, RTKs dimerize and initiate an autophosphorylation process that recruits adaptor proteins like Shc, which in turn mediate Ras activation. Three main downstream effector pathways can be triggered by Ras: the Raf/Mitogen Activated Protein Kinases (MAPKs) signalling cascade controls cell proliferation and differentiation, the phosphotidylinositol-3 kinase (PI3K) / protein kinase B (PKB/Akt) pathway promotes cell survival, and the CDC42/Rho signalling mediates gene expression and cytoskeletal organization (Heldin, 1996; Hunter, 2000; Sahai and Marshall, 2002). Despite being one of the most frequently mutated genes in human cancers, *ras* aberrations are not found in gliomas (Bos, 1989; Sahai and Marshall, 2002). Instead, platelet-derived growth factor (PDGF) seems to be a key regulator of glial cells development,

particularly oligodendrocytes (Yeh et al., 1993), and low-grade astrocytomas overexpress both PDGF ligands and receptors, possibly through the onset of an autocrine stimulatory loop synergizing with p53 inativation (Hermanson et al., 1992; Hermanson et al., 1996). In low-grade tumors, uncontrolled PDGF signalling is however not exerting a proliferative *in vivo* effect, perhaps because PDGF isophorms can associate in different dimeric receptors complexes and may preferentially activate the Ras-independent PI3K- and phospholipase C gamma (PLCγ) - signalling pathways, which trigger cell migration (Maher et al., 2001). Epidermal growth factor receptor (EGFR) may have a role in astrocyte differentiation, and appears to be necessary for sustained proliferation of neural stem cells. EGFR activation might be responsible for driving the transformation process toward high-grade gliomas, since EGFR gene is amplified in 50% of glioblastomas and in around 7% of anaplastic astrocytomas (Bigner et al., 1990; Lang et al., 1994; Schlegel and al., 1994). In addition, almost 40% of gliomas with EGFR gene amplification express a variant activated mutant form that lacks part of the extracellular ligand-binding domain and is constitutively autophosphorylated (Humphrey et al., 1990; Wong et al., 1992).

1.2.3. Disruption of pRb-mediated cell cycle regulation

The retinoblastoma family of tumour suppressors (pRb_f) consists of the three proteins pRb, p107 and p130 that are key regulators of the G1/S cell cycle checkpoint. In quiescent cells, pRb_f are maintained in a hypophosphorylated status that sequester E2F-family transcription factors, whereas in response to proliferative stimuli pRb_f get phosphorylated by cyclin-dependent kinases (CDK2/4/6), thus releasing E2F transcription factors that promote G1/S transition and DNA synthesis initiation. CDKs activity is positively regulated by formation of functional complexes with specific cyclins. Negative regulators of the pRb_f pathway are generic cyclin-dependent kinase inhibitors (CKIs) such as p21 and p27, and specific cyclin D - CDK4/6 complex inhibitors like $p16^{INK4a}$ and $p15^{INK4b}$ (Malumbres and Barbacid, 2001). The pRb regulatory circuit is disrupted in 80% of glioblastomas (Zhu and Parada, 2002), mainly via either loss of INK4a locus, loss of pRb or CDK4 amplification (He et al., 1995), but also through CDK6

and cyclin D1/E overexpression (Buschges, 1999; Costello, 1997) or E2F1 downregulation (Chakravarti, 2001). These genetic alterations are frequently mutually exclusive, but are rare in low-grade astrocytomas and therefore considered to be implicated not in glioma initiation but rather in tumour progression (Zhu and Parada, 2002).

1.2.4. Loss of PTEN

PTEN (phosphatase and tensin homology) gene encodes for a protein with a double phosphatase activity. PTEN controls cell migration and invasion by focal adhesion kinase (FAK) dephosphorylation through its N-terminal domain, which is highly homologous to tensin and auxilin, two proteins involved in interactions with actin filaments (Tamura et al., 1998). Besides, PTEN regulates cell proliferation and survival by blocking phosphatidylinositol (3,4,5)-triphosphate-dependent activation of the PKB/Akt pathway through its central domain, which shows homology to the catalytic region of protein tyrosine phosphatases (Maehama and Dixon, 1998). Loss of heterozigosity (LOH) on the long arm of chromosome 10, where PTEN maps, is found in 75–90% of high-grade gliomas, and *PTEN* mutations occur in 30–44% of anaplastic astrocytomas and glioblastomas (Maher et al., 2001). Furthermore, a recent study has proven that PTEN heterozygosity accelerates astrocytoma progression with a possible dose-dependent effect, and that Akt is regionally activated by serine 437 phosphorylation in 72% of these tumors (Xiao et al., 2002a). The discrepancy between LOH of 10p and mutation frequency of *PTEN* gene suggests that other glioma suppressor genes reside in this region. In this perspective, on the long arm of chromosome 10 maps a member of the Mad family of proteins, which are potent antagonists of c-Myc *in vivo* (Schreiber-Agus et al., 1998) and appear to be involved in neuronal differentiation (Queva et al., 1998). The impact of c-Myc disruption on gliomagenesis is controversial, since *c-myc* amplification is rarely detected in high-grade gliomas (Maher et al., 2001), but c-Myc is expressed at elevated levels in 78% of glioblastomas compared to normal brain tissues (Herms et al., 1999).

1.2.5. Integrins overexpression and cell migration

To migrate away from the tumor formation site, a glioma cell has to detach from neighbouring cells, digest the extracellular matrix (ECM) and move along a supporting surface (Uhm et al., 1999). Virtually all ECM proteins are localized in the normal adult brain at the perivascular space to form the blood–brain barrier (Carbonetto, 1984). High-grade glioma migration may take advantage of the ECM secreted by the normal surrounding tissue, even if ECM production is stimulated in these tumor cells. On the contrary, many members of the integrin family, in particular integrin $\alpha_3\beta_1$, are found overexpressed in gliomas, and control cellular locomotion by disassembling focal adhesions and reorganizing the actin filaments (Tysnes and Mahesparan, 2001). This effect is further enhanced by the cross-talk between the ECM/integrin and the RTKs signalling pathways via direct proximal clustering among integrins and growth factors receptors or through connecting proteins such as the focal adhesion kinase (FAK) (Uhm et al., 1999). Moreover, gliomas degrade different ECM basement components by overexpressing matrix metalloproteinases (MMP) 2 and 9 (Nakagawa et al., 1994), cysteine proteinases cathepsin B and L (Rempel et al., 1994), and the urokinase-type plasminogen activator (uPA) (Landau et al., 1994).

1.3. Standard therapies for glioma treatment

Currently, the standard therapeutic protocol for patients with high-grade malignant glioma is maximal resective surgery complemented by radiation therapy. However, total tumor resection is often not possible due to the critical localization of the neoplasm, which spreads in and surrounds the healthy portions of the brain. Incomplete removal of the cancerous mass has been shown to potentially induce early and aggressive tumor recurrence phenomena (DeAngelis, 2003; Giese and Westphal, 2001; Tysnes and Mahesparan, 2001). Radiotherapy is used as adjuvant treatment to increase patient survival expectances. Iridium and iodine-125 are the most commonly used radionuclides. In conventional radiotherapy protocols, patients are exposed to external beam radiation doses of 60 Gy (gray) in single daily

fractions of 1.7-2 Gy, five times a week. Approximately 50% of atrocytomas and 25% of glioblastomas decrease in size following radiotherapy (Leibel et al., 1989). In the treatment of tumor recurrence, stereotactic techniques are used to place catheters containing high-energy radioactive isotopes within brain tumors; this procedure is called brachytherapy and delivers an additional radiation dose of 50-60 Gy without tumoricidal effect on normal brain tissues (Castro et al., 2003). Stereotactic radiosurgery is a more recent and less invasive approach to treat well-defined lesions with a first 50 Gy dose and a second 15 Gy re-irradiation respectively applied right after tumor resection and 10 months later. Although preliminary results seem promising (Combs et al., 2005), adjuvant radiotherapy increases patients' lifespan of 14–36 weeks only (Grossman and Batara, 2004).

Nitrosoureas are lipid-soluble agents that cross the blood-brain barrier and have been employed in the chemotherapy of brain tumors as adjuvant treatment. Several strategies have been established for local delivery of chemotherapeutic compounds, including intra-arterial placement of microcatheters, implantation of slow-release biodegradable polymers, and use of convection-enhanced catheters to force the drug into non-resectable tumors (Castro et al., 2003). Despite being non curative and causing haematological, hepatic and pulmonary toxicity, the use of chemotherapeutics has been associated with improved survival (Giese and Westphal, 2001). Recently, the oral alkylating agent temozolomide has been demonstrated to increase survival when used in concomitance to radiation therapy in newly diagnosed glioblastoma patients. In addition, several biological modifiers such as anti-angiogenic agents and matrix metalloproteinase inhibitors have been tested in different clinical trials (Chamberlain, 2006). Nonetheless, the median overall survival of patients treated according to this advanced chemoradiotherapy protocol is improved of 2 months only compared to individuals receiving radiotherapy alone (Siker et al., 2006).

1.4. Gene therapy and glioma treatment

Given the striking resistance of brain tumors to conventional therapy, alternative therapeutic approaches are urgently required. Gene therapy is the insertion of genetic material into an individual's cells and tissues to treat a disease. Current attempts in this direction imply the direct *in vivo* delivery of therapeutic agents at the time of tumor resection by means of stereotactic injections, or the *ex vivo* genetic manipulation of the target cells that are subsequently implanted at the tumor site (King et al., 2005). Replacement gene strategy aims at the incorporation of functional genes to restore the activity of defective or absent genes, such as the tumor suppressors p53 or pRb (Riley et al., 1996). Another approach is to induce the expression of beneficial proteins, as angiostatin or endostatin, to inhibit endothelial proliferation and tumor-promoted angiogenesis (Ma et al., 2002; Peroulis et al., 2002). Antisense cDNA or oligodeoxynucleotides constructs have been also designed to block the expression of cancer genes by anti-parallel hybridization of the mRNA of interest. Several antisense constructs have been tested in glioma gene therapy, particularly against cellular growth factors such as bFGF, IGF-1, TGF-β, and VEGF (Engelhard, 1998). In parallel, gene therapy strategies have been studied with the aim to stimulate immune system activation against the tumor. Among others, have been evaluated the activation of T cells via tumor antigens upregulation (Parney et al., 1997) or induction of interleukins production (Okada et al., 1999), and the mobilization of dendritic cells through *ex vivo* manipulation (Aoki et al., 2001) or co-delivery with an interferon-α expressing vector (Tsugawa et al., 2004). Finally, a slightly different approach is to deliver a non toxic prodrug to the patient, and to force in tumor cells the expression of a specific enzyme that will convert the prodrug into a toxic product. A number of non-viral gene delivery systems have been developed for this technique (GDEPT - Gene-Directed Enzyme Prodrug Therapy), including naked DNA, liposomes, peptides and polymers. However, non-viral gene transfer has been increasingly replaced by the use of viral vectors modified by recombinant DNA technology to be more selective and efficient (Schepelmann and Springer, 2006). The prodrug ganciclovir has been administered in several glioma pre-clinical and clinical trials in combination with thymidine kinase

(TK) gene expression. Thymidine kinase phosphorylates the prodrug generating a nucleotide analogue that blocks DNA polymerase, thus inducing target cell death. Replication-defective adenoviruses (Immonen et al., 2004), bicistronic adeno-associated viral vectors (Okada et al., 1996), *ex vivo*-transduced retroviruses (Ezzeddine et al., 1991), intratumorally-implanted retrovirus-producing cells (Rainov et al., 1996), and replication-defective herpes simplex viral vectors (Miyatake et al., 1997; Moriuchi et al., 2005) have been all tested as conveyance methods.

1.5. Oncolytic viruses

Oncolytic viruses are replicating microorganisms endowed, either naturally or through appropriate engineering, with the capacity to infect and kill tumour cells. Oncolytic viruses selectively target cancer cells because they can take advantage of frequent tumour-specific mutations, be dependent on signalling pathways or transcriptional programmes that are constitutively activated in tumours, recognize cellular antigens that are unique or overexpressed on the tumour cell surface (Chiocca, 2002). Tumour cell is killed by the oncolytic virus as it takes over the cellular translational and/or transcriptional machinery, eventually inducing cell death through the activation of either necrotic or apoptotic pathways. Over the last decade, due to a better understanding of the molecular events ruling tumorigenesis, an oncolytic virotherapy platform has been developed (Bell et al., 2003). Up to the year 2005, an increasing number of different types of oncolytic viruses have been tested in preclinical trials, more than 50 phase I or II clinical trials have been conducted (Parato et al., 2005) and a phase III study has been published (Xia et al., 2004). The first tumor-selective lytic virus to demonstrate antitumor activity in a clinical setting was the adenoviral derivative ONYX-015; numerous viruses have been afterwards explored as tumor-selective replicating vectors, including herpes simplex virus (HSV), adenovirus, Newcastle disease virus (NDV), reovirus, poxvirus, vesicular stomatitis virus (VSV), and poliovirus (Nemunaitis and Edelman, 2002; Parato et al., 2005).

1.5.1. HSV-1

Herpes simplex virus type 1 (HSV-1) is a nuclear replicating, icosahedral, enveloped, double-stranded DNA virus belonging to the *Herpesviridae* family. HSV-1 is a human pathogen that generally causes gingivo-stomatitis and chronic skin keratitis (Lerner, 1980). HSV-1 is a neurotropic virus that has been therefore investigated for gene therapy treatments of malignant gliomas. Viral genome is linear, 152 kbp long, encoding for approximately 90 unique genes, half of which are essential for viral replication in permissive tissue cultures. Multiple transgenes can be simultaneously delivered and expressed in engineered HSV-1 mutants by deleting up to 30 kbp of viral genome. HSV-1 has a very wide host range, is highly infectious and efficiently expressing transgene products, and does not integrate its DNA into the cellular genome, eliminating the problem of insertional mutagenesis side effects. Two types of HSV-1 vectors have been used in cancer treatment. Replication-defective vectors express therapeutic genes inserted in amplicon plasmids containing HSV-1 packaging/cleavage signals and origin of replication, but are able to replicate only in cells that complement the deleted viral functions *in trans*. Conditionally replicating vectors are deleted for non-essential viral genes and preferentially infect, replicate in, and kill tumor cells (Shen and Nemunaitis, 2006). On the one hand, HSV-1 has been used for the delivery of suicide or therapeutic genes, such as native HSV-1 thymidine kinase combined with ganciclovir (Spencer, 2000), p53 (Rosenfeld et al., 1995), tissue inhibitor of metalloproteinase 2 (Hoshi et al., 2000), interleukins and interferons (Kim et al., 2000a). On the other hand, different strategies have been studied to genereate tumor-selective replication-conditional oncolytic HSV-1. Mutations and/or deletions have been inserted in one or more of the genes encoding thymidine kinase, DNA polymerase, uracil DNA glycosylase, and ribonucleotide reductase (Lou, 2003). Intratumoral administration of the TK-deleted strains has been shown to induce tumor necrosis and regression in several rat glioma models (Jia et al., 1994), but to lack tumor specificity in immunodeficient mice (Valyi-Nagy et al., 1994). ICP34.5 gene deletion results in the production of strains that are replication-defective in neurons but replicate efficiently in transformed cells (Andreansky et al., 1996). One of these, HSV1716, has been tested in

clinical trials involving high-grade glioma patients, and has proved to reduce the tumor size and to partially spread at distal tumor sites, without exerting clinical toxicity (Harrow et al., 2004). G207 is a second generation oncolytic HSV-1 virus that carries deletions in both ICP34.5 alleles and contains a detectable histochemical marker (Mineta et al., 1992). Animal experiments have demonstrated that multiple injections can be applied without inducing immune resistance to the virus, and phase I clinical trials in patients with malignant glial tumors are giving promising results (Markert et al., 2000). Further improvements consider the use of tumor- and/or tissue-specific promoters or surface proteins to alter viral tropism (Chung et al., 1999; Laquerre et al., 1998).

1.5.2. From Onyx-015 to chimeric adenoviruses

Most oncolytic adenoviral vectors are based on wild-type adenovirus serotype 5 (Ad5), a non-enveloped, double-stranded DNA virus with a 36 kbp linear genome (Bett et al., 1993). Onyx-015 is a modified adenovirus deleted for the E1B-55K gene product that replicates only in cells lacking p53, thus being selective for cancerous tissues (Heise et al., 1997). Onyx-015 potential efficacy and safety have been evaluated in different phase I and II studies for the treatment of malignant glioma and head & neck cancers (Chiocca et al., 2004; Khuri et al., 2000). The tropism of second generation conditionally replicating adenoviruses has been successfully redirected towards cancer cells by constraining essential viral genes under the control of heterologous promoters, enhancers, and silencers that are specifically active in tumour cells (Ko et al., 2005). In example, adenoviral derivatives have be engineered to be transcriptionally regulated by the prostate-specific antigen (PSA) (Schuur et al., 1996), E2F transcriptional factor (Parr et al., 1997) or the human telomerase reverse transcriptase (hTERT) (Iwamaru et al., 2006). In addition, chimeric viruses between different adenovirus serotypes have been generated to overcome Ad5 virus limited efficacy, due to the wide expression of its natural surface receptor in normal cells. For instance, have been tested chimeras based mainly on Ad5 genome, but expressing Ad3 fibre-knob protein that can selectively and efficiently infect ovarian cancer cells (Breidenbach et al.,

2004). The first gene therapy-associated death occurred in a patient suffering from a genetic disease and treated with a high dose of adenovirus (Lehrman, 1999); since then adenoviral vectors have been improved to limit their potential capacity to trigger a strong immunoresponse (Yla-Herttuala and Alitalo, 2003). To date, a subset of transcriptionally regulated selectively replicating oncolytic adenoviruses has proceeded to the completion of a phase III trial in China and a proposed phase III study in the United States, thus representing one of the most advanced candidates for virotherapy (Aghi and Martuza, 2005).

1.5.3. NDV and reoviruses

Newcastle disease virus (NDV) is an avian enveloped single-stranded 16 kb RNA paramyxovirus. Despite being potentially fatal to birds, NSV can infect humans causing only minor illnesses such as fever or flu-like symptoms (Alexander and Allan, 1974). Several lytic strains show enhanced replication efficiency in cancer cells and have been therefore tested for a possible clinical application. It has been proposed that NSV strains display a conditionally replication-competent behaviour because of Ras signaling pathway activation in neoplastic cells, as it has been demonstrated for reoviruses (Lorence et al., 1994a). NDV has undergone preclinical animal studies to test its efficacy against human gliomas (Lorence et al., 1994b), and is currently studied for the treatment of various cancers with different administration regimens (Schirrmacher et al., 2001). Some serious adverse events, including a patient's death, have been noted in a phase I trial with PV701 oncolytic strain (Phuangsab et al., 2001). On the contrary, MTH-68/H strain has been successfully used in a desperate case of a 14-year-old boy with a recurrent glioblastoma (Csatary and Bakacs, 1999). NDV has shown much more potential as a cancer vaccine therapy, where the virus is used as adjuvant to boost the host immune system (Schirrmacher et al., 1998). NDV replicates in the host cell cytoplasm with strong cell-binding properties, and enhances in infected tumors cells the recruitment and activation of natural killer and cytotoxic T cells (Haas et al., 1998).

Reoviruses are non-enveloped viruses with a 18-30 kbp segmented, double-stranded RNA genome (Fields et al., 1996). In infected cells, a

specific protein kinase (PKR) recognizes the double-stranded RNA and activates a host response against the virus. Hence, reoviruses have tropism for tumors cells where Ras signaling pathway, which interferes with PKR activation, is disrupted. Indeed, cells that are resistant to reovirus infection become susceptible after transformation with factors involved in the Ras signaling cascade (Strong et al., 1998). Reovirus infections tend to be asymptomatic, despite some cases of reported mild respiratory or gastrointestinal tract symptoms (Fields et al., 1996). Thus, reovirus efficacy as potential replication-competent virus in the treatment of gliomas has been investigated *in vitro*, *in vivo*, and *ex vivo*, using an immune-competent mouse model and even challenging animals with reovirus prior to tumor implantation (Coffey et al., 1998; Wilcox et al., 2001). Albeit a serious toxicity has been reported in immunodeficient mice, no dose-limiting toxicities have been recorded in phase I trials of reovirus (Reolysin™) administered via direct intratumoral inoculation in patients with recurrent malignant glioma (Oncolytics Biotech Press, 2006).

1.5.4. Vaccinia and myxoma viruses

Poxviruses are a family of enveloped, double-stranded, linear DNA viruses that present the uniqueness of remaining in the cytoplasm of infected cells for the entire life cycle, without translocating in the nucleus during DNA replication and transcription. Producing their own transcriptional machinery, poxviruses resist host cell's attempts to counteract infection, and have been studied for application in virotherapy (Moss, 1996).

Vaccinia virus (VV) is a member of the *Poxviridae* family that has been scrutinized as agent against malignant gliomas. The genome consists of 192 kbp with inverted terminal repeats and is packed in a particle of 200 nm in diameter and 300 nm in length (Smith and Vanderplasschen, 1998). VV has a distinct history as a vaccine used for the eradication of smallpox, and no known natural host has been identified so far (Baxby, 1985; Parrino and Graham, 2006). This virus has tropism for the ovary and tumor tissues, perhaps because the leaky vascular barrier surrounding tumors and ovarian follicles allows a better viral escape from the circulation (Goede et al., 1998). Though VV was extensively studied for its utilization as vaccine for anti-

cancer therapy, researchers have more recently evaluated the oncolytic ability of this virus to make vaccinia an anti-tumor agent (Parato et al., 2005). A recombinant vaccinia virus expressing p53 (rVV-p53) has been positively tested as potent apoptosis inducer and direct oncolytic agent in several human and rat glioma cell lines (Timiryasova et al., 1999a) and in the C6 rat glioma model in combination with radiation therapy (Gridley et al., 1998; Timiryasova et al., 1999b). Other recombinant vaccinia vectors expressing the murine interleukins IL-2 and IL-12 have been proven *in vitro* and *in vivo* to enhance VV oncolytic effect (Chen et al., 2000; Chen et al., 2001). Furthermore, vaccinia growth factor (VGF) and thymidine kinase genes single or double deletions have been used as tool to attenuate vaccinia virus and render the vector tumor-selective (Buller et al., 1985; Puhlmann et al., 1999).

Myxoma virus is an oncolytic poxvirus that has a narrow rabbit-specific tropism and is non-pathogenic in humans (McFadden, 2005), but has been shown to kill several human tumor cell lines (Sypula et al., 2004), including glioblastoma-derived cultures (Lun et al., 2005). Myxoma virus expresses gene products that counteract rabbit interferons but are unable to antagonize interferons from other species, including mice and humans. Hence, in normal human cells myxoma virus infection is blocked at a very early stage while the virus is able to replicate and exert a lytic effect in interferon-non-responsive human tumour cells (Wang et al., 2004a).

1.5.5. VSV and poliovirus

Vesicular stomatitis virus (VSV) is a 11 kilobases negative-stranded RNA virus belonging to the *Rhabdoviridae* family. VSV glycoprotein (G) is a major antigen responsible for type specificity and provides the virus with a wide tropism, ranging from vertebrates to invertebrates and even plants. VSV replicates in the cytoplasm of infected cells, does not undergo genetic recombination or reassortment, has no known transforming potential and does not integrate any part of its genome into the host (Dietzschold et al., 1996). Evidences indicate that VSV endocytosis occurs via binding to phosphatidylserine (PS) residues on the target membrane, while a drop in endosomal pH causes the release of the viral core into the cytoplasm

(Carneiro et al., 2002). VSV non-lethal infections may occur in farm animals, while the virus exerts a neuropathic action in mice (van den Pol et al., 2002) but is essentially asymptomatic for humans (Dietzschold et al., 1996). Besides, VSV replicates to high levels in numerous types of transformed and malignant tissue cultured cells, and rapidly induces apoptotic cell death. VSV has been now reported to be efficacious against cancers that are associated with persistent viral infection, such as hepatocellular carcinoma (Suriawinata and Xu, 2004), but also malignant glioma, melanoma, breast adenocarcinoma, and prostate cancers (Livingston et al., 1985; Obuchi et al., 2003). VSV oncolytic action has been correlated to defects in the innate immune responses of these cell types (Balachandran and Barber, 2004; Stojdl et al., 2003), either in interferon induction or signalling cascade activation (Dunn et al., 2002; Persing and Prendergast, 1999).

Poliovirus is a non-enveloped, RNA enterovirus with a positive-stranded genome of 7500 nucleotides in length, and can cause illnesses ranging from minor symptomatic infection to aseptic meningitis or paralytic poliomyelitis (Racaniello, 2006). Replacing the internal ribosomal entry site (IRES) located at the 5´-untranslated region of viral genome with a non-pathogenic but structurally related element of human rhinovirus type 2 attenuates the virus without affecting its ability to grow in non-neuronal cells (Gromeier et al., 1996). This intergeneric poliovirus, designated as PV1(RIPO), is not neuropathogenic and has been tested as oncolytic virus in brain cancer therapy. It has been demonstrated that human glioma cells susceptibility to poliovirus PV1(RIPO) depends on the expression of the human poliovirus receptor CD155 (Mendelsohn et al., 1989) but the virus has to be inoculated intratumorally in order to exert an efficient anti-tumor action (Gromeier et al., 2000). To improve poliovirus vectors´ safety for administration in the central nervous system as a possible treatment for gliomas, alternative candidate oncolytic agents derived from poliovirus have been designed (Jackson et al., 2001). Poliovirus genome is engineered to generate replicons that have deleted the gene encoding the capsids protein P1 and are therefore incapable to generate infectious progeny particles (Hagino-Yamagishi and Nomoto, 1989). Replicons are produced by complementation *in trans* of the capsid protein from a recombinant vaccinia virus (Ansardi et al., 1991) and induce oncolysis in different human

glioblastoma cell lines and low-passage cultures from patients (Ansardi et al., 2001). Besides, replicon oncolytic effects have been further potentiated by replacing the capsids P1 gene with a therapeutic gene, like the murine TNF-α (Bledsoe et al., 2000) or interleukin-2 (Basak et al., 1998).

1.6. Autonomous parvoviruses

The work conducted in the past by the group of Prof. J. Rommelaere has shown that autonomous parvoviruses circumvent tumor cell escape mechanisms and do kill tumor cells, thus yielding promising candidates for innovative cancer virotherapy (Rommelaere and Cornelis, 2001).

Parvoviruses are among the smallest animal DNA viruses. The family *Parvoviridae* is divided into two subfamilies: the *Densovirinae*, which infect invertebrates, and the *Parvovirinae*, which infect vertebrates and include the three genera *Parvovirus*, *Erythrovirus*, and *Dependovirus* (Muzyczka and Berns, 2001). Dependoviruses or adeno-associated viruses (AAVs) incorporate several different serotypes, and infect many types of cells and tissues of various species, including humans (Shaughnessy et al., 1996). AAVs efficiently infect the host cell but require a helper adenovirus or herpesvirus to supply functions needed for complete replication. When inoculated in cells alone, the genome may integrate into the host chromosome in a site-specific manner thus establishing latency (Chiorini et al., 1996). On the contrary, autonomous parvoviruses, *Parvovirus* and *Erythrovirus*, do not need a helper virus but require factors of the cellular S-phase for their replication (Rommelaere and Cornelis, 2001).

The erythrovirus B19 is the only family member demonstrated to be pathogenic in humans. This virus causes arthropathy in adults, aplastic anaemia in patients with sickle cell disease or childhood fifth disease (erythema infectiosum), and rare foetal infections (Brown et al., 1994). Rodent parvoviruses [minute virus of mice (MVM), LuIII, parvovirus H-1 (H-1PV) and Kilham rat virus (KRV)] generally infect rodent cells, but are able to replicate in a variety of different cells from other species, including humans. In the natural host, parvovirus infections of foetuses or neonates often term fatally, and are characterized by several symptoms such as

enteritis, hepatitis, cerebellar hypoplasia and ataxia, leucopoenia, haemorrhages of the liver, lung, brain, and intestine (Brownstein et al., 1991; Jacoby et al., 1996). On the contrary, parvoviruses are non-pathogenic in adult animals and seem to be associated with low or no immunogenicity. In humans, rodent parvoviruses infection causes viremia and seroconversion without pathological implications (Rommelaere and Cornelis, 2001). Parvoviruses are unique among DNA viruses in that they do not have any tumorigenic members (Berns, 1996). Moreover, these viruses have been shown to have oncosuppressive effects, inhibiting the formation of spontaneous as well as chemically or virally induced tumours in laboratory animals (Rommelaere and Cornelis, 2001).

1.6.1. Capsid structure and genome organisation

Autonomous parvoviruses are small (20-25 nm in diameter), non-enveloped, nuclear-replicating single-stranded DNA viruses. Parvoviral capsid is icosahedral, consisting of 60 subunits assembled with the three structural proteins VP1 (83-86 kDa), VP2 (64-66 kDa), and VP3 (60-62kDa) (Agbandje-McKenna et al., 1998). VP2 and VP3 constitute the majority of capsid proteins in full virions, VP2 being the most abundant (80%) and VP3 absent in empty particles (Tattersall et al., 1976). VP1 comprises the C-terminal 64 kDa region of VP2, and its N-terminal domain is required for the nuclear export of full virions (Valle et al., 2002). VP3 is formed in full capsids by cleavage of 25 amino acids at the N-terminus of VP2, which are exposed outside the particle (Weichert et al., 1998). Despite only about nine VP1 molecules are inserted per viral capsid, this polypeptide has been show to be essential for infectivity because its N-terminal sequence contains a phospholipase A_2 (PLA_2) motif which appears active when released from the capsid (Zadori et al., 2001). X-ray crystallography studies on parvovirus three-dimensional conformation have revealed the presence of a cylindrical structure surrounded by a canyon-like depression at about the fivefold axes, spike protrusions at the icosahedral threefold axes, and a dimple-like depression at the twofold axes (Agbandje-McKenna et al., 1998; Hernando et al., 2000) (Fig. 3A). The expression of specific sequences on the capsid surface allows some parvoviruses to infect several hosts and a variety of

tissues, in contrast to many parvoviruses that have tightly controlled host ranges and tissue tropisms (Agbandje-McKenna et al., 1998; Hueffer et al., 2003).

Viral genome consists of approximately 5150 nucleotides linear DNA, flanked by palindromic sequences that can form hairpin structures and serve as self-priming origins of DNA replication. The open reading frame (ORF) at the left side is controlled by the strong P4 promoter and directs the expression of the non-structural proteins NS1 and NS2. The coding sequences for the N-terminus of NS1 and NS2 overlap and the two proteins are produced after differential messenger RNA (mRNA) splicing. The right-hand promoter (P38) controls a second ORF driving the expression of the structural proteins of the capsid, which are translated using two distinct initiation codons as a result of differential splicing of the precursor mRNA (Cotmore and Tattersall, 1987) (Fig. 3B). NS1 is a 83 kDa nuclear

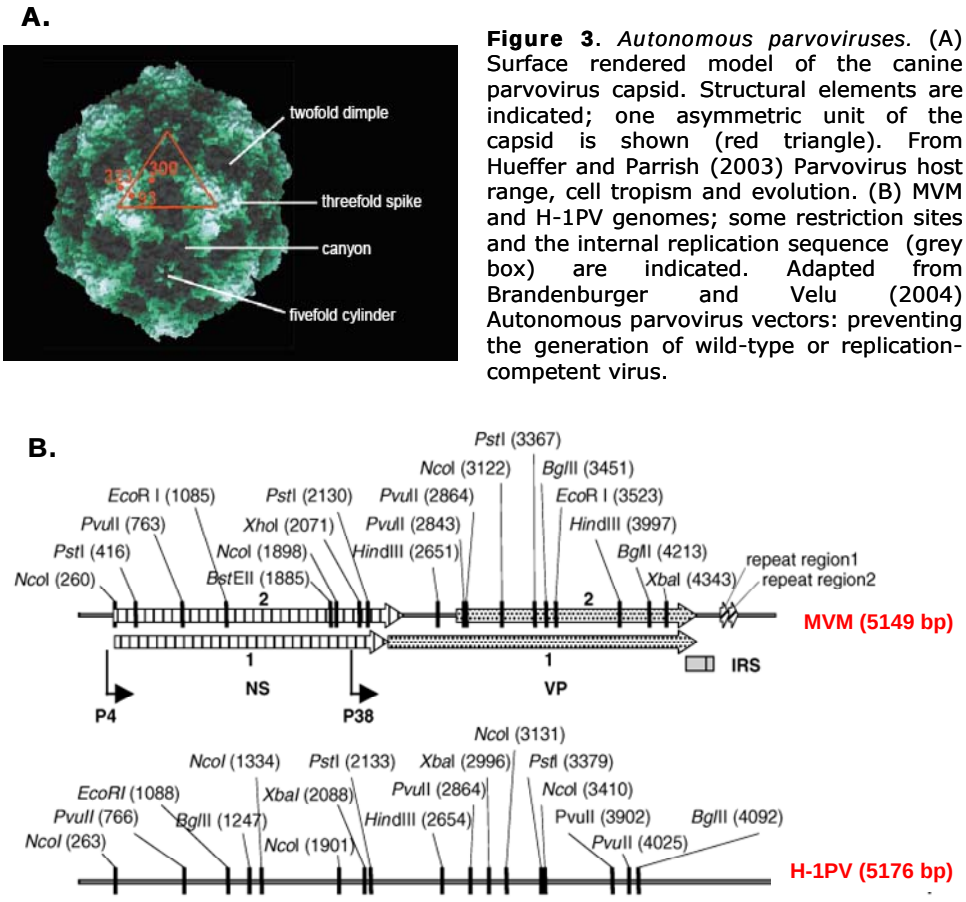


Figure 3. *Autonomous parvoviruses.* (A) Surface rendered model of the canine parvovirus capsid. Structural elements are indicated; one asymmetric unit of the capsid is shown (red triangle). From Hueffer and Parrish (2003) Parvovirus host range, cell tropism and evolution. (B) MVM and H-1PV genomes; some restriction sites and the internal replication sequence (grey box) are indicated. Adapted from Brandenburger and Velu (2004) Autonomous parvovirus vectors: preventing the generation of wild-type or replication-competent virus.

phosphoprotein that is required for replication of the viral genome after incoming single-stranded DNA has been converted into a duplex form. NS1 is

a multifunctional protein that exerts helicase, DNA nicking, ATPase, sequence specific DNA binding, and endonuclease activities. In addition, NS1 is a transcriptional factor trans-activating the P38 promoter (Rhode, 1985), and is considered the major mediator of parvovirus-induced cytotoxicity (Caillet-Fauquet et al., 1990; Li and Rhode, 1990). Importantly, NS1 phosphorylation has been demonstrated to be mediated by members of the protein kinases C family (PKC) and to control both the replicative and cytotoxic functions of this viral protein (Corbau et al., 2000; Lachmann et al., 2003; Nüesch et al., 1998). NS2 function has been studied mainly in mouse cells infected with MVM, where it has been observed to be involved in the synthesis of viral DNA replicative form, in progeny production, in VP1 and VP2 proteins assembly (Cotmore et al., 1997). NS2 seems to be non-essential in cells from other species and therefore influence parvoviral life-cycle in a host-specific manner (Naeger et al., 1990).

1.6.2. Parvovirus life cycle

Autonomous parvovirus infection occurs upon recognition of a target cell surface rerceptor. The human parvovirus B19 virus replicates only in human erythroid progenitor cells after binding to the erythrocyte P antigen (globoside) cellular receptor (Brown et al., 1993). Both canine parvovirus (CPV) and feline panleukopenia virus (FPV) bind to *N*-glycolyl neuraminic acid-containing glycoproteins expressed by most of their host cells in a temperature and pH dependent manner (Barbis et al., 1992; Tresnan et al., 1995). However, cell infection by CPV and FPV is dependent on the interaction with the transferrin receptor and following clathrin-mediated formation of endocytic vesicles (Suikkanen et al., 2002). Parvoviruses H-1 and MVM bind to ubiquitous *N*-acetylneuraminic acid (sialyl)-containing glycoproteins located at the cell surface. Viral genome is likely to be transported via late endosomes to perinuclear vesicular compartments, and released from capsids into the nucleus by the VP1-specific PLA_2 activity (Zadori et al., 2001). The initiation of parvovirus genome replication is S-phase dependent, as it requires the activity of the host cyclinA/CDK2 kinase complex to convert input single-stranded DNA into a duplex replication form (Bashir et al., 2000). Duplex parvoviral DNA serves as a

template for P4 promoter-driven synthesis of NS proteins mRNAs. A continuous elongation mechanism, promoted by NS1 and other host factors, results in the formation of multimeric double stranded intermediates that do not integrate in the host genome (Rommelaere and Cornelis, 2001). NS1 is covalently attached to the 5'-end of viral DNA and operates the production of a single stranded full length DNA progeny, in synchronization with the encapsidation process (Berns, 1996). Despite packaging signals have not been described for autonomous parvoviruses, CPV viral genome is bound to the interior of the capsid by 11 nucleotide oligomers (Chapman and Rossmann, 1995) that could be involved in the correct packaging of viral progeny (Tsao et al., 1991). Parvovirus replication in permissive cells leads to the release of progeny virions and is usually associated with cell death (Rommelaere and Cornelis, 2001).

1.6.3. Parvovirus oncotropism

Parvoviruses, in particular H-1PV and MVM, preferentially infect highly proliferating and generally poorly differentiated cells (Cotmore and Tattersall, 1987). Anyway, transformed cells are more sensitive to virus-induced cell killing because they set up an environment favourable to parvovirus multiplication. Indeed, the proportion of cells enabling NS1 nuclear accumulation is considerably lower in non-transformed cultures than in their transformed derivatives (Dinsart et al., 1996). However, in normal cells parvovirus uptake is not enhanced by the transformation process, indicating that transformed cells are more sensitive to viral infection because they are capable to better sustain a productive parvovirus cycle by influencing in particular DNA amplification, gene expression and cell killing (Rommelaere and Cornelis, 2001). As matter of fact, *ras* oncogene transformation mediates a strong induction of the P4 promoter via binding of transcription factors of the ATF/CREB, Ets, and Sp1 families (Fuks et al., 1996; Perros et al., 1995), while Simian vacuolating virus 40 (SV40) large T antigen activates P4 through the modulation of a distinct subunit of the transcription factors nuclear factor Y (NF-Y) (Gu et al., 1999). Moreover, P4 promoter activity is dependent on cellular transcription factors of the E2F and ATF family that normally control the G1/S cell cycle transition (Deleu et

al., 1998). Hence, oncogenic transformation is accompanied by cell cycle and differentiation deregulations (Hanahan and Weinberg, 2000) that stimulate the generation and expression of viral transcription templates and explain autonomous parvoviruses privileged tropism for cell of tumoral origin. Parvovirus oncotoxicity has been well documented in tumor-derived and *in vitro*-transformed human and rodent cell lines, but also in short-term cultures of human breast and liver carcinoma cells and malignant gliomas. Remarkably, parvoviruses selective oncolytic effect has not been described in the corresponding normal non-transformed counterparts (Cornelis et al., 1988; Faisst et al., 1989; Herrero y Calle et al., 2004; Lin et al., 1989; Salomè et al., 1990; Van Pachterbere et al., 1993).

1.6.4. Recombinant parvoviruses for gene therapy

Genetic engineering of parvoviruses has been tested with the aim of retaining the oncolytic and oncotropic properties while improving the infection efficacy for applications in cancer prevention or established tumors eradication. Recombinant vectors have been derived from the autonomous parvoviruses H-1, LuIII and MVM, but LuIII-derived vectors are less efficient since do not express NS proteins and therefore do not amplify (Maxwell et al., 2002). Parvoviral genome dimensions limit the insertion of therapeutic or reporter genes to a capacity of 800 bases without consequences, or up to 1600 bases but affecting vector production by markedly reducing viral titer (Kestler et al., 1999). All currently proposed recombinant vectors (NS^+VP^-) maintain the NS transcriptional unit in order to exert all the oncolytic and oncotropic features of parvoviruses. The capsid proteins encoding region is removed or truncated and replaced with target transgenes under the control of the P38 promoter, so that their expression is restrained to cells that also express NS1; VP proteins are produced either from a helper plasmid or in packaging cells (Brandenburger and Velu, 2004). The major problem encountered with these vectors is the generation of wild-type or replication-competent virus (RCV) through recombination with helper plasmids (Brandenburger et al., 1999). To decrease homology, sequence modifications in the vector upstream as well as downstream of the transgene have been studied. Alternative approaches have been also taken into consideration,

since autonomous parvoviruses limited genome and the presence of overlapping coding regions render it difficulty to remove all homology. Recombination phenomena have been partially limited by the combined use of vectors carrying minimal sequences required for their replication and helper plasmids with degenerated codons (Dupont et al., 2001). Besides, the construction of chimeric H-1PV/MVM vectors (Palmer and Tattersall, 2000), the use of two separate helpers for VP1 and VP2 expression (Brown et al., 2002), and vector pseudotyping has been investigated (Wrzesinski et al., 2003).

Suicide genes encoding for toxins like HSV-1 thymidine kinase (TK) and chicken anemia virus Apoptin are within the size limits required for proper capsid packaging, and have been tested *in vitro* to evaluate their potential in increasing parvovirus lytic action (Dupont et al., 2000; Olijslagers et al., 2001). In addition, H-1PV or MVM vectors have been designed to express immune modulators in order to expose cells that are not directly hit by the vector to the cytotoxic response elicited by neighbouring cells. In example, parvoviral vectors harbouring interleukin-2 (IL-2) gene have been used to inhibit tumor formation in both nude and immuno-competent mice, upon implantation of human or mouse carcinoma cells. When vectors are inoculated to tumor cells prior to implantation (*ex vivo*), IL-2 is secreted within the tumor and induces natural killer (NK) cells recruitment and activation, while tumor-free mice develop anti-tumor immunity and are protected against a second challenge with tumor cells (El Bakkouri et al., 2000; Haag et al., 2000). A similar tumorsuppressive effect is observed when mouse melanoma cells are grafted in nude mice after infection with an H-1PV-based vector expressing the human monocyte chemotactic protein 3 (MCP-3), which triggers macrophages and NK cells activation (Wetzel et al., 2000). Furthermore, the mouse anti-angiogenic chemokine IP-10 (interferon γ – inducible protein 10), has been introduced in a MVM-backbone and the anti-metastatic potential of this parvoviral vector has been evaluated in a mouse hemangiosarcoma model. While wild-type MVM inhibits the formation of metastases only when tumor cells are infected *ex vivo*, recombinant MVM transducing IP-10 has a protective effect even when intratumorally administered *in vivo* to mice bearing neoplastic lesions (Giese et al., 2002).

1.6.5. Basis for the use of parvovirus H-1 in glioma therapy

Given the properties of autonomous parvoviruses outlined so far, wild-type parvovirus H-1 has been scrutinized for its possible use in oncolytic brain tumour therapy. Cell permissiveness to H-1PV infection has been analysed in established glioblastoma cell lines of human and rat origin as well as in short-term and low-passage cultures of human brain tumours. In the malignant glial cells tested, parvovirus H-1 induces an efficient and dose-dependent killing even at low multiplicities of infection. Moreover, progeny virions are released from these infected cells, indicating that H-1PV might spread its oncolytic effect through successive rounds of infection (Herrero y Calle et al., 2004). Parvovirus H-1 infections have been further analyzed in extracts from normal tissue to confirm the specificity of viral cytopathic effect for neoplastic cells. Supporting the rationale that the normal adult brain is not a mitotically active organ and therefore not prone to parvovirus cytotoxicity, human brain cells isolated from epileptic patients are not permissive to H-1PV, and intracerebral injections of the virus do not cause damage in the brain of immunocompetent adult rats (Geletneky et al., 2005). Finally, no pathologic side effects have been reported in early times clinical trials with parvovirus H-1 in patients with advanced disseminated cancer (LeCesne et al., 1993; Toolan et al., 1965).

1.7. Programmed cell death

Tumor cells differently react to autonomous parvoviruses infections; according to cell type and to limiting environmental conditions, different death pathways can be triggered by these viruses. Besides, several programmed cell death mechanisms can be activated by glioma cells in response to diverse stimuli, such as chemotherapy toxic action or ionizing radiation-induced damage.

Programmed cell death (PCD) is a term used to define a genetically controlled death program, executed by active cellular processes that can be intercepted by interfering with intracellular signalling. PCD is by definition

opposed to passive or accidental cell death that is necrosis induced by pathological stimuli. Initially referred to phenomena occurring during development or aging, the expression ´programmed cell death´ was for a long time considered as synonym of apoptosis (Danial and Korsmeyer, 2004). Indeed, a first cell death classification based on ultrastructural data suggesting the existence of alternative types of PCD was almost ignored by the scientific community (Schweichel and Merker, 1973), and only 20 years later it became evident that the classic distinction between apoptosis and necrosis was an oversimplification of the processes promoting cell death (Clarke, 1990). More recently, the editors of Cell Death and Differentiation have established the Nomenclature Committee on Cell Death (NCCD) and proposed unified criteria for the definition of different types and morphologies of cell death (Kroemer et al., 2005).

Type I cell death refers to apoptosis, which is characterized by cell shrinkage, chromatin condensation, nucleosomal DNA degradation, little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing, and cell disintegration into small fragments called apoptotic bodies (Kerr et al., 1972). The appearance of these morphological features is often accelerated by or even dependent on the activation, at biochemical level, of the caspase family of cysteine proteases. At the end of the apoptotic process, apoptotic cellular debris are phagocyted by neighboring cells. Anoikis is historically defined as death in response to loss of attachment to the matrix or to neighbouring cells, but this process is at a molecular level undistinguishable from apoptosis (Kroemer et al., 2005).

Type II cell death corresponds to autophagy, which is distinguished by the absence of chromatin condensation or caspases activation, and the appearance of double or multimembrane vesicles engulfing bulk cytoplasm and organelles that are eventually degraded after the fusion of autophagic vesicles with lysosomes (Levine and Klionsky, 2004). Autophagy main task is to recycle long-lived proteins and organelle components, and cells that undergo excessive autophagy are triggered to die in a non-apoptotic manner (Gozuacik and Kimchi, 2004). At this regard, the NCCD pinpoints that autophagic cell death may be a misleading term as it may imply that cell death is occurring through autophagy, while it simply describes cell death with autophagy (Kroemer et al., 2005). As counterproof, *in vivo* knockdown

or knockout of genes required for autophagy does not reduce cell death (Boya et al., 2005).

Type III cell death is defined as non-lysosomal vesiculate degradation or paraptosis. This death process is dependent on the activation of RTKs or a member of the tumor necrosis factor (TNF) receptor family, which trigger MAPKs-mediated swelling of mitochondria and the endoplasmic reticulum, and final cytoplasmic diffuse vacuolization. In addition, paraptosis occurs independently of caspases and seems to be initiated in response to variations in intracellular calcium concentrations (Fombonne et al., 2006; Sperandio et al., 2000; Sperandio et al., 2004; Wang et al., 2004b).

Mitotic catastrophe is triggered by mitotic failure caused by defective cell cycle checkpoints or mitosis dysregulation in proliferating aneuploid cells. Mitotic catastrophe results in the formation of micronuclei, which often are misallocated chromosomes or chromosome fragments, or multinuclei due to deficient separation during cytokinesis (King and Cidlowski, 1995). This death pathway has been observed to be promoted upon DNA damage or treatment with microtubule stabilizing or destabilizing agents, and occurs either independently or partially dependently on p53 activation (Roninson et al., 2001). Whether mitotic catastrophe is fundamentally different from apoptosis or not, it is still a matter of debate. Indeed, this process has been reported to be associated with mitochondrial membrane permeabilization and caspase activation, but caspase inhibition or Bcl-2 overexpression fails to prevent its initiation (Castedo et al., 2004; Lock and Stribinskiene, 1996).

Three very specific forms of programmed cell death are excitotoxicity, Wallerian degeneration and cornification. Excitotoxicity is a form of death occurring in neurons as consequence of increase of cytosolic calcium in response to excitatory aminoacids-mediated opening of the N-methy-D-aspartate (NMDA) channel. Wallerian degeneration is a poorly characterized death process where part of a neuron or axon degenerates without affecting the main cell body (Orrenius et al., 2003). Cornification or keratinisation is different from apoptosis, and results in the formation of the cornified envelope of the epidermis (Candi et al., 2005). In addition, attempts have been made to catalogue PCD according to different criteria, such as ordering caspase-independent cell death according to the cellular organelles involved (Ferri and Kroemer, 2001), or defining four subclasses of PCD (apoptosis,

apoptosis-like PCD, necrosis-like PCD and necrosis) in relation to nuclear morphology and chromatin condensation extent (Leist and Jäättelä, 2001).

1.7.1. The apoptotic machinery

Apoptosis is an energy-dependent type of cell death (Leist et al., 1997) that plays an active role in cell turnover during adult life, embryonic development and normal functioning of the immune system (Thompson, 1995). On the opposite, disruption of the apoptotic pathway is involved in the pathophysiology of different disorders like cancer, autoimmunity and neurodegenerative diseases (Meier et al., 2000). At the biochemical level, apoptosis of mammalian cells is triggered by variety of biological, chemical or physical stimuli, through different pathways that finally converge on massive caspase activation (Adams, 2003; Green and Kroemer, 1998). At present, 15 different mammalian caspases have been described and classified into initiator (caspase 1, 2, 4, 8, 9, 10 and 12) and effector (caspase 3, 6, 7, 11 and 13) caspases according to function and size of the N-terminal pro-domain. Caspases are synthesized as inactive zymogens and become active enzymes after proteolytic removal of the pro-domain and oligomerization into tetramer complexes. Initiator caspases possess a long N-terminal pro-domain responsible for interaction with adaptor proteins that regulate their activation. On the other hand, effector caspases are activated by other proteases, usually upstream active caspases, since their short pro-domain does not interact with adaptor proteins (Ho and Hawkins, 2005). Two principal pathways control apoptosis, the intrinsic or mitochondria-mediated pathway that is triggered in response to intracellular insults, and the extrinsic pathway, responding to extracellular stimuli and initiated by the engagement of death receptors (Fig. 4). In addition, other intracellular organelles have been demonstrated to promote apoptosis via the activation of either the extrinsic and/or intrinsic signalling pathways (Guicciardi et al., 2004; Orrenius et al., 2003). In example, upon severe or prolonged stress in the endoplasmic reticulum (ER), the unfolded protein response (UPR) causes Ca^{2+} release from the ER lumen, which activates calpain in the cytosol and triggers apoptosis via either direct Ca^{2+}-mediated collapse of the inner

membrane potential, or sequential cleavage of caspase 12, 9 and 3 (Schroder, 2006).

The extrinsic pathway is a plasma membrane receptor-mediated mechanism that results in the activation of caspase 8 and/or 10. Is triggered by self-association of cell surface receptors belonging to the TNF superfamily,

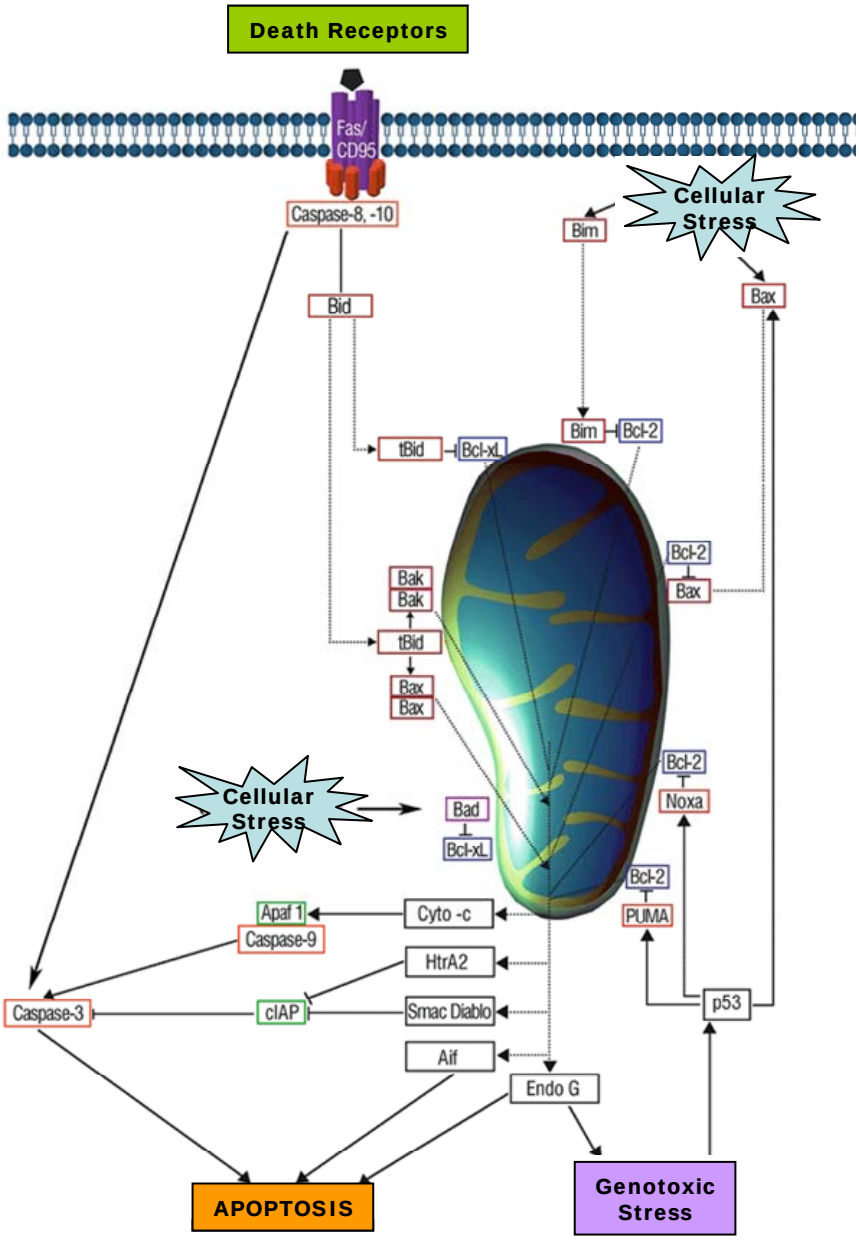


Figure 4. *Integration of the apoptotic pathways*. Adapted from Kutuk and Basaga (2006) Bcl-2 protein family: implications in vascular apoptosis and atherosclerosis. For abbreviations refer to text.

among which the best known in apoptosis are CD95 (designed also Apo-1 or Fas), TNF-α receptor 1 (TNFR1), and TRAIL (TNF-related apoptosis inducing

ligand) receptors DR4 and DR5. The extrinsic pathway is initiated in response to different stimuli like microbial and viral infections or oncogenic transformation. Upon binding of the respective ligand (i.e. Fas-L, TNF, TRAIL/Apo-2), the receptor undergoes oligomerization and recruits intracellular adaptor proteins, such as FADD and TRADD (Fas- and TNFR-associated death domain, respectively). TNF receptor ligation is rarely enough on its own to initiate apoptosis as is the case with Fas-L and TRAIL, whose binding triggers rapid apoptosis in many cells. Adaptor proteins are associated with the receptor through an interaction between homologous death domains (DD) and recruit the initiator caspases 8 and 10 via a death effector domain (DED). The complex of receptor, adaptor proteins and pro-caspases is known as death-inducing signalling complex (DISC) and is required for the efficient autoproteolytic activation of caspase 8 and 10 (Chen and Wang, 2002). Along the TNFR1-mediated apoptotic pathway, TRADD also associates with FADD, which on the contrary has been shown to be not essential for TRAIL-promoted apoptosis, where no adaptor molecule has been identified yet. Cells that constitutively express TRAIL have developed an apoptosis protection mechanisms based on decoy receptors (DcR1 and DcR2) that compete with the DR4 and DR5 receptors. These receptors bind TRAIL, but do not initiate apoptosis since DcR1 lacks a cytoplasmic domain, while DcR2 has a truncated inactive death domain (Chaudhari et al., 2006). Signalling downstream of caspase 8/10 is cell type-dependent: initiator caspases directly activate the effector caspases 3 and 7 in type I cells, while converge on the intrinsic signalling pathway in type II cells through the cleavage of Bid, a member of the Bcl-2 family proteins (Scaffidi et al., 1998; Sprick and Walczak, 2004).

The intrinsic pathway is functioning in response to various types of intracellular stimuli, like stress-inducing agents, DNA damage, and growth factor withdrawal (Adams, 2003). This apoptotic pathway is controlled by the balance between pro- and anti-apoptotic members of the Bcl-2 family. Majority of these proteins operate on the level of mitochondria, participating in the stabilization or destabilization of these organelles, but fulfill other functions in the ER and nucleus. Bcl-2 family members contain one or more conserved homology domains (BH1 to BH4) and a C-terminus transmembrane domain that anchors them to membranes. Pro-apoptotic Bcl-

2 family proteins are divided into a first subgroup having two or three BH regions and sharing extensive structural similarity (i.e. Bax, Bcl-X_s, Bak), and a second subgroup containing only the short BH3 domain (i.e. Bid, Bad, Noxa, Puma) (Antonsson and Martinou, 2000). Following the identification of stress signals, pro-apoptotic Bcl-2 family proteins are translocated from the cytosol to the mitochondrial outer membrane to form pore-like structures that promote the release of apoptogenic factors, like cytochrome *c*, AIF (apoptosis inducing factor), Smac/DIABLO (second mitochondria-derived activator of caspases), endonuclease G and Omi/HtrA2 (Cory et al., 2003). Anti-apoptotic members of the Bcl-2 family (i.e. Bcl-2 and Bcl-x_L) exhibit all four homology domains, with the BH1, BH2 and BH3 domains forming a hydrophobic pocket that is further stabilized by BH4 domain (Huang et al., 2002). Structural analyses suggest that the anti-apoptotic Bcl-2 members act as functional traps by blocking the BH3 domain of pro-apoptotic members in the hydrophobic groove formed by their BH1-3 domains (Sattler et al., 1997). Through the formation of dimers, oligomers or high-order multimers, pro-apoptotic members of the Bcl-2 family undergo conformational changes that prevent this inhibitory mechanism (Griffiths et al., 1999; Ruffolo and Shore, 2003). Once released in the cytosol, cytochrome *c* forms the apoptosome complex by associating with the adaptor protein Apaf-1 (apoptotic protease-activating factor-1) and pro-caspase 9, which upon cleavage directly activates the effector caspases 3 and 7 (Wang, 2001).

Effector caspases participate in the cell disassembly by both cleaving and inactivating inhibitors of apoptosis, dismantling cellular structures or regulating the activity of other proteins resulting in chromatin condensation, DNA fragmentation, and all the other morphological and functional changes associated with apoptosis (Degterev et al., 2003). Inhibitors of apoptosis proteins (IAPs) are a family of regulatory proteins controlling caspase processing and activation. All IAPs present baculoviral repeats domains (BIRs) that selectively mediate the binding to distinct caspases, while some members contain an additional zinc-binding motif (RING domain) that catalyze caspase ubiquitination for proteasome degradation (Vaux and Silke, 2005). IAPs basal expression is ubiquitous while the high expression level of different family members is tissue specific. The effect of IAPs is antagonized

by Smac/DIABLO and Omi/HtrA2 after mitochondrial release (Saelens et al., 2004).

1.7.2. Macroautophagy

Two major degradative pathways subsist in higher eukaryotic cells (Ciechanover and Schwartz, 1994): the proteasome is involved in the degradation of short-lived proteins (Wang and Maldonado, 2006), whereas the lysosomal system is responsible for the degradation of long-lived proteins and the turnover of organelles by several mechanisms collectively known as autophagy (Holzman, 1989). Beside the general default mechanism that delivers cell surface macromolecules to the lysosomes through endocytic vesicles, at least three other routes have been described for the lysosomal degradation. In the chaperone-mediated transport, cytosolic proteins are selectively imported into the lysosomal lumen upon the recognition of a KFERQ sequence motif by the lysosomal associated membrane protein Lamp2a and in presence of the heat-shock protein Hsc73. In microautophagy, cytoplasmic macromolecules and organelles such as peroxisomes are taken up by invaginations of the lysosomal membrane in a GTP-dependent process. Macroautophagy is the major non-selective vacuolar-mediated transport responsible for the turnover of mitochondria and other organelles, as well as the delivery of cytoplasmic material to lysosomes during periods of starvation. Macroautophagy, usually referred to simply as autophagy, is also involved in several pathophysiological processes such as cancer, cardiomyopathies and neurodegenerative diseases (Cuervo, 2004).

The molecular mechanism controlling the onset of autophagy has been initially discovered in yeast with the cloning of different genes that have been unified in the nomenclature as Atg (autophagy-related) genes (Klionsky et al., 2003). Mammalian orthologues of some of the Atg genes have been identified, and their function has been demonstrated to be preserved from yeast to mammals (Mizushima et al., 2003). The initiating event of macroautophagy is the formation of a membrane boundary called autophagosome that enwraps cytoplasmic constituents and organelles. The origin of the autophagosomal membrane is still unclear, as it has been

proposed to form from the endoplasmic reticulum, the Golgi, a not characterized membrane compartment called phagophore, or *de novo* through nucleation, assembly and elongation of small membrane structures (Noda et al., 2002). A multiprotein complex comprising class III PI3K, Beclin1, a p150 myristylated protein kinase, and UV irradiation resistance-associated gene product (UVRAG) is involved in the formation of the autophagosome (Kihara et al., 2001; Liang et al., 2006; Petiot et al., 2000). In a second moment, the cytosolic protein MAP1LC3 (microtubule-associated protein 1 light chain 3), shortly LC3, undergoes ubiquitin-like post-translational modifications and subsequent phosphatidylethanolamine conjugation that allow its tight association with the autophagosomal membrane (Kabeya et al., 2004). In the end, autophagosomes fuse with the outer membranes of endosomes or lysosomes to form autolysosomes where lysosomal hydrolases degrade the autophagosomal cytoplasm-derived content and inner membrane (Kondo et al., 2005) (Fig. 5).

Autophagic response to amino acid starvation is regulated by the Ras/Raf-1/ERK1/2 signaling pathway. Raf-1 undergoes an amino acid-dependent inhibitory phosphorylation, while in absence of amino acids ERK1/2 (extracellular signal-regulated kinases) get phosphorylated and trigger the activation of heterotrimeric G proteins localized to Golgi/ER membranes that promote the initiation of autophagy (Houri et al., 1993; Ogier-Denis et al., 2000; Pattingre et al., 2003). This signaling pathway can also be differently modulated by growth factors through the RTKs-mediated activation of both the ERK1/2 and Akt signalling cascades. Despite ERK1/2 activation, under these circumstances autophagy is blocked since Akt on one side inactivates Raf-1 (Pattingre et al., 2003) while on the other side triggers mTOR (mammalian target of rapamycin) that inhibits autophagy via the phosphorylation of downstream proteins such as p70S6 kinase (p70S6K) and its target the ribosomal protein S6 (Blommaart et al., 1997). Since autophagy depends on both continuous protein synthesis and continuous presence of ATP, it is not surprising that mTOR, being an integrator of growth factor induced signals and a sensor for amino acids and ATP, acts as gatekeeper for initiation of the autophagic pathway (Kim and Klionsky,

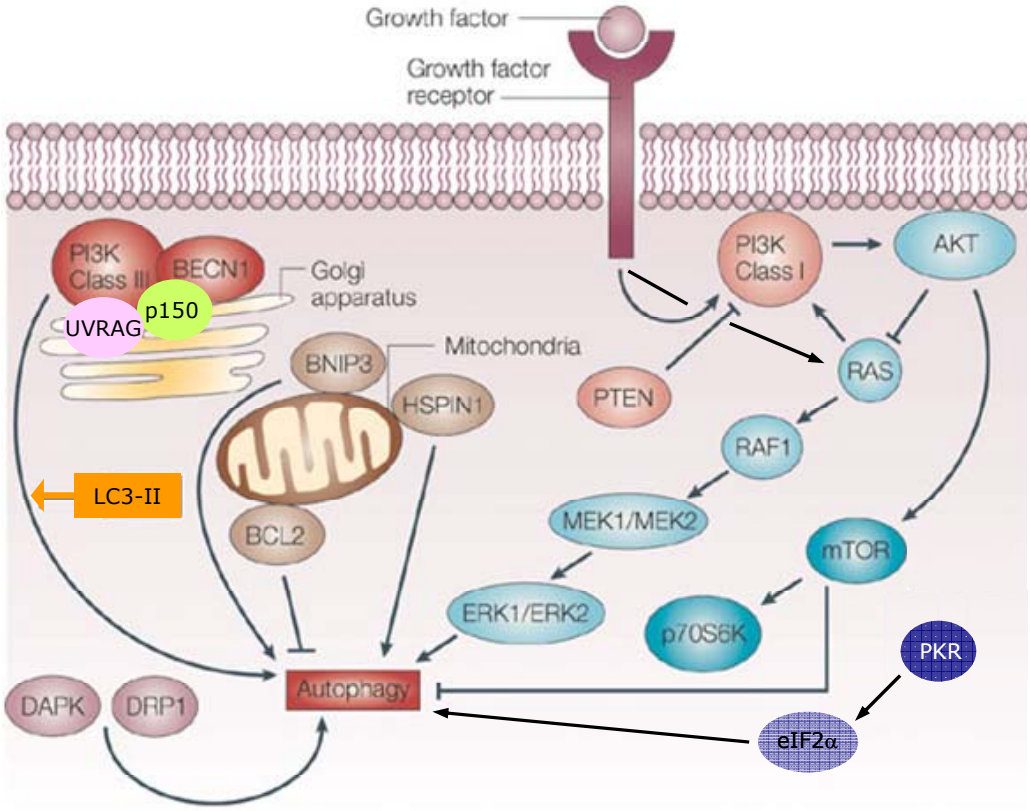


Figure 5. *Signalling pathways involved in the regulation of macroautophagy*. Adapted from Kondo et al. (2005) The role of autophagy in cancer development and response to therapy. MEK; mitogen-activated protein kinase kinase. For other abbreviations refer to text.

2000b). Moreover, PTEN, which is a negative regulator of Akt, has been shown to stimulate autophagy (Arico et al., 2001) (Fig. 5).

The regulation of autophagy in response to nutrient starvation and viral infection is dependent on the phosphorylation of the eukaryotic initiation factor 2α (eIF2α) on serine 51. This process is mediated by the eIF2α family of protein kinases and represents a central mechanism that induces a global arrest in translation under stress conditions. The mammalian family consists of four eIF2α kinases, among which the double-stranded RNA-dependent protein kinase (PKR) has been observed to be essential for autophagy induction in response to HSV-1 infection (Talloczy et al., 2002) (Fig. 5). Interestingly, mRNAs translation is not completely shut down when autophagy is induced, but continues in a selective manner (Fernandez et al., 2002).

Death Associated Protein kinase (DAPk) and its highly homologous DAPk related protein (DRP-1) are two Ca^{2+}/calmodulin-regulated serine/threonine kinases associated with the actin cytoskeleton (Kogel et al., 2001). DAPk is a positive mediator of cell death in response to various stimuli such as inferferon γ, activation of Fas receptors, TNF-α/β (Cohen and Kimchi, 2001; Jang et al., 2002). DAPk and DRP-1 are two novel regulators of autophagy, and DRP-1 in particular has been found to localize in the lumen of the autophagic vesicles, raising the possibility that this kinase may have a direct role in autophagic vesicle formation (Inbal et al., 2002) (Fig. 5).

Finally, autophagic type II cell death is promoted by BNIP3, a hypoxia-inducible BH3-like domain-containing protein that integrates into the mitochondrial outer membrane and induces the opening of the permeability transition pore without consequent cytochrome *c* release (Vande Velde et al., 2002). A similar effect is promoted by the human orthologue of Drosophila Spin (HSpin1), a transmembrane protein that is necessary for programmed cell death in Drosophila reproductive and nervous systems (Yanagisawa et al., 2003). More complex is the role of Bcl-2 in autophagy regulation (Fig. 5), since this anti-apoptotic molecule blocks Beclin1 by direct binding (Pattingre et al., 2005), triggers autophagy when overexpressed in human leukaemic cells (Saeki et al., 2000), but enhances etoposide-induced autophagy when overexpressed in mouse embryonic fibroblasts (Shimizu et al., 2004).

Past and present evidences demonstrate that autophagy is an adaptive response with a double potential, promoting programmed cell death or maintaining continual cell survival under stress conditions. The liver, for example, constitutes an extreme case in which amino acid deprivation-induced autophagy provides essential elements for biosynthetic pathways in the entire organism. Besides, hepatocytes rapidly remove depolarized mitochondria by autophagy, suggesting that this process may be a shielding mechanism that prevents apoptosis by increasing the threshold of mitochondria-dependent cell death (Elmore et al., 2001; Lemasters et al., 1998). On the contrary, autophagy plays a role in higher organism development, being involved in the death of central cells during intestine cavity formation, of Mullerian duct palatal epithelial cells during male sexual

development, and in hormone deprivation-induced regression of mammary glands or atrophy of prostate (Bursch, 2001).

In several cellular contexts, apoptosis and autophagy may be interconnected and even simultaneously regulated by the same trigger. In neural growth factor-deprived primary sympathetic neurons, the execution of apoptosis is preceded by and dependent on the onset of autophagy, which can even induce a caspase-independent cell death if apoptosis is blocked (Xue et al., 1999). In HT-29 colon carcinoma cells, autophagy antagonizes sulindac sulfide-induced apoptosis, and the inhibition of autophagy along the ERK1/2 pathway increases the sensitivity of these cells to apoptotic signals by allowing a faster cytochrome *c* release (Bauvy et al., 2001). Finally, apoptosis and autophagy may occur in mutually exclusive manner; several malignant glioma cell lines exert autophagic cell death in response to arsenic trioxide but die from apoptosis when pre-treated with the autophagy inhibitor bafilomycin A1 (Kanzawa et al., 2003). In this perspective, the role of several proteins capable of inducing both apoptosis and autophagy, like DAPk and anti-apoptotic Bcl-2 family members, is currently under investigation to identify the molecular regulators controlling the switch between these two type of programmed cell death (Kondo et al., 2005).

Bimodal is the role of autophagy in cancer development and maintenance. Cell growth is influenced by a balance between the rates of protein synthesis and autophagic degradation of long-lived proteins, the latter being considerably higher in stationary/quiescent than in proliferating cells (Lee et al., 1992). Indeed, in several cell lines tested *in vitro* and during animal experimental carcinogenesis, autophagic capacity and lysosomal enzymes activities have been observed to be often lower in transformed cells than in their normal counterparts (Canuto et al., 1993; Gronostajski and Pardee, 1984; Kisen et al., 1993; Yucel et al., 1989). Nonetheless, in rat pancreatic carcinogenesis cells show an increased autophagic capacity during premalignant stages and a decreased autophagic activity during malignant/terminal stages, suggesting that autophagy is necessary and/or compatible with malignant transformation but must be downregulated for cancer preservation (Toth et al., 2002). Along this line, autophagic cell death can be activated in cancer cells in response to various agents used in cancer

therapy, such as for instance radiation treatments in breast, glioma, prostate and colon cancer cell lines (Paglin et al., 2001).

1.7.3. Cathepsins and the lysosomal pathway

Lysosomes control cell death mainly at two levels. In the autolysosome, hydrolases participate in organelle and macromolecules turnover under physiological conditions and are thus actively involved in autophagic cell death. In response to endogenous or exogenous stress, lysosomal membrane permeabilization (LMP) leads to the release of catabolic hydrolases that mediate different types of cell death. In this case, lysosomal leakage can be a downstream event involved in the clean-up phase of apoptosis or in cellular autolysis during uncontrolled necrosis, but it can also initiate programmed cell death (Guicciardi et al., 2004; Shintani and Klionsky, 2004). Studies using detergents, lysosomotropic antibiotics and oxidative stress agents have demonstrated that LMP can trigger classical apoptosis as well as caspase-independent apoptosis and necrosis according to the amount of lysosomal rupture: a limited release of lysosomal contents to the cytoplasm induces apoptosis or apoptosis-like cell death, whereas a generalized lysosomal rupture results in rapid cellular necrosis (Brunk et al., 1997; Kagedal et al., 2001) (Fig. 6).

Among lysosomal hydrolases, cathepsin proteases have a central role in lysosomal control of cell death due to the fact that they have optimal enzymatic activity at the acidic pH of lysosomes (pH 4–5), but many of them can also function at neutral pH outside of the lysosomes, though with a decreased stability and/or altered specificity (Turk et al., 2001). Depending on their active-site functional amino acid, these proteins are generally divided into cysteine (B, C, H, F, K, L, O, S, V, W and X/Z), aspartate (D and E) and serine (G) cathepsins. The 11 human cysteine cathepsins are predominantly 40 kDa endopeptidases belonging to the papain subfamily of cysteine proteases. Some cathepsins are expressed constitutively and are thought to participate in protein turnover, in example cathepsin B and L (Rawlings et al., 2006). The expression of other cysteine cathepsins is regulated and cell type specific, for instance cathepsin K in osteoclasts

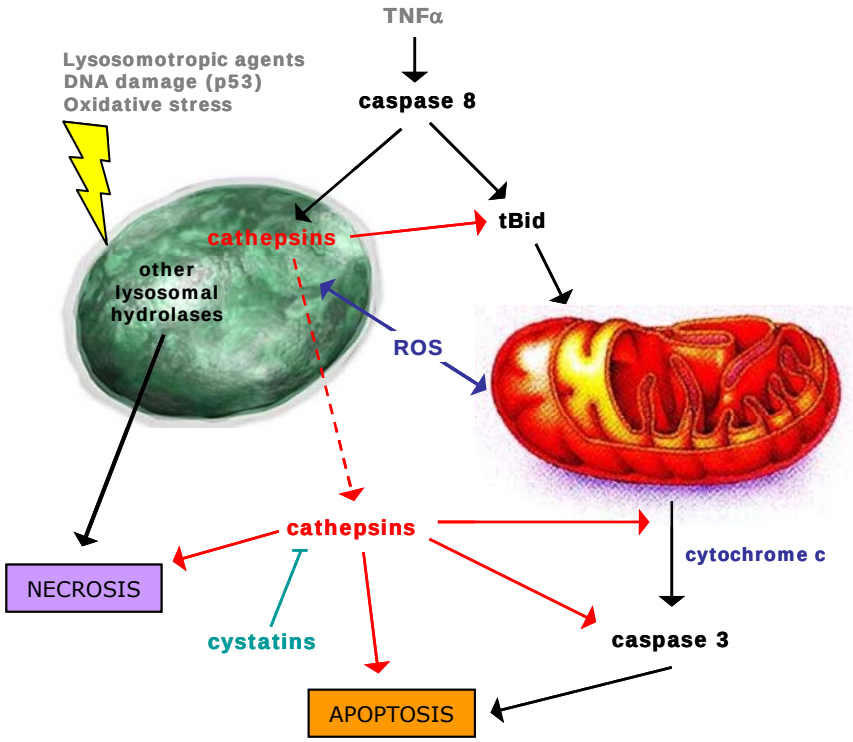


Figure 6. *Lysosomes in cell death.*

(Saftig et al., 1998) and cathepsin S in immune cells (Shi et al., 1999). Despite cathepsins function primarily intracellularly within endolysosomal compartments, some of them are also secreted extracellularly: cathepsin K is involved in bone remodelling (Delaisse et al., 2000; Xia et al., 1999) while cathepsins B and L digest the extracellular matrix in physiological processes like wound healing as in pathological conditions such as cancer invasion (Buth et al., 2004; Mohamed and Sloane, 2006). Cathepsins are physiologically regulated by cystatins, a large group of evolutionary related competitive, reversible, tight-binding protease inhibitors (Rawlings et al., 2006; Turk et al., 2002). Cystatins do not directly interact with the enzyme catalytic centre but bind adjacent to the protease active site, thus obstructing the access to the substrate (Bode and Huber, 2000). On the basis of sequence homology, the cystatin superfamily is divided into three groups that display considerable differences in amino acid sequence but present a conserved functional tertiary structure (Bode et al., 1988; Stubbs et al., 1990). Type 1 cystatins (or stefins, A and B) are 98 amino acid residues polypeptides which possess neither disulfide bonds nor

carbohydrate side chains and are located mainly intracellularly. Type 2 cystatins (C, D, E/M, F, S, SN, and SA) are 120 amino acid residues polypeptides characterized by two conserved disulfide bridges and the presence of a signal peptide for extracellular targeting. Type 3 cystatins (kininogens) are 60–120 kDa multifunctional plasma proteins containing three type 2 cystatin-like domains (Rawlings et al., 2004).

Whether LMP results in the selective release of certain cathepsins or whether all proteins are released simultaneously from the lysosomal lumen is still unknown, even if a selective release based on molecular size has been observed (Bidere et al., 2003). LMP might in part be triggered by oxidation of membrane lipids, resulting from the Fenton reaction between intra-lysosomal iron and H_2O_2 accumulating from oxidative stress (Brunk et al., 2001). The cytotoxic effects of LMP often rely on the activation of the mitochondrial cell death pathway through reactive oxygen species (ROS) and lipid mediators such as arachidonic acid, generated in a cathepsin B-dependent manner (Boya et al., 2003; Kagedal et al., 2001) (Fehrenbacher et al., 2004; Guicciardi et al., 2000). A possible links between cathepsins and the loss of mitochondrial membrane potential might be represented by Bid, which can be processed and activated by several cysteine cathepsins at cytosolic pH (Cirman et al., 2004). Remarkably, the lethal effects of LMP and cytosolic cathepsins are not limited to the activation of the intrinsic apoptotic pathway. LMP and cathepsins also participate in the execution of cell death that is induced by classic apoptotic stimuli such as death receptors and p53 activation (Foghsgaard et al., 2001; Nakayama et al., 2002; Yuan et al., 2002). Following TNFR1 internalization, caspase 8 activation promotes LMP while Bid is cleaved by cathepsin D in the endo-lysosomes (Heinrich et al., 2004; Schneider-Brachert et al., 2004). In cells treated with TNF in combination with inhibitors of transcription or translation, cathepsin B is responsible for apoptotic cell death, whereas the inhibition of caspase activity sensitizes TNF-treated cells to caspase-independent LMP and necrotic cell death (Khwaja and Tatton, 1999).

2 Aim of the Thesis

Current experimental data have demonstrated that parvovirus H-1 (H-1PV) can efficiently kill different low-passage glioma cultures and established glioblastoma cell lines of human origin, but exerts no cytopathic effect on normal astrocytes. Moreover, the virus has proven to be effective *in vivo*, inducing full regression of implanted rat brain tumours into recipient animals. H-1PV seems therefore to be a promising candidate for brain cancer treatment.

Several gliomas are initially sensitive to standard therapeutic protocols but rapidly become resistant, mainly by escaping apoptosis execution. To define the application range of parvovirus H-1 in brain cancer virotherapy is first of all important to scrutinize the molecular mechanisms responsible for virus-mediated cell killing, and secondly to verify whether H-1PV is able to neutralize glioma cells attempts to evade induced death. Hence, this work has evaluated the death pathways triggered by the virus in NCH82 cells, a low-passage culture isolated from a glioma patient. In particular, the cleavage of caspase 3 and the role of anti-apoptotic molecules like Bcl-2 have been considered to ascertain the activation of classic apoptosis. The activity of cathepsins has also been investigated, as these lysosomal proteases can be triggers or central effectors of apoptosis as well as of alternative non-apoptotic death pathways. Additionally, the onset of autophagy upon H-1PV infection has been elucidated since this degradative pathway has been shown to be involved in both cell survival and death, in response to pathogens and during tumorigenesis. Ultimately, the reciprocal role of apoptosis, autophagy and lysosomal cathepsins activation has been determined to establish the sequence of events achieving virus-induced cell death. The activity of cathepsin B has been analyzed in tissue extracts of H-1PV-treated rat brain tumors, to complete the study with an overview on the *in vivo* oncosuppressive properties of the virus.

The capacity of glioma cells to acquire resistance towards conventional death-inducing agents also correlates with the appearance of multiple genetic alterations associated with and accumulating during tumor development. In order to evaluate H-1PV as possible therapeutic tool, it is crucial to identify the molecular changes that are associated with astrocytes malignant transformation and are necessary for the acquisition of sensitiveness to H-1PV-induced cell death. In addition, it is important to determine if the expression of different mutations correlates with the virus-mediated activation of different killing mechanisms, to understand whether H-1PV could jeopardize cell survival by adapting to the host genetic profile. To these purposes, rat embryo fibroblasts (REFs) have been used as a well established cellular model for oncogene cooperation in malignant transformation, to conduct a preliminary study for unravelling the effects of cell immortalization and/or transformation on viral permissiveness. In particular, the aim has been to investigate whether cell transformation is strictly required or if cell immortalization in sufficient to render REFs permissive to H-1PV-induced killing. Furthermore, this simple study model has been used to evaluate the nature of the intracellular pathways utilized by the virus to achieve cell death, and how key oncogenes could affect the accomplishment of parvovirus H-1 life cycle.

3 Material and Methods

3.1. Chemicals and reagents

Complete-mini™ protease inhibitor cocktail, FuGENE 6™ and X-tremeGENE™ transfection reagents were purchased from Roche, Mannheim, Germany. Fetal Calf Serum (FCS) and adult Calf Serum were from Biochrom, Berlin, Germany; iodexanol 60% (w/v) water solution (OptiPrep™) came from Axis-Shield, Dundee, Scotland. Bacto™Agar was purchased by BD Bioscience, Heidelberg, Germany. Gentamicin sulfate solution (50 mg/ml), Hanks' balanced salt solution (HBSS), kanamycin sulfate solution (10 mg/ml), uncoloured minimum essential medium (MEM) containing Hanks' salts and L-glutamine, OptiMEM medium, and trypsin 0.25% solution were obtained from GIBCO, Karlsruhe, Germany. Caspase 3 inhibitor (DEVD-CHO, cell permeable), cathepsin B inhibitor (Ca-074 Me) and cathepsins inhibitor (E64d) were acquired from Calbiochem, Darmstadt, Germany. 3-methyladenine (3-MA), acridine orange dye, Dulbecco's modified Eagle medium (DMEM) high glucose (4.5 g/l), Neutral Red solution, paraformaldeyde (PFA), polybrene, puromycin dihydrochloride, Triton X-100, Trypan blue solution, and all other non mentioned chemicals, salt solutions and antibiotics were purchased from Sigma, Schnelldorf, Germany.

3.2. Cell cultures

Rat embryo fibroblasts (REFs) were isolated from Fisher rats (Charles River Laboratories, Sulzfeld, Germany) at 13-14 days gestation. Animals were sacrificed in a CO_2 tank and dissected under sterile conditions; uteri were identified, cleaned from fat and resected. Embryos were then extracted, head and liver depleted, and tissue was minced with a scalpel in 5 ml PBS 1x (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.47 mM, pH 7.4). Tissue was digested 20 minutes at 37°C by adding 5 ml trypsin

0.25% and proteolysis was stopped with 5 ml DMEM. Tissue was afterwards disintegrated into single cells by forcing the homogenate throughout a narrow mouth pipette. Living cells were counted using a hemocytometer (0.1 ml cell suspension, 0.9 ml PBS 1x and 1 ml Trypan blue) and plated in number of $3x10^7$ per 15 cm (∅) dish. Cell stocks were prepared after 3-4 days culturing by collecting cells at confluence from a 15 cm (∅) dish in 6 cryovials (1 ml volume) and freezing them in liquid N_2 in DMEM supplemented with 10 % (v/v) FCS and 1% (v/v) DMSO [Dimethyl Sulfoxide].

NCH82 low-passage cell culture was prepared from a glioblastoma patient and characterized at the Neurosurgery Department, Heidelberg University, as previously described (Herold-Mende et al., 1999). The RG2 rat glioma cell line was established almost 40 years ago from an ethylnitrosourea-induced glioma in Fischer 344 rats (Wechsler et al., 1969), and was reported to provide a reproducible model for experimental neuro-oncology when inoculated into the brains of syngenic rats (Aas et al., 1995). RG2 cells were provided by Dr. K. Geletneky, Neurosurgery Department, Heidelberg University.

Cells were cultured in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 μg/ml) and gentamicin (50 μg/ml), maintained at 37°C temperature in 5% CO_2 atmosphere.

3.3. Retrovirus infection and plasmids transfection

Murine leukaemia virus (MLV)-based pBabe-puro retroviral vectors were used to overexpress the following genes in NCH82 glioma cells: human *Bcl-2* complete coding sequence (cds) inserted in the EcoRI site, human *cystatin B* complete cds cloned into BamHI-EcoRI restriction sites, and BamHI-EcoRI-inserted human *Flip* complete cds. The cystatin B expression vector, a gift from Dr. E. Spiess, DKFZ, Heidelberg, was tagged by green fluorescent protein (GFP – Clontech, St-Germain-en-Laye, France). For safety reasons, these replication-deficient ecotropic vectors comprise *cis*-acting sequences (long terminal repeats [LTRs], primer binding sites and packaging signals) and need to be produced in packaging cells expressing

the *trans*-complementing genes (*gag*, *pol* and *env*). In this study, retroviruses were produced by transfection of helper-free ecotropic virus-producing BOSC 23 cells using FuGENE 6™ reagent according to the manufacturer's instructions. To allow the infection of human NCH82 cells, BOSC 23 cells were co-transfected with the pseudotyping vector pVSVg (Invitrogen, Karlsruhe, Germany) in the ratio of 3:1 (w/w), and supernatants were collected 48 h post-transfection. Recipient cells were incubated for 4 h with 3 ml inoculum supplemented with polybrene (6 μg/ml). NCH82 transfected cells were selected in DMEM supplemented with 10% (v/v) FCS and puromycin (2.5 μg/ml); selection was terminated as control untransfected cells were killed.

To transfect REFs, the following vectors have been used: pcDNA3.1(-) empty (Invitrogen), pEGFP-C1 (Clontech), pBabe-neo with Simian vacuolating virus 40 (SV40) *large T antigen* cds cloned in the BamHI-SalI site, pJ4Ω with human *c-myc* complete cds inserted in the BglII-HindIII site, pED-1 with EcoRI-XbaI-cloned *p53* cds carrying a substitution of cysteine to serine at position 135 that encodes for a protein that oligomerize with wild-type p53 thus rendering it biologically non-functional, pMOD-puro (Invivogen, Toulouse, France), and pUC with BamHI-EcoRI-cloned human *ras* cds carrying a substitution of glycine to valine at position 12 that renders the oncoprotein constitutively active. Despite more recent transfection procedures, for rat embryo fibroblasts the calcium phosphate precipitation method proved to be the most efficient. REFs were transfected by adapting a previously well established protocol (Land, 1995). Briefly, cells were seeded in number of $1.5x10^6$ cells per 10 cm (Ø) dish in order to get 60–80% cell confluence after 24 hours. At this time point, 8-10 μg of plasmid DNA per 10 cm (Ø) dish were mixed with 62.5 μl $CaCl_2$ 2 M in a final H_2O volume of 500 μl, and the $CaCl_2$/DNA solution was added drop wise to 500 μl HBS 2x buffer (Hepes [4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid] 50 mM, NaCl 280 mM, Na_2HPO_4 1.5 mM, pH 7.05) while gently shaking. The solution was incubated 10–15 minutes at room temperature (RT) to allow the formation of the DNA/phosphate precipitates; in the meanwhile cells were re-fed with 10 ml DMEM supplemented with 10% (v/v) FCS without antibiotics. DNA/phosphate precipitates mixture was afterwards distributed drop wise over the cells and incubated at 37°C temperature in 5% CO_2 atmosphere.

Culture medium was changed at 24 h post transfection with DMEM supplemented with 10% (v/v) FCS and puromycin (1.5 μg/ml) to select stable transfectants; selection was terminated as control untransfected cells were killed.

Small interfering RNA oligonucleotides targeting human cathepsin B (5'-GCU UGG AAC UUC UGG ACA ATT-3') or L (5'-GAA CAU GAA GAU GAU UGA ATT-3') transcripts (MWG, Ebersberg, Germany), and Luc (5'-AAC GUA CGC GGA AUA CUU CGA-3') control oligonucleotide (Xeragon, Zürich, Switzerland) were transiently transfected using X-tremeGENE™ reagent according to the manufacturer's instructions. In brief, cells were seeded in number of $5x10^3$ cells per well (96-well plate) or $1x10^5$ cells per 6 cm (Ø) dish in DMEM supplemented with 10% (v/v) FCS without antibiotics. After 24 hours, 0.1 or 2 μg siRNA were combined with 0.8 or 10 μl X-tremeGENE™ reagent in a final volume of 30 or 200 μl in OptiMEM medium (per well or dish, respectively), incubated 15 minutes at RT, and subsequently added to cells. Culture medium was replaced at 24 h post transfection with DMEM supplemented with 10% (v/v) FCS, kanamycin (100 μg/ml) and gentamicin (50 μg/ml).

Transfection vectors were prepared from mother stocks by transformation of *E. coli* DH10b strain bacteria (Invitrogen) using the heat-shock method. 0.1 μg of purified plasmid DNA was added to 50-100 μl of competent bacteria (stored at -80°C in 10% [v/v] glycerol solution), incubated on ice for 30 minutes, then heated at 42°C for 2 minutes and rapidly cooled on ice for additional 2 minutes. The reaction mixture was afterwards diluted in 500-800 μl of Luria-Bertani (LB) broth (NaCl 5 g, yeast extract 5 g, bacto-trypton 5 g per H_2O litre, autoclaved, pH 7.0), and incubated for 60-90 minutes at 37°C under shaking at 300-400 rpm. Finally, 200-300 μl transformed bacteria were plated on LB-agar Petri dishes (1.5% [w/v] Bacto™Agar in LB broth) supplemented with kanamycin (50 μg/ml – pEGFP-C1 and pBabe-neo transformations) or ampicillin (100 μg/ml), and incubated overnight at 37°C. The following day, single colonies were picked from agar plates with a scraper or a pipette tip and cultured in suspension in 5 ml LB broth with antibiotic, at 37°C under shaking at 200 rpm, till the culture reached an OD_{600} (optical density at 600 nm) value of 0.6-0.8. A first 1 ml aliquot was taken apart for mini-isolation of plasmid DNA, while a

second aliquot was diluted 1:250 in 250 ml LB broth with antibiotics, cultured overnight at 37°C under shaking at 200 rpm, and ultimately treated for plasmid DNA maxi-isolation. Plasmid DNA isolations and purifications from 5 or 250 ml bacterial cultures were respectively performed with a Mini- or Maxi-kit (Qiagen, Düsseldorf, Germany), according to manufacturer's instructions. To control the quality of plasmid DNA, samples from mini-preparations were digested with specific restriction enzymes to cut the inserted cds out of the vector. Plasmid digestion was carried out for 1h at 37°C: 3 µl of enzyme buffer 10x were mixed with 1 µl of each restriction enzyme (10.000 U/ml) and 2 µg of plasmid DNA in a final volume of 30 µl H_2O. All restriction enzymes and buffers were purchased from New England Biolabs (Frankfurt, Germany). At the end of the digestion period, 6 µl of agarose gel loading buffer 6x (EDTA [ethylenediaminetetraacetate disodium salt] 1 mM, 2% [w/v] SDS [sodium dodecyl sulphate], 0.5% [w/v] Bromophenol blue, 50% [v/v] glycerol) were added to all samples, 10-20 µl of which were separated and analyzed on an ethidium bromide (0.5 µg/ml)-stained 1% (w/v) agarose gel. DNA concentration (µg/ml) was calculated by multiplying the samples absorbance at 260 nm by the dilution factor times 50; DNA purity was evaluated considering that pure samples should have an absorbance ratio of around 1.8, measured at 260 versus 280 nm. Plasmids were stored at -20°C.

3.4. H-1PV production and titering

H-1PV was amplified on human NBK cells and purified on iodexanol gradients as previously described (Wrzesinski et al., 2003). Briefly, NBK cells were seeded in 20 dishes (10 cm ∅) in number of 10^6 cells/dish, and infected the following day in medium without serum or antibiotics for 1h at 37°C with parvovirus H-1 at a multiplicity of infection of $3x10^{-3}$ plaque forming units (pfu) per cell. Afterwards, cells were cultured for 4h at 37°C in DMEM supplemented with 10% (v/v) FCS and split 1:1 in 15 cm (∅) dishes. Cells were maintained in culture for 4-5 days, till they showed the first sights of lysis. Cells were then scraped in the medium, collected, lysed in 10 ml VTE buffer (Tris-HCl 50 mM, EDTA 0.5 mM, pH 8.7) and undertaken to three

cycles of freezing at –20°C and thawing at 37°C. Viral suspension was stored overnight at –20°C. The following day a purification gradient was generated by overlapping iodexanol 15%, 25%, 40% and 60% solutions to the virus suspension. Sample was centrifuged under vacuum at 50000 K for 2 hours at 4°C, and the virus was extracted from the iodexanol 40% fraction with a syringe, avoiding mixing with the upper iodexanol 25% fraction, containing the empty capsids. The viral suspension was stored at 4°C, and H-1PV was then titrated on NBK indicator cells by plaque assay. Cells were seeded in 6 cm (∅) dishes in number of $4.5x10^5$ cells/dish, and infected the following day in medium without serum or antibiotics for 1h at 37°C with H-1PV at 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} pfu/cell. Afterwards, cells were cultured for 3-4 days in 5 ml MEM supplemented with 10% (v/v) FCS and 0.65% (w/v) Bacto™Agar. Plaque formation was detected by adding 2 ml of a PBS 1x solution containing 0.18% (v/v) Neutral Red and 0.8% (w/v) Bacto™Agar. Every single plaque was considered as coming from one viral particle; H-1PV was therefore used at multiplicities of infection expressed in pfu/cell.

3.5. Flow cytometry analyses

A FACScan (argon-ion lamp, 488 nm excitation laser line - BD Biosciences) was used to evaluate cell cycle perturbation, mitochondrial membrane depolarization, cell acidification, and NS1 viral protein expression in infected versus mock-treated cells by flow cytometry analysis. In all the assays, 10^6 cells per 10 cm (∅) dish were seeded, and infected the following day with H-1PV (5 pfu/cell) in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 μg/ml) and gentamicin (50 μg/ml). Measurements were carried out at 24 h and/or 48 h after infection; data were acquired on homogeneous populations gated according to cell size (forward scatter – FSC – values) and granularity (side scatter – SSC – values), and processed with the CellQuest™ software (BD Bioscience).

Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4.5 base pairs of DNA (Waring, 1965). PI was therefore used to quantify the amount of cellular DNA, thus determining the cell cycle profile of the

different cell populations. Briefly, cells were trypsinized, washed and resuspended in 200 μl PBS 1x, then fixed 30 minutes at –20°C in 800 μl MeOH 100% cold added drops wise. Cells were afterwards pelletted and treated with RNAse A (0.1 mg/ml final) in 500 μl PBS 1x for 30 minutes at 37°C. Finally, cells were incubated on ice for 30 minutes with PI (20 μg/ml final) in 500 μl PBS 1x, passed through a mesh and analyzed at the flow cytometer. When bound to nucleic acids, the absorption maximum for PI is 535 nm and the fluorescence emission maximum is 617 nm. DNA content distribution (PI fluorescence) was analyzed on the FL2-H (pulse high) histogram to distinguish apoptotic cells that have DNA content inferior to 2n. In parallel, data analysis was carried out on cells gated on an FL2-A (pulse area) channel versus FL2-W (pulse width) channel to exclude cell debris and clumped cells, and plotted on the Fl2-A histogram to better discriminate 2n from 4n subpopulations.

Mitochondrial membrane depolarization assays were performed by measuring the potential-dependent accumulation of JC-9 cationic dye (Molecular Probes, Leiden, The Netherlands). A positive control of cells treated with H_2O_2 (5 mM) for 30 minutes was included. After 24 h infection, cells were gently harvested with trypsin, washed in PBS 1x, and incubated with JC-9 (5 μM) in PBS 1x for 10 minutes at 37°C. Cells were subsequently washed again in PBS 1x, passed through a mesh, and analyzed at the flow cytometer. JC-9 dye potential-dependent accumulation in mitochondria is indicated by a fluorescence emission shift from green (525 nm) to red (590 nm), and mitochondrial depolarization is consequently associated to a decrease in the red/green fluorescence intensity ratio (Reers et al., 1991). Mitochondrial membrane depolarization was analyzed on a FL1-H versus FL2-H dot blot, and indicated by an increase in green fluorescence on the FL1-H histogram.

Identification of acidic compartments by acridine orange staining was performed to estimate initially the occurrence of cell acidification and secondly the appearance of autophagic vesicles. In parallel to H-1PV infection, cells were treated with rapamycin (1 μM) for 24 h as positive control for autophagy. In cell rescue experiments, cells were incubated with 3-MA (autophagy inhibitor, 500 μM), E64d (cathepsins inhibitor, 1 μM), Ca-074 Me (cathepsins B inhibitor, 10 μM), or DEVD-CHO (caspase 3

inhibitor, 20 μM) at 5 h post treatment. After 24 h infection, cells were harvested with trypsin, and incubated with acridine orange (10 μg/ml) in DMEM for 1 h at 37°C. Cells were then washed in PBS 1x, passed through a mesh, and studied at the flow cytometer on the FL3-H channel. Acidic compartments increase in number/size or leakage were respectively marked by a shift to the right or to the left in the red fluorescence (630 nm).

To determine NS1 expression, $3x10^6$ cells were collected in conical tubes, fixed 15 minutes on ice in 200 μl PFA 4% (w/v) in PBS 1x, washed with 1 ml SM buffer (HBSS 1x, calf serum 5%, Hepes 25mM), and re-fixed 20 minutes on ice in 200 μl MeOH. Cells were subsequently washed in 1 ml PBS 1x and in 1 ml SM buffer, permeabilized 2 minutes on ice in 200 μl Triton X-100 0.1%, washed again in 1 ml PBS 1x and in 1 ml SM buffer and incubated 30 minutes on ice in 50 μl primary antibody solution (mouse monoclonal α-NS1 clone 3D9, diluted 1:25 in SM buffer). Afterwards, cells were diluted in 1 ml SM buffer plus 250 μl calf serum, pelletted and incubated 20 minutes on ice in secondary antibody solution (goat polyclonal α-mouse FITC-conjugated, diluted 1:250 in SM buffer). Finally, cells were diluted in 1 ml SM buffer plus 250 μl calf serum, pelletted and resuspended in 500 μl SM buffer, passed through a mesh and analyzed on the FL1-H channel. FITC (fluorescein isothiocyanate) fluorescence emission maximum is at 494 nm.

3.6. Foci formation and soft agar cloning

Compared to their normal derivatives, transformed cells are characterized by higher division speed, loss of cell contact growth inhibition, and survival in environmental prohibitive conditions. Two simple methods to verify the acquisition of a transformed phenotype are to evaluate the capacity to form foci in the culture dish and colonies in soft agar.

In the first test, REFs transfectants were seeded in number of 10^6 cells per 10 cm (∅) dish and maintained at 37°C temperature, 5% CO_2 atmosphere in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 μg/ml) and gentamicin (50 μg/ml). Fresh medium was replaced every 3 days, without splitting the cell cultures. Transformed cells rapidly reached

dish confluence and further developed tridimensional spherical structures (foci) that were counted at 14 days post seeding.

In the second analysis, cells were resuspended in MEM supplemented with 10% (v/v) FCS, kanamycin (100 μg/ml), gentamicin (50 μg/ml) and 0.4% (w/v) Bacto™Agar, and seeded in number of $5x10^5$ cells per 6 cm (∅) dish on a bottom agar surface of MEM supplemented with 10% (v/v) FCS, kanamycin (100 μg/ml), gentamicin (50 μg/ml) and 0.625% (w/v) Bacto™Agar. Cultures were maintained at 37°C temperature and 5% CO_2 atmosphere, adding 0.2 ml of MEM supplemented with 10% (v/v) FCS, kanamycin (100 μg/ml) and gentamicin (50 μg/ml) every 5 days. Transformed cells were able to spread in the bottom agar stratum, expand clonogenically and form tridimensional spherical structures (clones) that were counted at 21 days post seeding.

3.7. Cell fractionation and protease activity measurements

In these analyses, 10^6 cells per 10 cm (∅) dish were seeded, and infected the following day with H-1PV (5 pfu/cell) in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 μg/ml) and gentamicin (50 μg/ml). At 24 h post infection, cells were collected in PBS 1x, pelleted by centrifugation (500 g, 10 minutes, 4ºC) and resuspended in 0.5 ml PBS 1x for the measurement of caspases activities or in 1.5 ml hypotonic buffer (sucrose 0.25 M, Hepes-NaOH 10 mM, EDTA 1 mM, pH 7.4) for cathepsin B, hexosaminidase, acid phosphatase and catalase activities.

One of the commonly used ways to detect caspases activities is to incubate synthetic substrates such as peptides conjugated to a fluorochrome (i.e. 7-amino-4-trifluoromethyl coumarin [AFC]), with lysates of apoptotic cells, and measure their cleavage. Substrate processing gives rise to increased fluorescence intensity, whose signal is proportional to the amount of cleaved substrate, which in turn is dependent on caspase activity and is related to the percentage of apoptotic cells in a cell population (Kohler et al., 2002). Caspases activity was determined by adding 25 μl of whole-cell extract to caspase buffer (Hepes 50 mM pH 7.4, NaCl 150 mM, 0.1% [v/v] CHAPS, 0.1% [v/v] Triton X-100, EDTA 1 mM, 10% [v/v] Glycerol, DTT 10

mM) supplemented with caspase 3 (Ac-Asp-Glu-Val-Asp-AFC), 8 (Z-Ile-Glu-Thr-Asp-AFC), or 9 (Ac-Leu-Glu-His-Asp-AFC) substrates (1 mM, Calbiochem). The reaction was monitored for 1 h on a Fluoroskan & FL (Thermolabsystem) measuring the emission at 510 nm wavelength with the excitation wavelength set at 390 nm.

In cell fractionation assays for cathepsin B activity, cells were homogenized in a cell-cracker (bead diameter of 8.006 mm, 10 strokes). Nuclei and heavy mitochondria were pelleted by centrifugation at 2500 g for 10 minutes at 4ºC. A 200 μl aliquot of the supernatant (post nuclear supernatant [PNS]) was kept and used to determine enzyme latency. The light mitochondrial fraction (LMF) was obtained by centrifugation of 1 ml PNS at 17000 g for 20 minutes at 4ºC. The supernatant was kept as a cytosolic extract and the LMF was either resuspended in 1-1.3 ml hypotonic buffer for the determination of enzyme activity or further fractionated. Lysosomes were purified from the LMF fraction (1.3 ml) on a self-generated gradient of iodixanol by adding 0.7 ml of 50% iodixanol in EDTA 1 mM, Hepes-NaOH 10 mM pH 7.4, sucrose 42 mM, and centrifuging at 380000 g for 1 h 30 minutes at 4ºC in a TLV100 rotor. Twelve 150-μl fractions were collected and analyzed by immunoblotting. Acidic phosphatase, hexosaminidase, cathepsin B and catalase activities were determined by adding 25 μl of whole cell extract, cytosolic extract or LMF sub-fractions to the respective reaction mixtures. For the measurement of acidic phosphatase activity, this mixture consisted of 0.2 ml of p-nitrophenyl phosphate 8 mM, sodium acetate-acetic acid 90 mM, pH 5.0; the reaction was stopped after 1 h at 37ºC by adding 0.6 ml of NaOH 0.25 M, and absorbance was measured at 410 nm. Hexosaminidase activity was determined using 1 volume of p-nitrophenol-N-acetyl-β-D-glucosaminide 3.75 mM in citrate buffer 0.05 mM, pH 5.0 containing 0.25% (v/v) Triton X-100. The reaction was stopped after 3 h at 37ºC by adding 1.5 volumes of glycine 50 mM, EDTA 5 mM, pH 10.4 and absorbance was read at 405 nm. For the measurement of catalase activity, the assay buffer was obtained by diluting 8.5 ml of H_2O_2 30 mM in 91.5 ml of Tris-HCl 20 mM, pH 7.0 containing bovine serum albumin (BSA – 1 g/l). Cell fractions (25 μl) were mixed with an equal volume of 0.5% (v/v) Triton X-100 in Tris-HCl 20 mM, and 0.5 ml of assay buffer. After 10 minutes at room temperature, 1 ml of titanium oxysulphate

(2.25 g/l) in H_2SO_4 1M was added, and absorbance was measured at 405 nm. Cathepsin B activities were determined by adding 25 µl whole-cell extract, cytosolic extract, or LMF subfraction to the reaction mixture, consisting of MES 50 mM pH 6.0, sucrose 0.25 M, EDTA 1mM, N-acetyl-L-cysteine 2 mM. After a 10-minutes incubation, the substrate Z-Arg-Arg-AMC (Calbiochem) was added (1 mM) and the reaction was monitored for 1 h on a Fluoroskan & FL (Thermolabsystem) measuring the emission at 455 nm wavelength, 360 nm excitation wavelength.

3.8. Immunoprecipitation and immunoblot analyses

Cells were seeded in number of 10^6 per 10 cm (∅) dish, and infected 24 h later with H-1PV (5 pfu/cell) in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 µg/ml) and gentamicin (50 µg/ml). Cells were harvested with trypsin, washed in PBS 1x, and lysed 20 minutes on ice in 400 µl RIPA buffer (Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 1 mM, 1% [v/v] NP-40, 0.25% [w/v] Na-deoxycholate, Complete-mini™ protease inhibitor cocktail). Samples were centrifuged at 13000 rpm for 20 minutes at 4ºC and supernatants were stored at -20°C; protein concentrations were estimated over bovine serum albumin (BSA) standards (0.2, 0.5, 0.8, 1.2, 1.5 µg/µl) by using the DC Protein Assay (Bio-Rad Laboratories, Munich, Germany).

For immunoprecipitation analyses, 200 µg total protein extracts were pre-cleaned with 40 µl Protein G Sepharose 80% (v/v) bead slurry (Upstate Biotech, Hamburg, Germany) for 1 h at 4°C. Pre-cleaned extracts were subsequently incubated 2 h at 4°C with 1 µg antibody against wild-type (Ab-6) or mutated p53 (Ab-3) (Calbiochem) bound to 50 µl Protein G Sepharose bead slurry (80%), and then separated by SDS-PAGE (SDS polyacrylamide gel electrophoresis).

For the SDS-PAGE, a 5% polyacrylamide stacking gel (Tris-HCl 125 mM pH 6.8, 0.1% [w/v] SDS, 0.1% [w/v] APS [ammonium persulfate], 0.1% [v/v] TEMED) and 10-15% polyacrylamide resolving gels (Tris-HCl 375 mM pH 8.8, 0.1% [w/v] SDS, 0.1% [w/v] APS, 0.04% [v/v] TEMED) were prepared; polyacrylamide here refers to a 30% (v/v) acrylamide and

0.8% (v/v) bis-acrylamide solution. Sample buffer (10% [v/v] 2-mercaptoethanol, 10% [w/v] SDS, 10% [v/v] glycerol, 0.1% [v/v] bromophenol blue) was mixed 1:1 with 40 up to 100 µg of total protein extracts, denaturated 5 minutes at 95°C and loaded on the gel. Chambers were filled in with reservoir buffer (Tris-HCl 25 mM pH 8.3, glycine 192 mM, 0.1% [w/v] SDS), and proteins were separated overnight at 50 V, 100 mA.

To perform the immunobloting, gels were detached from the glasses of the electrophoretic apparatus, cut in the required size and equilibrated in cathode buffer (Tris-HCl 25 mM pH 9.4, 10% [v/v] MeOH, glycine 40 mM) for at least 15 minutes. Nitrocellulose membranes (Protran, Schleicher & Schuell, Dassel/Relliehausen, Germany) were cut of the same sizes of the gel, hydrated 2 minutes in H_2O, and equilibrated in anode buffer II (Tris-HCl 25 mM pH 10.3, 10% [v/v] MeOH). 18 sheets of chromatography 3MM paper (Whatman, Dassel, Germany), were cut approximately 1cm larger than the gel sizes, and drenched in anode buffer I (Tris-HCl 300 mM pH 10.7, 10% [v/v] MeOH – 6 sheets), in anode buffer II (3 sheets), or in cathode buffer (9 sheets). A blotting sandwich was assembled by stratifying the anode buffer I sheets, the anode buffer II sheets, the nitrocellulose membrane, the gel and the cathode buffer sheets, and by wiping any bubble away with a roller. Proteins were transferred using a semidry blotting apparatus (Bio-Rad Laboratories) for 1 hour and 30 minutes at 24V, 250-300 mA. Membranes were afterwards stained 15 minutes in Ponceau Red Solution (0.2% [w/v] Ponceau-S red, 3% [v/v] trichloroacetic acid 30%, 3% [v/v] sulfosalicylic acid), cut according to protein marker weights, and incubated in blocking solution (4% [w/v] milk powder, 0.1% [v/v] Tween20 in Tris Buffered Saline – TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 7.6) for 1 hour at room temperature. Then, membranes were incubated with a primary antibody diluted 1:1000 in blocking solution for 1 hour at room temperature, and washed twice in washing buffer (0.1% [v/v] Tween20 in TBS) for 10 minutes. Membranes were incubated with a secondary antibody diluted 1:2500 in blocking solution for 1 hour at room temperature, washed twice in washing buffer for 10 minutes, and shortly air-dried. Immunodetection was performed with Chemiluminescence Reagent Plus/Western Lightning (PerkinElmer Life Sciences, Rodgau - Jügesheim, Germany).

The primary antibodies used for immunostaining were directed against β tubulin (Sigma), cystatin C (Upstate Biotech), Beclin1, GFP, Lamp2, LC3-goat (for REFs transfectants), c-myc clone 9E10, Ha-ras (Santa Cruz, Heidelberg), Bcl-2 (Calbiochem), Flip (Apotech, Epalinges, Switzerland), SV40 large T antigen (Covance, Freiburg, Germany), LC3-rabbit (for NCH82 cells - Biozol, Eching, Germany), NS1 (rabbit antiserum αSP8), or VP (rabbit antiserum polypeptide, nt 3610–4310 region of H-1PV genome). Human cathepsin B (clone CB 59-4B11), cathepsin L (clone CPLH 33/2), and cystatin B (clone RJMW 2E7) antibodies were a gift of Dr. E. Weber (Martin Luther University, Halle, Germany). Peroxidase-conjugated secondary antibodies were all purchased from Santa Cruz. Antibodies for immunoprecipitation against p53 (Ab-7), biotin-conjugated anti-sheep antibody, and peroxidase-conjugated streptavidin were purchased from Calbiochem.

3.9. Southern blot analyses

Low-molecular-weight DNA was extracted from H-1PV and mock-infected cell pellets by the Hirt procedure (Hirt, 1967). Cells were seeded in number of 10^6 per 10 cm (∅) dish, and infected the following day with H-1PV (5 pfu/cell) in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 µg/ml) and gentamicin (50 µg/ml). 24 or 48 hours post infection cells were harvested, washed in PBS 1x, resuspended in 200 µl VTE buffer, and lysed by adding 200 µl Hirt buffer 2x (Tris-HCl 20 mM, 1.2% [w/v] SDS, EDTA 20 mM, pH 7.4). Samples were incubated with Proteinase K (400µg/ml final) overnight at 46°C under shaking at 200 rpm, and DNA was then fractionated with a syringe (0.5 and 0.4 µm needles, 5 strokes each). 2 µg DNA were diluted in 20 µl H_2O, mixed with 4 µl agarose gel loading buffer 6x, and run 5 h at 120 mA on a 0.8% (w/v) agarose gel. After electrophoresis, gel was incubated 30 minutes in Denaturation buffer (NaOH 500 mM, NaCl 1.5 M), washed twice in H_2O, incubated 1 h in Neutralization buffer (Tris-HCl 500 mM, EDTA 1 mM, NaCl 1.5 M, pH 7.2), and blotted overnight onto Hybond N+ nylon membranes (Amersham Pharmacia, Little Chalfont, United Kingdom). In synthesis, SSC 20x buffer (NaCl 3 M, trisodium citrate 300 mM) moves up by capillary action through moist 3MM

papers, carrying the single-stranded DNA from the gel to the membrane, where it is immobilised in the same position relative to where it had migrated in the gel. DNA was fixed on the nylon membrane by UV-crosslinking at 1200 Joule (Stratalinker™ – Stratagene, Amsterdam, The Netherlands) and subsequently pre-hybridized in 50 ml hybridisation buffer (SSC 5x, Hepes 50 mM pH 7.4, 1% [w/v] SDS, herring sperm DNA 100 µg/ml, Denhardts solution 1.5x [a 10x solution was prepared by dissolving 2 g Ficoll™ 400, 2 g polyvinylpyrrolidone, and 2 g BSA in 1 litre water]) for 3 hour in agitation at 65°C. Membrane was hybridized with a specific ^{32}P-labeled NS1 DNA probe consisting of a 703-bp *Eco*RV-*Eco*RI restriction fragment of H-1PV-derived phH1Δ800 vector (Wrzesinski et al., 2003). The probe was labelled with the Megaprime Labeling Kit (Amersham Pharmacia), collected in TE Buffer (Tris-Cl 10 mM, EDTA 1 mM, pH 7.5), directly added to the membrane in hybridisation buffer, and incubated overnight in agitation at 65°C. The following day, membrane was washed 15 minutes in 50 ml washing buffer I (SSC 2x, 2% [w/v] SDS) and 15 minutes in 50 ml washing buffer II (SSC 0.2x, 0.2% [w/v] SDS), air dried, and exposed to a radiographic film (Amersham Pharmacia) for at least 5 h at -80°C; the radiographic cassette was finally de-frozen 1 hour at -37°C and the film was developed in a Classic E.O.S. processor (AGFA, Cologne, Germany).

3.10. Assessment of cell viability and lysis

To determine cell viability and lysis upon H-1PV infection, cells were seeded in number of 3000 per well (96-well plates) and infected after 24 h with H-1PV at different multiplicities of infection in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 µg/ml) and gentamicin (50 µg/ml). In cell rescue experiments, cells were treated with 3-MA (1 mM, 500 or 100 µM), E64d (1 µM), Ca-074 Me (10 µM), or DEVD-CHO (20 µM) 5 h post infection.

Viability was evaluated using the hexosaminidase assay, performed at 48 h post infection according to a previously well established protocol. Briefly, cultures in 96-well plates were washed twice in PBS 1x, and lysed in 100 µl reaction buffer (p-nitrophenol-N-acetyl-β-D-glucosaminide 3.75 mM in sodium citrate 0.05 mM, 0.25% [v/v] Triton X-100, pH 5.0) per well. After 1

h (REFs transfectants) or 3 h (NCH82 cells and derivatives) incubation at 37ºC, the assay was terminated and the color developed by adding 150 µl STOP solution (glycine 50 mM, EDTA 5 mM, pH 10.4) per well. The absorbance was read at 405 nm.

Cell lysis was determined by measuring the release of glucose 6-phosphate dehydrogenase (G6PD) into culture medium, using the Vybrant Cytotoxicity Assay Kit (Molecular Probes) according to the manufacturer's instructions. This assay was preferred to the more frequently used lactate dehydrogenase (LDH) assay (Promega, Mannheim, Germany) because low-passage glioma cells needed to be maintained in DMEM supplemented with 10% (v/v) FCS, a serum concentration that often results in a high background noise from control cells LDH basal release, thus compromising the data processing.

3.11. Immunofluorescence analyses, electron and optic microscopy

To study cathepsin B intracellular localization, cells were seeded in 8 chambers/slides in number of $1x10^4$ cells per chamber, and infected or not with H-1PV (5 pfu/cell) in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 µg/ml) and gentamicin (50 µg/ml) for 24 h. Cells were then washed twice in 200 µl PBS 1x, fixed for 10 minutes on ice in 400 µl 2% (v/v) PFA in PBS 1x, washed once in 200 µl PBS 1x, and re-fixed 10 minutes on ice in 500 µl MeOH cold. Cells were permeabilized 5 minutes in 400 µl 0.1% (v/v) Triton X-100 in PBS 1x, washed once in 200 µl PBS 1x, and incubated 30 minutes at RT in 150 µl 2% (v/v) NGS (normal goat serum – Santa Cruz) in blocking solution (1% [w/v] BSA in PBS 1x). Cells were afterwards incubated 30 minutes at RT with the cathepsin B (clone CB 59-4B11) antibody diluted 1:200 in 50 µl blocking solution, washed three times in 200 µl PBS 1x and once in 200 µl MeOH cold. Cells were then incubated 30 minutes at RT with α-rabbit FITC- or Cy3-conjugated antibody diluted 1:250 in 50 µl blocking solution, washed three times in 200 µl PBS 1x and once in 200 µl MeOH cold. Finally, cells were incubated 30 minutes at RT in 200 µl Dapi (4',6-diamidino-2-phenylindole; 1µg/λ in MeOH solution), washed three times in 200 µl MeOH cold, air dried and sealed in ProLong™

Gold antifade reagent (Molecular Probes). Slides were analyzed with a DM-RBE automated fluorescence microscope (Leica, Wetzlar, Germany) and images were processed with the Openlab™ software (Improvision, Tübingen, Germany).

Electron microscopy analyses were performed by Dr. H. Zentgraf (DKFZ, Heidelberg). Briefly, cells were seeded in number of 10^6 per 10 cm (∅) dish, infected h with H-1PV (5 pfu/cell) in DMEM supplemented with 10% (v/v) FCS, kanamycin (100 µg/ml) and gentamicin (50 µg/ml), and fixed after 24 h with 2.5% (v/v) glutaraldehyde in sodium cacodylate 0.1 mM (pH 7.2), overnight at 4°C. After washing in sodium cacodylate 50 mM, cells were post-fixed in 2% (w/v) osmium tetroxide and 0.5% (w/v) uranyl-acetate, dehydrated through ascending alcohol concentrations and propylene oxide, and embedded in Epon (Carl Roth, Karlsruhe, Germany). Ultrathin sections were cut with a Reichert-Jung microtome (Leica) and examined with an EM-10A electron microscope (Carl Zeiss, Esslingen, Germany) at 80 kV. The magnification indicator was routinely controlled by the use of a grating replica.

To identify lysosomes and autophagic compartments, mock-treated and H-1PV-infected cells were stained with acridine orange (10 µg/ml) in PBS 1x for 2-3 minutes, washed in PBS 1x, and directly analyzed with a DM-IL inverted microscope (Leica) using filters for red fluorescence. Images were acquired with a DFC350-FX monochrome digital camera (Leica) and processed with the Openlab™ software (Improvision).

3.12. Animal treatment

Rat glioma (RG2) cells were intracerebrally implanted (3000 cells/animal) into Wistar Kyoto inbred rats, and tumor development was monitored by magnetic resonance imaging. On day 11 post-implantation, both the tumor and its corresponding portion in the tumor-free hemisphere were stereotactically injected with H-1PV (1 pfu/cell) or mock treated. Animals were sacrificed on day 3 post-infection and protein extracts from resections of tumors and healthy brains were prepared for measuring cathepsin B activity and expression.

4 Results

Parvovirus H-1 kills permissive glioma cells by triggering an autophagic cell death dependent on cathepsins cytosolic activation

4.1. Apoptosis activation is a minor event occurring during parvovirus H-1 infection of glioma NCH82 cells

Parvovirus H-1 (H-1PV) exerts an efficient lytic action on primary cultures of human glioma cells that had proven to be resistant to DNA damaging agents and soluble death ligands (Di Piazza et al., 2007). Thus, to strengthen the feasibility of H-1PV application in brain cancer virotherapy, a priority was to scrutinize the death pathways activated by the virus in order to determine whether human glioma cells could acquire resistance and escape H-1PV-mediated killing. Rodent parvovirus infections can induce either necrosis or apoptosis, depending on the tumor model considered. After infection with H-1PV, human monoblastic leukaemia cells (U937) and several hepatocarcinoma cell lines die from apoptosis (Moehler et al., 2001; Rayet et al., 1998), while transformed rat fibroblasts and human keratinocytes show signs of necrosis (Ran et al., 1999). The question arose whether parvovirus H-1 would trigger an apoptotic death in NCH82 cells, a low-passage human glioma culture that was chosen in this study because of its sensitiveness towards the virus, cisplatin and, to some extent, TRAIL (Di Piazza et al., 2007).

A mechanism developed by tumor cells to prevent caspases activation is the overexpression of polypeptides that directly inactivate caspases or their activating complexes. In particular, Flip was demonstrated to interact with the cytoplasmic domain of death receptors, preventing the cleavage and activation of caspase 8 (Xiao et al., 2002b). Bcl-2 instead prevents the outer mitochondrial membrane depolarization and the relocation of cytochrome *c* to the cytosol, and had been shown to interfere, depending on the cell type,

with both the intrinsic and extrinsic apoptotic pathways (Fulda et al., 2001; Scaffidi et al., 1998). It was therefore tested if H-1PV-induced glioma cell death could be modulated by the overexpression of these inhibitors. Flip or Bcl-2 were overexpressed in NCH82 cells, and the expression of these polypeptides was confirmed by immunobloting not to be affected by cells treatment with H-1PV at a multiplicity of infection of 5 infectious particles per cell, expressed in plaque forming units (pfu/cell) (Fig. 7A). The ability of H-1PV to trigger apoptosis was studied in the NCH82 transfectants by measuring the induction of caspase 3 activity in infected cells, in comparison to cultures treated with cisplatin or TRAIL (Fig. 7B). The ability of these agents to cause cell death (Fig. 7C) tightly correlated with the induction of caspase 3 activity. Bcl-2 overexpression prevented caspase 3 activation after exposure of the cells to H-1PV, cisplatin and TRAIL while Flip overexpression inhibited caspase 3 activation in response to TRAIL but not cisplatin, as expected, and only to a limited extent upon H-1PV infection. Caspase 3 activation after infection of the cells with H-1PV seemed therefore to be mostly dependent on the loss of the mitochondrial membrane potential ($\Delta\Psi m$).

Remarkably, $\Delta\Psi m$ resulted not to be strongly disturbed upon H-1PV infection of NCH82 cells (data not shown). Moreover, although Bcl-2 overexpression was generally able to block caspase 3 activation, it only protected the cells against the cytotoxic effect of the two drugs but not against H-1PV-induced killing (Fig. 7C). It was thus hypothesized that caspase 3 cleavage might be a side effect of H-1PV infection and reflect the activation of proteases in response to the viral infection of glioma cells. In agreement with this possibility, previous reports had shown that members of the cathepsin cysteine proteases family can participate in caspase 3 induction and, as a result, promote cell death (Hishita et al., 2001; Johansson et al., 2003; Nagaraj et al., 2006). To test this assumption, caspase 3, 8 and 9 activation was evaluated in NCH82 glioma cells infected with H-1PV with or without co-treatment with the cathepsin B inhibitor Ca-074 Me (Fig. 7D). Caspase 3 and 9 activation was promoted by viral infection, but greatly reduced after exposure to Ca-074 Me, suggesting that caspases were indeed direct or indirect targets for cellular cathepsins.

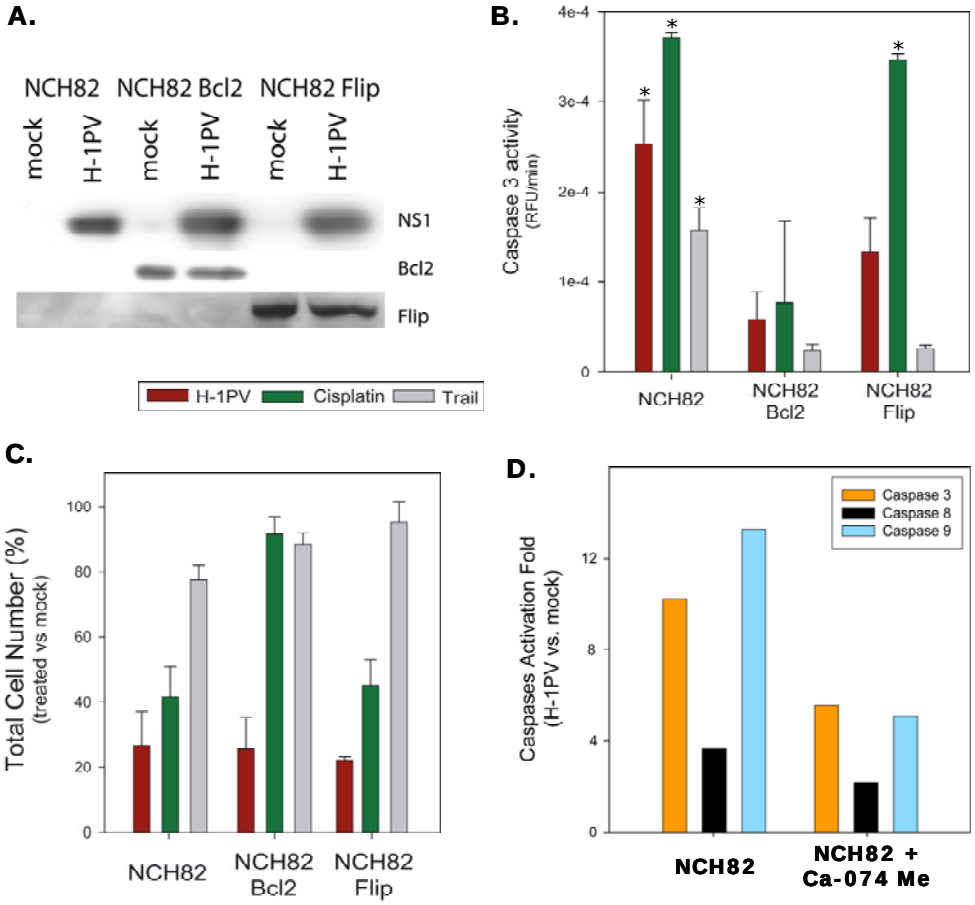


Figure 7. *H-1PV-induced glioma cell killing subsists when apoptosis is inhibited*. (A) Western blot analysis for the accumulation of Bcl-2, Flip, and the parvoviral protein NS1 after H-1PV or mock infection, in primary and Bcl-2 or Flip stable transductants of NCH82 glioma cells. (B) Caspase 3 activation after 24 h treatment of these cells with H-1PV (5 pfu/cell) or cisplatin (2.5 µg/ml) and TRAIL (100 ng/ml). Caspase 3 activity is expressed in relative fluorescence units (RFU) per minute. Statistically significant differences between treated and control samples ($p<0.05$) are indicated by a star (*). (C) Effect of TRAIL, cisplatin, and H-1PV on cell survival; hexosaminidase assay, average values and standard deviation bars from three independent experiments, each in triplicate. (D) Caspase 3, 8, and 9 activation in NCH82 glioma cells 24 h after H-1PV infection (5 pfu/cell), in presence or absence of Ca-075 Me (10 µM).

In summary, parvovirus H-1 promoted pro-caspase 3 cleavage through mitochondrial depolarization but this process was not required for H-1PV-induced glioma cell death. A non-apoptotic type of cell death, cathepsin B-dependent and accountable for efficient cell killing, was activated upon H-1PV infection of NCH82 glioma cells.

4.2. Organelles integrity and accumulation of acidic vesicles in H-1PV-infected NCH82 cells

The alteration of $\Delta\Psi m$ had been often related to the permeabilization of the outer mitochondrial membrane and the production of reactive oxygen species (ROS) and lipid peroxidation during the apoptotic process, a phenomenon that was observed to cause organelle swelling and morphological disruption (Kim et al., 2006; Simon et al., 2000). The integrity of several organelles was therefore analyzed in H-1PV-infected NCH82 glioma cells. Cell extracts were cleared from nuclei and further fractionated into cytosolic and organelle fractions by centrifugation. The integrity of organelles proved to be conserved in H-1PV-infected cells as shown by the confined activity of catalase to peroxysomes, and of acid phosphatase and hexosaminidase to lysosomes (Fig. 8A). However, it was noted that in H-1PV-infected cells the proportion of total cathepsin B activity present in the cytosol was increased by more than 2-fold. This could be due to a specific relocation of the lysosomal cathepsins in the cytosol, or to a disruption of the trafficking network between the Golgi and the lysosomes for the delivery of newly synthesized cathepsins. But acid phosphatase, whose molecular weight is similar to that of cathepsin B, remained inside the lysosome in H-1PV infected cells, arguing against a virus-induced early permeabilization of the lysosomal membrane.

To confirm this, cells were stained with acridine orange, a fluorogenic weak base that chelates nucleic acids emitting in the green field, but also accumulates inside acidic vesicles where it becomes protonated and undergoes a shift to red-orange fluorescence. As a result, acidic compartments fluoresce red-orange against a green background (i.e. cytoplasm). NCH82 cells were infected with H-1PV (5 pfu/cell) and acridine orange staining was monitored by flow cytometry (Fig. 8B) and visualized by microscopy (Fig. 8C) at different times points after infection. An increase in red fluorescence that could be explained as an augment in either the number or the size of acidic vesicles was detected at 24 h post infection. At later times (48 h) post infection a double peak became visible, indicating that when cells were actually dying, their vesicles content eventually dropped (Fig. 8B). Indeed, the accumulation of acidic vesicles in the cytoplasm of

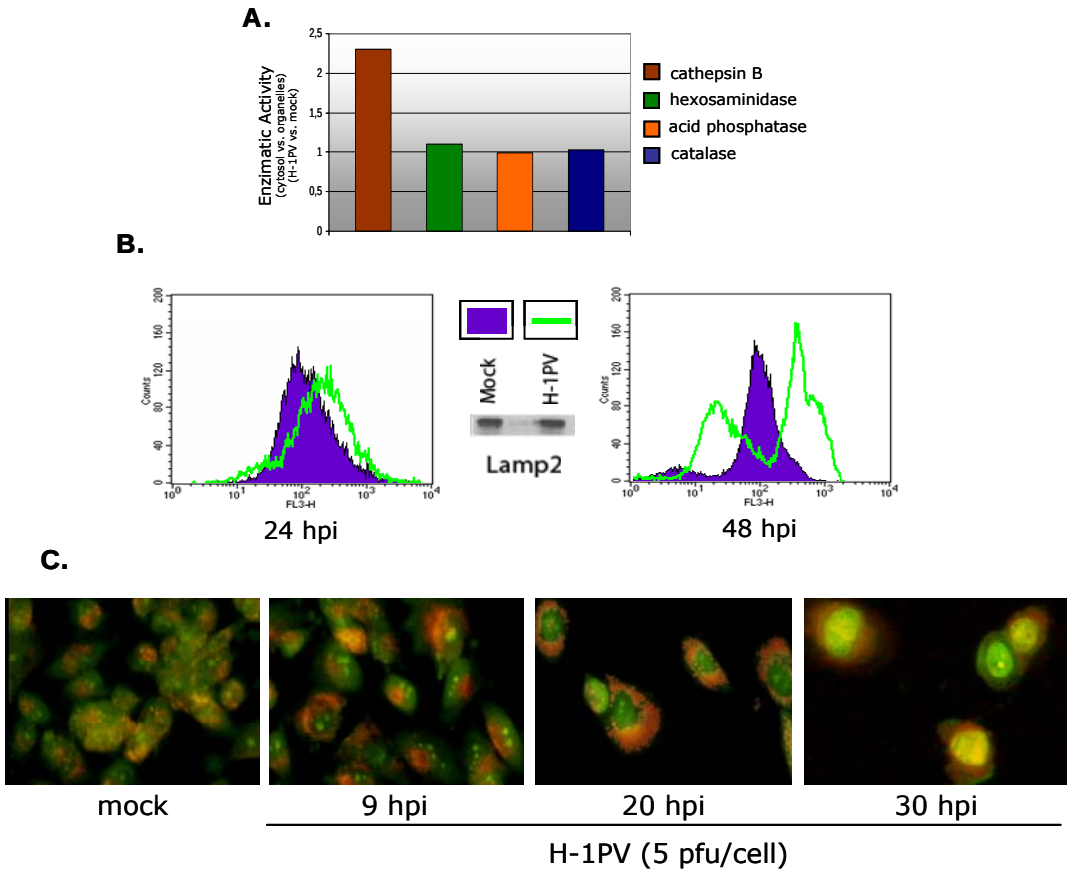


Figure 8. *Organelles integrity and accumulation of acidic vescicles in the cytosol of H-1PV-infected NCH82 cells*. (A) Relative enzymatic activities in the cytosol versus lysosomes (cathepsin B, hexosaminidase, acid phosphatase) or peroxysomes (catalase) of NCH82 cells, calculated as ratio in H-1PV-infected (24 hpi, 5 pfu/cell) over mock. (B) Acidic vesicles content, measured by flow cytometry after acridine orange staining [FL3-H channel], and Western blotting detection of Lamp2 in NCH82 glioma cells at 24 and 48 h after H-1PV (5 pfu/cell) or mock treatment. (C) Acridine orange staining of NCH82 cells that were either mock-treated or analyzed 9, 20 and 30 h after H-1PV infection. Fluorescence microscopy, magnification 800x; acidic vesicles appear as red dots, nucleic acids are stained in green.

infected NCH82 cells was observed as early as 9 h post infection and became massive at 20 h post infection. The cytosol appeared yellow in the majority of infected cells at 30 h post infection, as result of vesicles content release and dye shift of fluorescence in the more alkaline cytosolic environment (Fig. 8C). At this stage, the expression of lysosomal trans-membrane protein Lamp2 did not change in infected versus mock-treated cells, suggesting that the virus induced a leakage of acidic compartments rather than their blebbing (Fig. 8B). Thus glioma infection with H-1PV did not disrupt the membrane boundary of cellular organelles but increased the number of acid vesicles, a process that was reminiscent of macroautophagy.

4.3. Autophagic cell death is promoted after parvovirus H-1 infection of NCH82 glioma cells

The evidence of H-1PV-induced accumulation and later leakage of acidic vesicles potentially contributing to virus-mediated cell death required further analysis in order to ascertain the nature of these compartments. Acridine orange was previously used as lysosomotropic agent to detect the development of acidic vesicular organelles, which were subsequently identified as autophagic vesicles (Kanzawa et al., 2004).

Autophagy or type II cell death is characterized by the appearance of cytoplasmic vesicles called autophagosomes. These contain cytoplasm and/or cytoplasmic organelles and eventually fuse with lysosomes, leading to the formation of autophagic vacuoles where the autophagosome content is notably degraded by proteolysis. Autophagy was demonstrated to be either promoted and/or inhibited during tumor progression, depending on environmental conditions and the genetic alteration status of transformed cells (Kondo et al., 2005). Moreover, the formation of autophagic vesicles in response to human parvovirus B19 (Nakashima et al., 2006) and other pathogens (Kirkegaard et al., 2004) infection was reported to be a defense mechanism by which cells digest infectious agents. Besides, some viruses were shown to hijack the autophagic machinery to the benefit of their life cycle (Jackson et al., 2005). These observations raised the question whether H-1PV triggered autophagy to kill infection-permissive cells.

NCH82 glioma cells were infected for 24 h with parvovirus H-1 (5 pfu/cell) and subsequently prepared for electron microscopy analysis. Compared to mock-treated cells (Fig. 9A and B), infected NCH82 cells displayed a completely different morphology, appearing to be filled in with vesicles and vacuoles (Fig. 9C and F). In infected cells, autolysosomes were identified as single membrane structures containing electron-dense material, pinpointed as cytoplasmic components at various stages of degradation (Fig. 9E). Autophagosomes were characterized as transient double membrane structures containing undigested cytoplasmic material and including portions of mitochondria (Fig. 9H), and appeared rather different in comparison to normal mitochondria (Fig. 9G). Partial chromatin condensation, an event which had been defined as one of the hallmarks of autophagy (Bursch et al.,

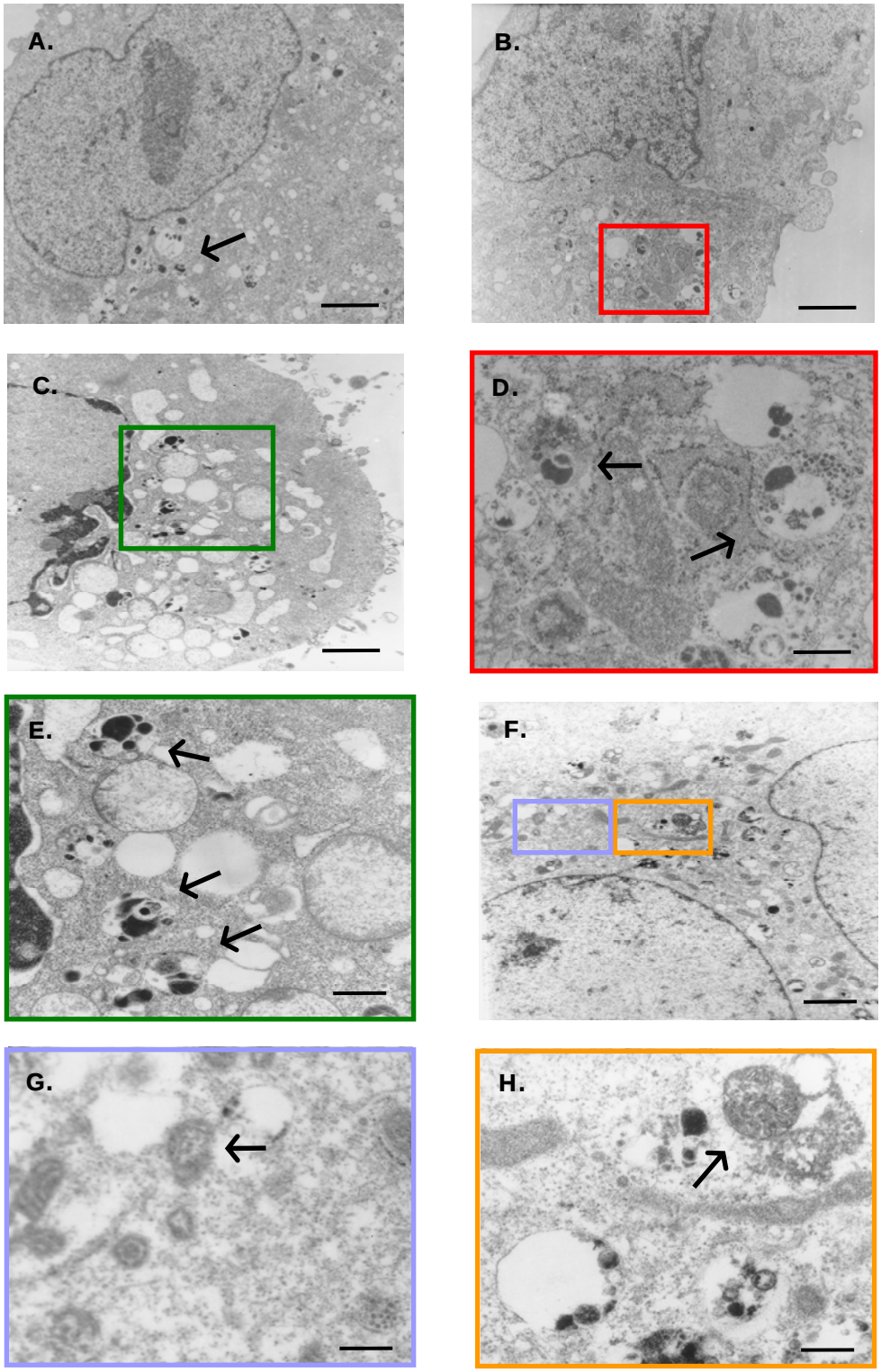


Figure 9. *Autophagy is triggered upon parvovirus H-1 infection of NCH82 glioma cells.* Electron microscopy images of cells (A, B) mock-treated or (C, F) infected 24 h with H-1PV (5 pfu/cell). Scale bar: 1,5 μm. Autolysosomes (D, E), a mitochondria (G) and an autophagosome (H) are indicated. Scale bars: 600 nm (E); 300 nm (D, G, H).

2000), was observed in the nuclei of NCH82 cells infected with H-1PV (Fig. 9C). Notably, the presence of structures resembling autolysosomes was detected even in mock-treated NCH82 cells (Fig. 9A and D), suggesting that this process was already activated in this cancer cell line, most likely at a basal level to sustain survival in low-oxygen and nutrient *in vivo* growth conditions (Kondo et al., 2005) and after cell adaptation to *in vitro* culturing. Anyway, autophagic compartments increased noteworthy in number after parvovirus H-1 infection.

To further support the hypothesis of type II cell death activation upon H-1PV infection, the expression of different proteins involved in autophagy was analyzed in NCH82 cell 24 h after treatment with H-1PV at 5 pfu/cell (Fig. 10). In parallel, it was decided to test as positive control the effect of rapamycin (1 μM), an inhibitor of the PI3K–Akt–mTOR signalling pathways that had been recently shown to induce autophagy in glioma cells (Iwamaru et al., 2006).

Microtubule-associated protein 1 light chain 3 (LC3) is a good marker protein for autophagic membranes since it is synthesized as cytosolic form (LC3-I, apparent mobility of 18 kDa) and specifically processed, upon activation by the mammalian Atg7 (Tanida et al., 2001), into a membrane-bound form (LC3-II, apparent mobility of 16 kDa) that associates tightly to the autophagosomal membrane. The amount of LC3-II or the LC3-II/LC3-I ratio correlates with the number of autophagosomes (Kabeya et al., 2000). The LC3-II isoform was already detected in the mock-treated NCH82 cells, confirming that this glioma cell population was prone to the activation of autophagy in absence of H-1PV infection. Nevertheless, in cells infected with the virus the conversion of LC3-I to LC3-II was almost total and markedly stronger than in cells treated with rapamycin (Fig. 10, 3rd lane).

The pro-autophagic factor Beclin1 was shown to localize in the trans-Golgi network where it might be involved in sorting putative autophagosomal components thus initiating the autophagic process (Kihara et al., 2001). In agreement with data in the literature (Liang et al., 1999), Beclin1 expression levels were low in cultured NCH82 tumor cells but increased 24 h after infection with parvovirus H-1 (Fig. 10, 2nd lane). Surprisingly, no changes in Beclin1 expression were observed upon treatment of these glioma cells with rapamycin. This compound blocks the activity of mTOR, a negative regulator

of autophagy considered a key factor for the initiation of the autophagic pathway. Nonetheless, despite the fact that Beclin1 siRNA was recently demonstrated to inhibit rapamycin-induced autophagy (Iwamaru et al., 2006), a direct relationship between mTOR and Beclin1 has not been yet proven.

Altogether, these data indicated that autophagy was already present in NCH82 glioma cells but that H-1PV infection triggered the activation levels of this process.

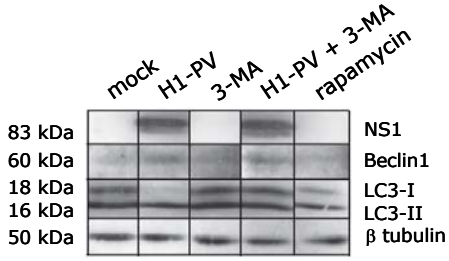


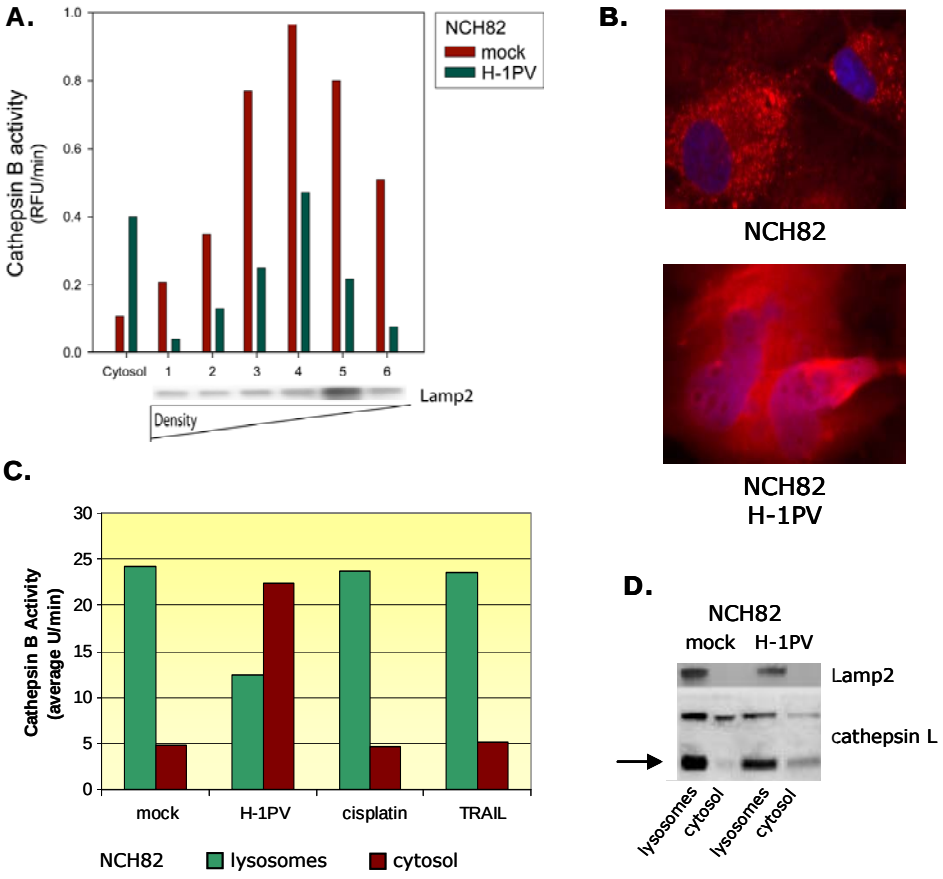
Figure 10. *Autophagy activation following parvovirus H-1 infection*. Western blot analysis for the expression of NS1, Beclin1, LC3 I and II isoforms in NCH82 cells at 24 h after treatment with H-1PV (5 pfu/cell) and/or 3-MA (500 μM) or rapamycin (1 μM); β tubulin was used as a standard for protein load matching.

4.4. Lysosomal cathepsins accumulate in the cytosol of parvovirus H-1-infected NCH82 cells

In H-1PV-infected NCH82 glioma cells, caspases 3 and 9 activation was demonstrated to be cathepsin B-dependent (Fig. 7D) and a 2-fold increase in the cytosolic fraction of total cathepsin B activity was observed (Fig. 8A). Cathepsins are normally located inside lysosomes, and their targeted transport from the endoplasmic reticulum to these organelles minimizes their impact on the cytosol. Under stress, i.e. exposure to lysosomotropic agents or starvation, the integrity of the lysosomal membrane is lost, and the release of cathepsins into the cytosol induces cell necrosis (Guicciardi et al., 2004; Jäättelä, 2004). This did not appear to occur in H-1PV-induced death, as immediately after infection acidic vesicles accumulated in, instead of disappearing from, the cytoplasm. It was nonetheless important to investigate what was the role of this imbalance in cytosolic vs. lysosomal cathepsin B activity in H-1PV-triggered cell killing, and whether it was a common parvovirus-induced phenomenon.

To this purpose, NCH82 glioma cells were infected with H-1PV (5 pfu/cell) and samples were analyzed by cell fractionation at 24 h after

infection. Post-nuclear extracts were centrifugated to separate the lysosomal/organelle and the cytosolic fractions, and cathepsin B activity was measured by a fluorescence-based enzymatic assay. H-1PV-infected NCH82 cells showed a much higher level of cathepsin B activity in the cytosolic fraction as compared to mock-treated cells (Fig. 11A), and immuno-fluorescence analysis was performed to further confirm H-1PV-induced



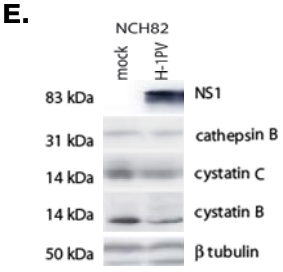


Figure 11. *Cathepsins cytosolic activation and cystatins down-regulation 24 h after H-1PV infection (5 pfu/cell) of NCH82 glioma cells.* (A) Distribution of cathepsin B activity between the cytosol and vesicle fractions after separation by iodexanol gradient centrifugation. RFU, relative fluorescence units. The lysosomal trans-membrane protein Lamp2 was used as marker to identify individual fractions (B) Immunofluorescence assay for cathepsin B localization. An anti-rabbit Cy3-conjugated secondary antibody was used; nuclei were stained with DAPI. (C) Effect of H-1PV, cisplatin (2.5 μg/ml) and TRAIL (100 ng/ml) on cathepsin B lysosomal and cytosolic activity in NCH82 cells. (D) Western blotting detection of Lamp2 and cathepsin L (black arrow, active form) proteins in the cytosolic and lysosomal fractions of mock-treated and infected cells. (E) Western blot analysis for the expression of cathepsin B and its inhibitors cystatin B and C; the parvoviral NS1 protein served as a marker for H-1PV infection while β tubulin was used as a standard for protein load matching.

cytosolic activation of cathepsin B. Mock-treated samples displayed a dotted pattern while infected cells showed a diffuse staining, indicating an intracellular relocation of cathepsin B from the lysosomes to the cytosol after H-1PV infection (Fig. 11B). Moreover, cathepsin B cytosolic activation was proved to be exclusively dependent on parvovirus H-1 infection, since any difference was observed in the levels of lysosomal and cytosolic cathepsin B activity of cells treated with cisplatin (2.5 μg/ml) or TRAIL (100 ng/ml) (Fig. 11C). Finally, also active cathepsin L was detected in the cytosol of infected but not of mock-treated NCH82 cells (Fig. 11D).

Altogether, these data lead to the conclusion that H-1PV infection resulted in an alteration of the distribution of lysosomal proteases, an enhanced proportion of these enzymes being present and active in the cytosol. It has to be specified that the modification of cathepsin distribution and concomitant death of glioma cells were not observed after treatment with empty (genome-free) capsids or UV-inactivated virus, nor after infection with full virions and co-treatment with bromodeoxyuridine (BrdU; 30μM) or aphidicolin (20μM), conditions allowing viral entry but preventing the onset of genome replication (Kwant and van der Vliet, 1980; Rhode, 1974) (data not shown). Thus, H-1PV DNA replication, gene expression, or both appeared to be required for these virus effects.

4.5. Physiological inhibitors of cathepsins are down-regulated in parvovirus H-1-infected NCH82 cells

Cathepsin activity is tightly regulated by pH and by polypeptides of the cystatin family. Among these, cystatin C has a signal peptide for extracellular transport and controls secreted cathepsins activity, while cystatin B is intracellularly located and inhibits cytosolic cathepsins accidentally released from or not delivered to the lysosomes (Rawlings et al., 2004). To evaluate whether parvovirus H-1 could regulate cathepsins activity through the modulation of their inhibitors, cathepsin B and cystatin B and C levels were measured by Western blotting in NCH82 glioma cells infected with H-1PV at 5 pfu/cell (Fig. 11E). The expression of both cystatin B and C was clearly down-regulated in infected versus mock-treated cells, while

cystatin C expression was even up-regulated after infection of human astrocytes that were demonstrated not to be permissive to H-1PV (data not shown). Moreover, cathepsin B levels were slightly reduced in infected NCH82 cells (Fig. 11E), and the overall expression of cathepsin L did not undergo any substantial change after infection (Fig. 11D), suggesting that parvovirus H-1 did not control the activity of lysosomal proteases by regulating their transcriptional levels.

It was therefore decided to investigate the reciprocal role of lysosomal proteases and their physiological inhibitors in H-1PV-triggered cell death to further substantiate their involvement during viral infection of gliomas. NCH82 cells were infected, or not, with a recombinant retrovirus driving overexpression of either a cystatin B-GFP fusion protein or control GFP alone (Fig. 12A). Afterwards, cathepsin B and L expression was transiently down-regulated by siRNA knockdown in NCH82 cells (Fig. 12B) and their derivatives overexpressing cystatin B or control GFP. Cells were infected 24 h after siRNAs treatment with H-1PV at 5 pfu/cell for other 48 hours, and H-1PV-induced cell lysis was measured by mean of the G6PD assay (Fig. 12C). NCH82 cells transfected with cathepsin-specific siRNA proved less sensitive to H-1PV-induced killing than the corresponding parental cells, confirming the involvement of cathepsins in viral cytopathic effects. Interestingly, the efficacy of cathepsin B or L siRNA in terms of cell rescue from H-1PV infection was roughly identical, and was not enhanced by the combined siRNAs treatment. Moreover, cells overexpressing cystatin B were also less sensitive than control cells to H-1PV-induced killing, and could not be further protected from the viral toxic effects by means of cathepsin-specific RNAi. Notably, cathepsin-specific siRNAs had no detectable consequence on viral protein expression (Fig. 12D) while reduced the efficacy of viral genome replication and progeny production (Fig. 12E), an effect that was considered not to be relevant, and related to a nonspecific outcome of the RNAi approach, as it could be detected in the control (Luc) siRNA as well.

Since NF-κB signalling pathway controls cell survival in response to a variety of immune and inflammatory stimuli (Baldwin, 2001; Ghosh et al., 1998), and NFκB-responsive genes were also reported to inhibit cathepsin B activation (Liu et al., 2003), NCH82 cells were examined to verify whether

H-1PV infection might modulate NFκB activity. No significant changes in NFκB activity were observed upon infection, indicating that virus-induced activation of cathepsins was not mediated by this factor (data not shown).

In summary, cathepsin inhibitors were down-regulated upon parvovirus H-1 infection. Both cathepsins inactivation and cystatin B overexpression were sufficient to rescue NCH82 glioma cells from H-1PV-triggered cell death.

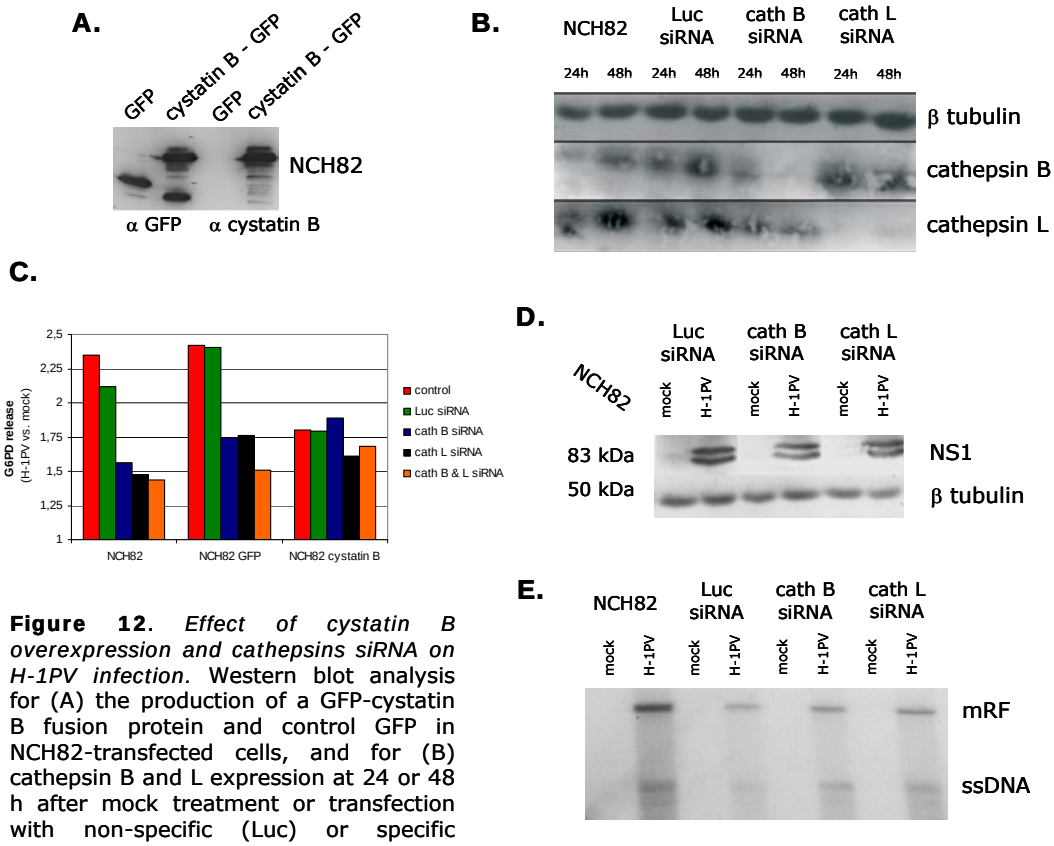


Figure 12. *Effect of cystatin B overexpression and cathepsins siRNA on H-1PV infection.* Western blot analysis for (A) the production of a GFP-cystatin B fusion protein and control GFP in NCH82-transfected cells, and for (B) cathepsin B and L expression at 24 or 48 h after mock treatment or transfection with non-specific (Luc) or specific siRNAs. (C) Effect of cathepsin B and/or L versus control (Luc) siRNAs on H-1PV-induced cell lysis, measured as G6PD release into the medium of NCH82 cells or NCH82 derivatives overexpressing either GFP or cystatin B. Data are expressed as total G6PD activity in mock versus H-1PV-infected (5 pfu/cell) cultures, normalized with respect to a fully lysed positive control. (D) Western blotting detection for NS1 expression and (E) Southern blotting analysis of viral DNA replication, 48 h after H-1PV infection (5 pfu/cell) and 72 h after mock treatment or transfection with non-specific (Luc) or specific siRNAs. mRF = monomer replicative form; SS = single-stranded DNA. β tubulin was used as a standard for protein load matching.

4.6. Both autophagy and cathepsins inhibitors protect glioma cells from H-1PV-induced oncolysis

It became at this point necessary to distinguish between the putative roles of cytosolic cathepsins and autophagic vesicles in the death of H-1PV-infected glioma cells. To this purpose, chemical inhibitors of both autophagy and cathepsins were compared for their ability to rescue cells from H-1PV-induced killing. 3-methyladenine (3-MA), a widely used inhibitor of autophagy, exerts its action by blocking the class III PI3 kinase complex, which promotes the formation of autophagosomes (Petiot et al., 2000; Seglen and Gordon, 1982). E64d [(2S,3S)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane Ethyl Ester Loxistatin], an analogue of the natural peptidyl epoxide E64, is a general inhibitor of cathepsins B, H, and L (Kirschke and Barret, 1987). It has to be noted that E64d is also used, at a 10-fold higher concentration, to inhibit the fusion between the autophagosome and the lysosome, and detect LC3-II accumulation at cellular level (Tanida et al., 2005). Ca-074 Me [[L-3-*trans*-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline Methyl Ester], methyl ester of the E64 derivative Ca-074, had been initially synthesized as cathepsin B specific inhibitor (Buttle et al., 1992). Despite recent *in vivo* observations on Ca-074 Me as non-selective inhibitor of lysosomal cysteine proteinases (Montaser et al., 2002), this compound is still considered as mainly even if not specifically inhibiting cathepsin B activity (Bang et al., 2004).

NCH82 cells were infected with H-1PV (5 pfu/cell) and cultured under standard growth conditions or in medium supplemented with 3-MA (1mM and 100 μM), E64d (1 μM), or Ca-074 Me (10 μM). In parallel, cells were treated with rapamycin 1 μM in presence or absence of 3-MA (500 μM), and the caspase 3 inhibitor DEVD-CHO (20 μM) was added as further control. Hexosaminidase measurements, performed at 48 h post treatment, demonstrated that both 3-MA and Ca-074 Me exerted almost full protection (Fig. 13A). E64d was less efficient but still significantly able to rescue cells from virus-induced death, while DEVD-CHO had no shielding effect. 3-MA showed a dose-dependent protection capacity (Fig. 13A), and interfered with the conversion of LC3-I to LC3-II after infection of NCH82 cells (Fig. 10), thus blocking H-1PV-induced accumulation of acidic vesicles (Fig. 13B).

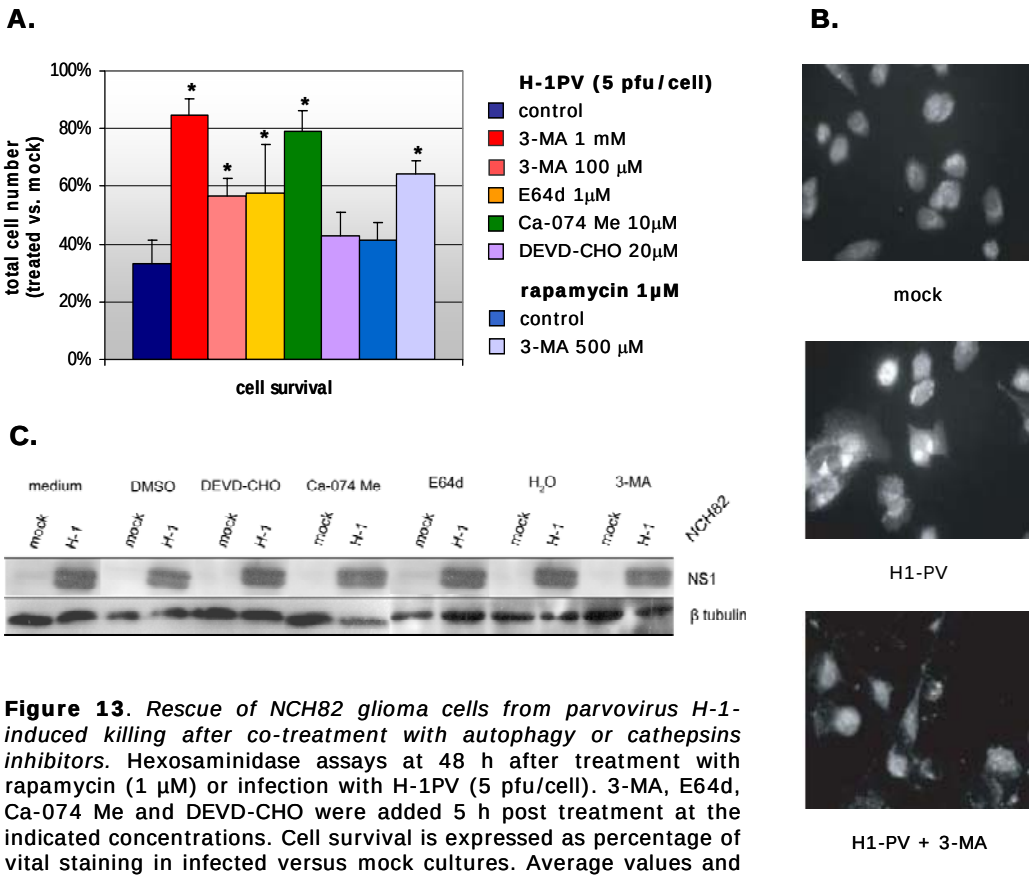


Figure 13. *Rescue of NCH82 glioma cells from parvovirus H-1-induced killing after co-treatment with autophagy or cathepsins inhibitors.* Hexosaminidase assays at 48 h after treatment with rapamycin (1 µM) or infection with H-1PV (5 pfu/cell). 3-MA, E64d, Ca-074 Me and DEVD-CHO were added 5 h post treatment at the indicated concentrations. Cell survival is expressed as percentage of vital staining in infected versus mock cultures. Average values and standard deviation bars from three independent experiments, each in triplicate. Statistically significant differences ($p<0.05$) are indicated by a star (*). (B) Acridine orange staining 24 h after treatment with H-1PV (5 pfu/cell) with or without 3-MA (500 µM). Fluorescence microscopy, red filter, magnification 800x. (C) Western blotting detection for NS1 to test the effect of above mentioned inhibitors on H-1PV protein expression. β tubulin was used for protein load matching.

Remarkably, 3-MA, E64d and Ca-074 Me were likely to interfere directly with the H-1PV-triggered death pathway rather than with production of cytotoxic viral intermediates, as no change in H-1PV protein synthesis was detected in the presence of these drugs (Fig. 13C).

Moreover, 3-MA counteracted rapamycin-mediated initiation of autophagy in NCH82 glioma cells, which proved to be sensitive to the drug, as denoted by a 60% decrease in cell survival (Fig. 13A). It has to be specified that 3-MA most commonly used working concentrations of 5 mM (Paglin et al., 2001) and 10 mM (Boya et al., 2005) turned to display an overall toxicity on the cells. In this analysis, 3-MA concentrations of 1 mM and 100 µM resulted to provide respectively the higher and lower protective effect while being essentially harmless. The intermediate 3-MA working concentration of 500 µM was therefore chosen for further tests.

Altogether these data indicated that newly formed autophagosomes and cytosolic cathepsins were both indispensable components of a distinct pathway leading to the death of H-1PV-infected glioma cells.

4.7. Cathepsins B cytosolic activation in H-1PV-infected NCH82 cells is prevented when autophagy is inhibited

In order to obtain a complete overview on the non-apoptotic cell death mechanism triggered by parvovirus H-1, the reciprocal role of autophagy activation and cathepsins B and L lysosomal release needed to be clarified.

It was first decided to investigate whether autophagy still occurred when virus-promoted cell death was blocked by the inhibition of cathepsins or the overexpression of cystatin B. NCH82 cells and derivatives overexpressing cystatin B were treated for 24 h with parvovirus H-1 (5 pfu/cell) or with rapamycin (1 µM) with or without co-incubation with 3-MA (500 µM). In parallel, NCH82 cells were infected for 24 h with parvovirus H-1 (5 pfu/cell) in medium complemented with E64d (1 µM) or Ca-074 Me (10 µM). Flow cytometry analysis was performed to quantify their acidic vesicles content (Fig. 14A). In agreement with the previous observations, an increase in the number/size of autophagic vacuoles was detected in NCH82 cells either infected with the virus or stimulated with rapamycin. Moreover, this phenomenon was prevented by the action of 3-MA while both E64d and Ca-074 Me did not exert an inhibiting effect on H-1PV-induced initiation of autophagy. More surprisingly, an augment in acidic vesicles content was noticed at 24 h post infection or rapamycin treatment of NCH82-cystatin B cells. Again, it was possible to identify acidic compartments as autophagic vacuoles since 3-MA treatment resulted in a back-shift of the red fluorescence to the mock peak position (Fig. 14A, right panels) and an increase of LC3-II isoform expression was detected by Western blot analysis (Fig. 14B). In synthesis, avoidance of cathepsins cytosolic activation by chemical inhibition or cystatin B overexpression did not impede the onset of autophagy after H-1PV infection.

Then, the question arose whether cathepsins were still present in the cytosolic fraction of infected NCH82 glioma cells when autophagy was

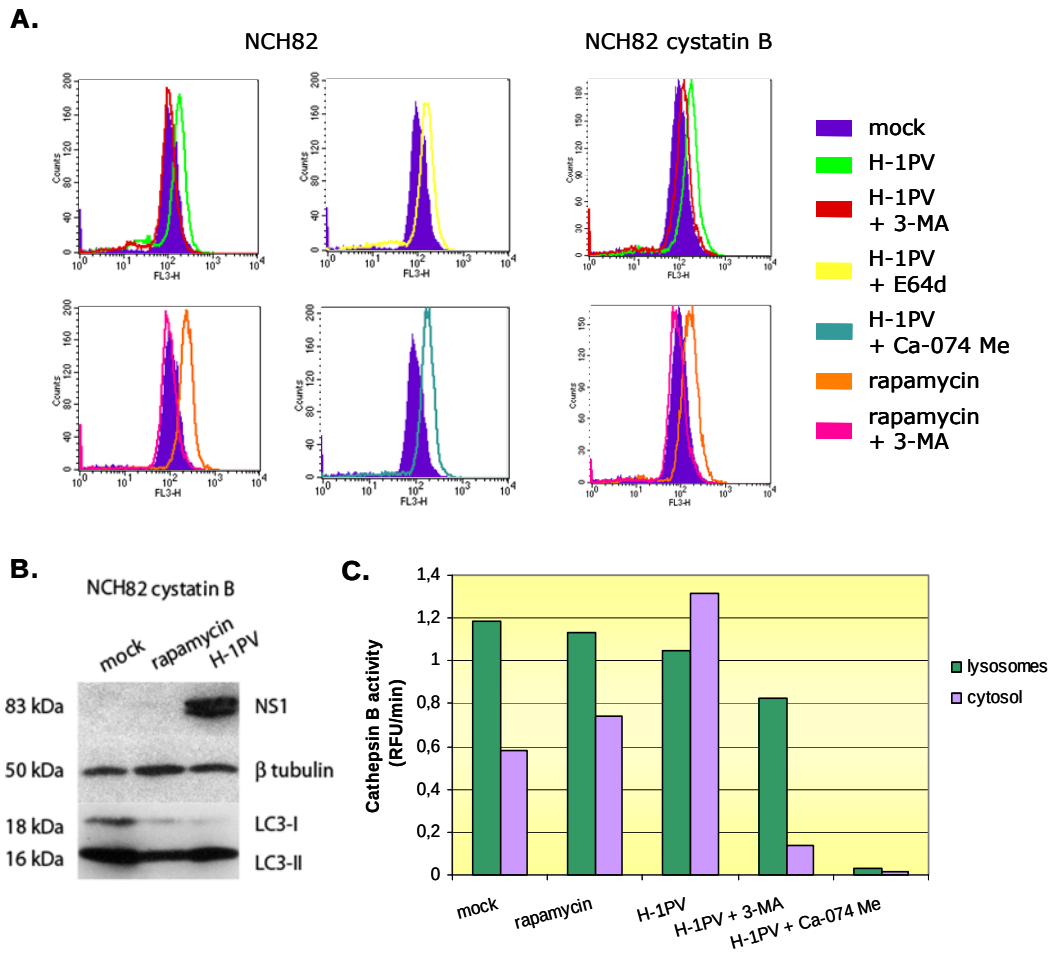


Figure 14. *Cathepsins inhibition or cystatin B overexpression does not prevent autophagy activation upon H-1PV infection.* NCH82 cells and derivatives overexpressing cystatin B were infected with parvovirus H-1 (5 pfu/cell) or treated with rapamycin (1 μM) for 24 h; 3-MA (500 μM), E64d (1 μM), and Ca-074 Me (10 μM) were added at 5 h post treatment. (A) Acidic vesicles content, measured by flow cytometry after acridine orange staining; FL3-H channel. (B) Western blot analysis for the expression of NS1, β tubulin, LC3 I and II isoforms. (C) Cathepsin B total activity in lysosomal and cytosolic fractions. RFU, relative fluorescence units.

inhibited. To verify this, cells were incubated with 3-MA (500 μM) or Ca-074 Me (10 μM), 5 h post infection with parvovirus H-1 at 5 pfu/cell. Cathepsin B activity was measured at 24 h post infection, after samples cell fractionation (Fig. 14C). In addition, the assay was conducted on cell treated for 24 h with rapamycin (1 μM), to ascertain if the outcome of autophagy on cathepsin B cytosolic activation was virus-specific or not. Cathepsin B activities in the lysosomal fractions of mock-, rapamycin- and H-1PV-treated NCH82 cells were almost equal, while an evident increase of activity in the cytosolic fraction occurred only in H-1PV-infected cells. 3-MA appeared to negatively affect cathepsin B overall activity, supporting the hypothesis of an intrinsic toxicity of this chemical. Indeed, 3-MA was reported to have some effects on

membrane trafficking including endocytosis (Punnonen et al., 1994), and to mildly alkalinise the lysosomal lumen (Caro et al., 1998). Nonetheless, autophagy inhibition drastically reduced cathepsin B activity in the cytosol of infected cells. Co-treatment with Ca-074 Me completely abolished cathepsin B lysosomal and cytosolic activities, confirming the efficiency of the compound in inhibiting this cysteine protease.

In summary, it was possible to establish the sequence of events leading to virus-induced cell death, demonstrating that autophagy played a primary role in the killing mechanism initiated by H-1PV infection. Cathepsins cytosolic activation occurred after and as consequence of the onset of autophagy.

4.8. H-1PV-induced tumor regression is associated with cathepsin B activation *in vivo*

A rat syngenic glioma model has been developed by Dr. K. Geletneky (Neurosurgery Department, University of Heidelberg) in the context of a collaboration to test parvovirus H-1 *in vivo* capacity of tumor cell killing. In this model, rat glioma cells (RG2) were intracerebrally implanted into recipient animals and tumor development was monitored by magnetic resonance imaging. After 11 days, a distinct tumoral mass was observed at the implantation site, and both the tumor and its corresponding portion in the tumor-free hemisphere were stereotactically injected with H-1PV (1 pfu/cell) or PBS (1x - mock). Parvovirus H-1 injection resulted in a striking and irreversible tumor regression, which was massive already at day 5 post infection and became almost complete by day 7 post infection (Fig. 15A) (Geletneky et al., 2007; manuscript in preparation).

For that reason, it was interesting to determine if the effect of H-1PV intratumoral injection on glioma regression correlated with a differential expression of cathepsin B in tumors versus healthy brains. Animals were sacrificed on day 3 post infection and cathepsin B expression (Fig. 15B) and activities (Fig. 15C and D) were measured in protein extracts from resections of the tumor-bearing and tumor-free hemispheres, either infected with H-1PV at 1 pfu/cell or mock-treated. H-1PV-induced glioma regression *in*

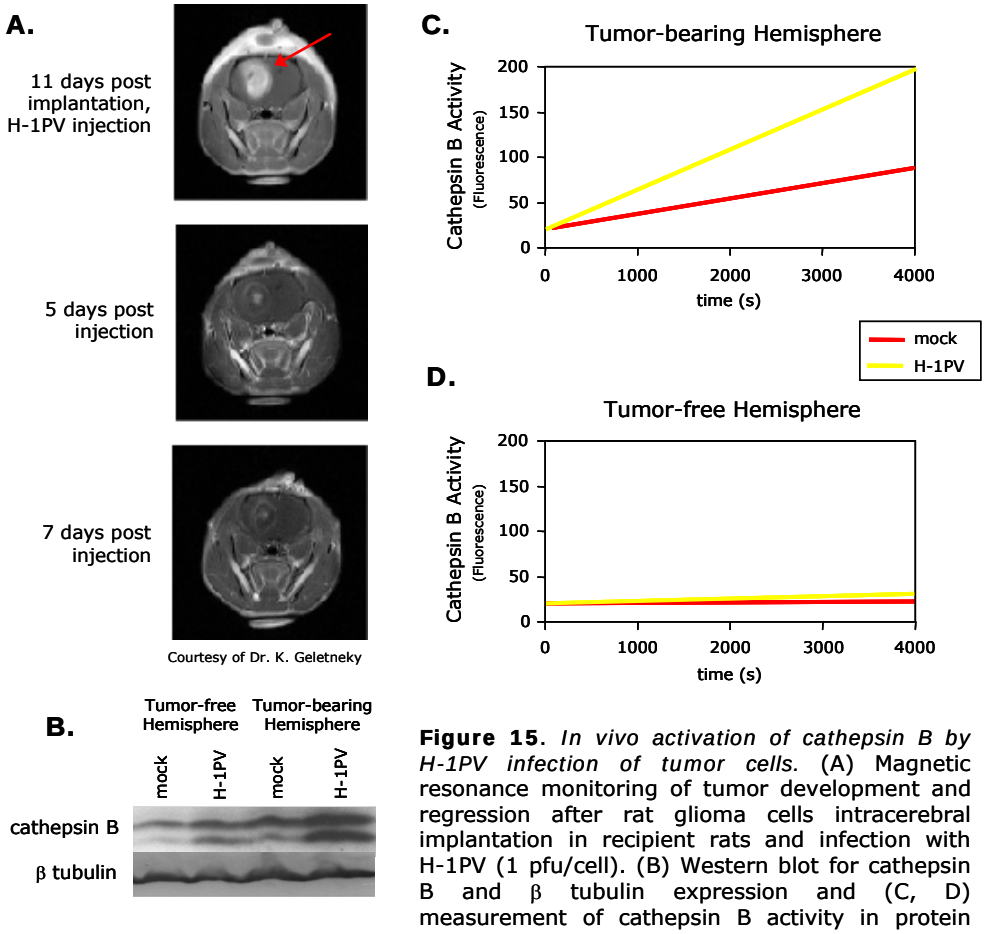


Figure 15. *In vivo activation of cathepsin B by H-1PV infection of tumor cells.* (A) Magnetic resonance monitoring of tumor development and regression after rat glioma cells intracerebral implantation in recipient rats and infection with H-1PV (1 pfu/cell). (B) Western blot for cathepsin B and β tubulin expression and (C, D) measurement of cathepsin B activity in protein extracts isolated 3 days after infection or mock treatment from the tumor and its corresponding portion in the tumor-free hemisphere. Protein amounts were normalized according to tissue masses and volumes of the extracts.

vivo was found to be associated with cathepsin induction. In contrast to the data obtained *in vitro*, the total amount of cathepsin B was increased in tumors infected with H-1PV (Fig. 15B). In addition, cathepsin B expression levels in the infected tissue extracts from the tumor-free hemisphere were similar to those in the mock-treated preparations from the tumor-bearing hemisphere, both relevantly higher than the mock-treated tissue extracts from the tumor-free hemisphere. Indeed, cathepsins had been observed to be highly expressed in high-grade gliomas and participate in malignant cells invasion and migration (Mohanam et al., 2001; Rempel et al., 1994). As confirmation, glioma-bearing hemispheres showed an enhanced cathepsin B activity, detected already in mock-infected tumors but resulted to be dramatically greater in regressing H-1PV-treated tumors (Fig. 15C). Remarkably, cathepsin B activity was very low and not significantly affected

by H-1PV intracerebral injection in tissue extracts from the tumor-free hemisphere (Fig. 15D) and from the healthy brains (data not shown).

Considering that cathepsins are often secreted by tumor cells to digest the extracellular matrix, it was possible to conclude that the observed cathepsin B *in vivo* accumulation, probably both intra- and extracellular (after cell death), might have contributed to tumor regression and participated to the formation of an empty cavity in the place of the tumor.

c-myc overexpression sensitizes rat embryo fibroblasts to the activation of a non-apoptotic death pathway upon parvovirus H-1 infection

4.9. Immortalization and transformation of rat embryo fibroblasts

Parvovirus H-1 is poorly infecting primary human fibroblast and without cytopathic effect, but malignant transformation increases their susceptibility to the lytic action of the virus, a possible component of its mediated oncosuppression observed *in vivo* (Cornelis et al., 1988; Op De Beeck et al., 2001). Nonetheless, it was still unclear how oncogenes expression renders transformed cells more permissive to H-1PV and if this is related to the acquisition of a precise transformed phenotype. A previous report claimed that *p53* mutation, in combination with *ras* activation, is necessary for rat embryo fibroblasts to become sensitive to H-1PV-induced killing (Telerman et al., 1993). It remained to be shown if cell full transformation by oncogene cooperation is generally necessary to sensitize REFs to H-1PV cytotoxicity and if, in this context, oncogenes of the same class (immortalizing versus transforming) are exchangeable or if the activation of specific oncogenes is strictly required.

Rat embryo fibroblasts isolated from 14 days old embryos were transfected with vectors expressing different oncogenes (*c-myc*, *Ha-ras*, *SV40 large T antigen*) and a dominant negative mutant form of the tumor suppressor *p53* (*p53dn*), alone or in different combinations, together with a puromycin resistance gene harbouring vector. Stable transfectants were

selected by culturing the cells in medium supplemented with FCS 10% (v/v) and puromycin (1.5 μg/ml) for 7-8 days, an incubation period sufficient for the drug to completely kill normal and GFP-transfected REFs control populations. Western blot and immunoprecipitation analyses were performed to confirm the expression of the respective proteins (Fig. 16A and B).

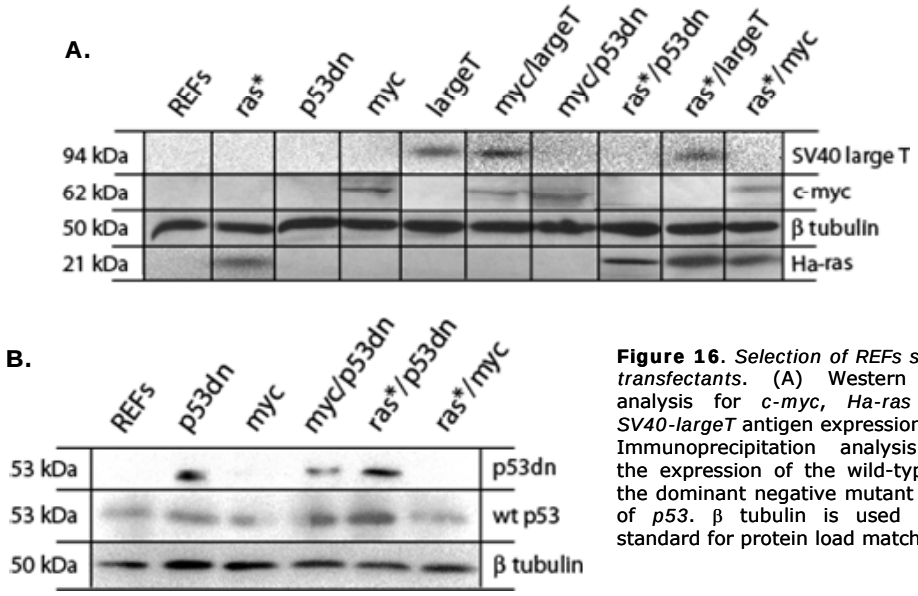


Figure 16. *Selection of REFs stable transfectants*. (A) Western blot analysis for *c-myc*, *Ha-ras and SV40-largeT* antigen expression. (B) Immunoprecipitation analysis for the expression of the wild-type or the dominant negative mutant form of *p53*. β tubulin is used as a standard for protein load matching.

REFs transfection with different combinations of oncogenes and oncosuppressor led to cell immortalization or transformation, and the acquisition of one or the other phenotype was determined according to several criteria. In terms of cell morphology, *Ha-ras*-transfected REFs (*ras**) appeared undistinguishable from the primary cells whereas cells transfected with *c-myc* (*myc*), *p53dn* or *SV40 large T antigen* (*largeT*), alone or in combination (*myc/p53dn* and *myc/largeT)* became immortalized (Fig. 17). These cultures were still dividing 35 days post transfection, and did not show signs of senescence when compared to control cells; interestingly, double oncogene transfection seemed to confer stronger immortalization features, as cells displayed a notably higher division speed in comparison to single transfectans (Fig. 18A). The acquisition of a transformed phenotype was discerned in *ras*/p53dn*, *ras*/myc* and *ras*/largeT* transfected REFs. These cells were able to proliferate in absence of growth factors, to spread in the culture dish thus developing foci at high density, and to form clones in soft agar (Fig. 17). However, *ras*/p53dn* transfected cells showed a less

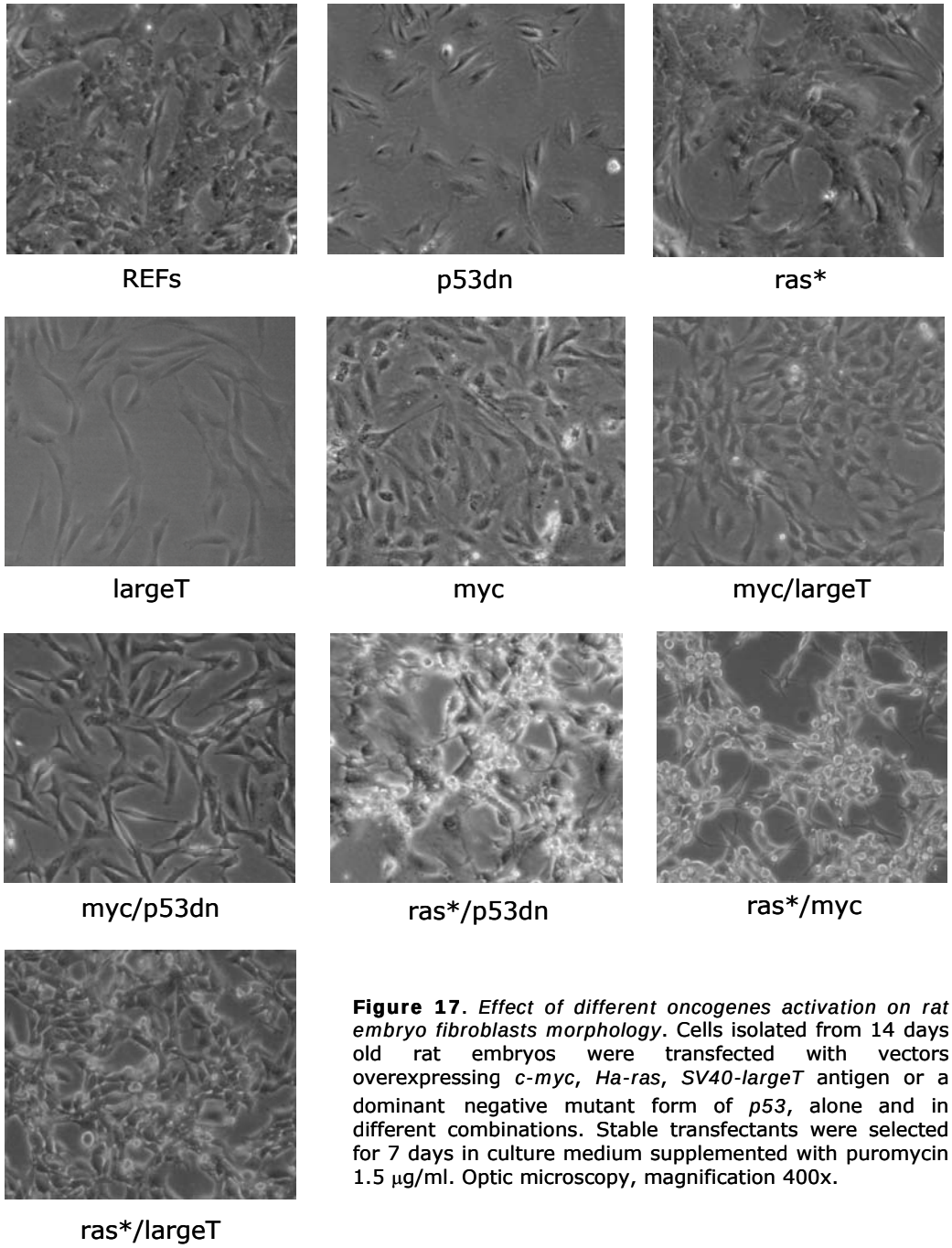
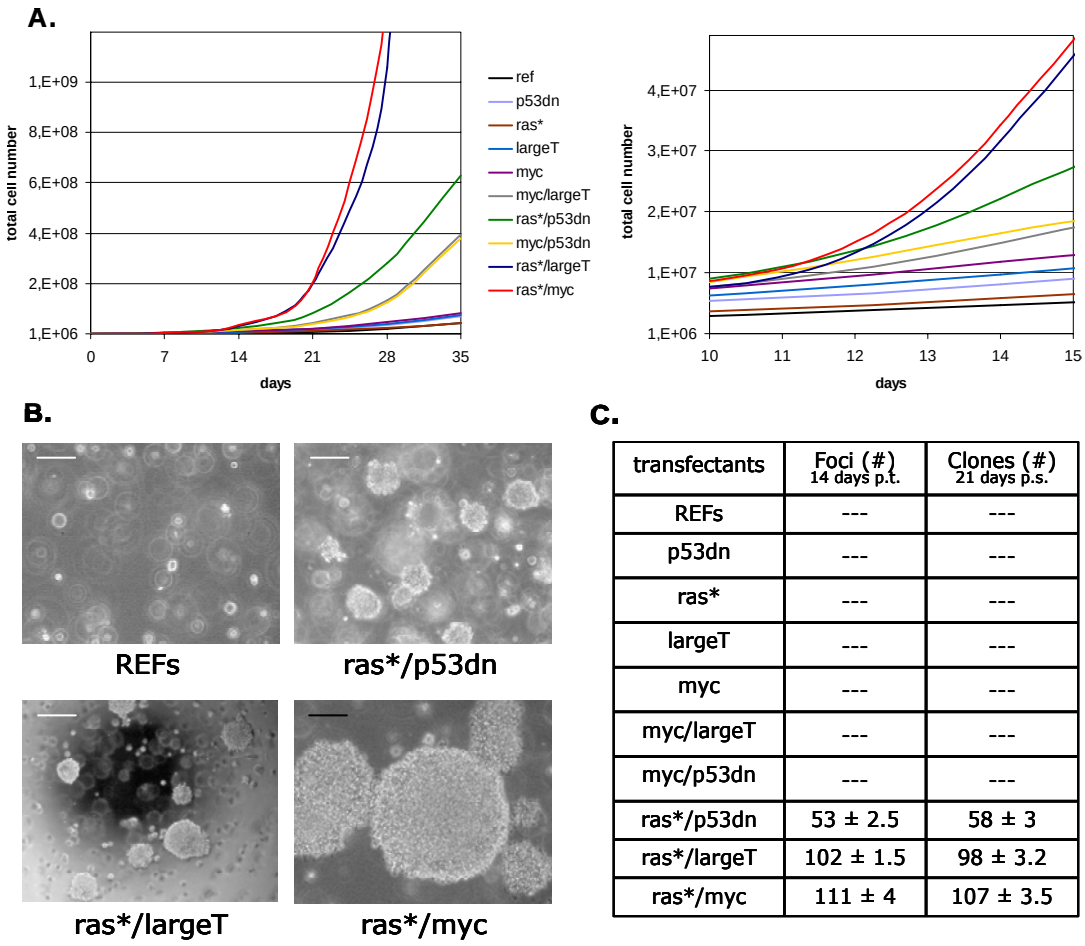


Figure 17. *Effect of different oncogenes activation on rat embryo fibroblasts morphology*. Cells isolated from 14 days old rat embryos were transfected with vectors overexpressing *c-myc*, *Ha-ras*, *SV40-largeT* antigen or a dominant negative mutant form of *p53*, alone and in different combinations. Stable transfectants were selected for 7 days in culture medium supplemented with puromycin 1.5 μg/ml. Optic microscopy, magnification 400x.

aggressive behavior, characterized by slower growth and weaker formation, in number and size, of foci in cell culture and clones in soft agar (Fig. 18B and C). Compared to *ras*/largeT* transfected cells, *ras*/myc* transfectans were found to stronger exhibit transformed features, having a similar growth speed but developing bigger foci and soft agar clones (Fig. 18B).

Altogether, these ten REFs subpopulations displayed diverse phenotypes due to the combined/differential expression of key oncogenes

involved in cell cycle control, apoptosis regulation, cell proliferation, survival and differentiation. This cell collection reflected a combination of mutation commonly observed in many types of tumours, and appeared therefore to be suitable to test the effect of genetic alterations on REFs permissiveness to parvovirus H-1-induced killing.



C.

transfectants	Foci (#) 14 days p.t.	Clones (#) 21 days p.s.
REFs	---	---
p53dn	---	---
ras*	---	---
largeT	---	---
myc	---	---
myc/largeT	---	---
myc/p53dn	---	---
ras*/p53dn	53 ± 2.5	58 ± 3
ras*/largeT	102 ± 1.5	98 ± 3.2
ras*/myc	111 ± 4	107 ± 3.5

Figure 18. *Effect of different oncogenes activation on rat embryo fibroblasts behaviour*. (A) Cell growth was followed up to 5 weeks after isolation of stable transfectants. (B) Transformed REFs spread and formed clones after 3 weeks seeding in agar 0.625% solution. Optic microscopy, magnification 100x and 200x. Scale bars: 600 μm (white) and 300 μm (black). (C) Foci formation and soft agar cloning properties of the different REFs transfectans. Average values ± standard deviations from three independent experiments. p.t. = post transfection; p.s. = post seeding.

4.10. *c-myc* overexpression sensitizes rat embryo fibroblasts to parvovirus H-1 oncotoxic action

To evaluate cell sensitivity to viral infection, transfectants and primary REFs were seeded in 96 wells/plates in number of 3000 cells/well and infected with H-1PV at multiplicities of infection of 0.625, 1.25, 2.5, 5 and 10 pfu/cell. Hexosaminidase and G6PD (glucose 6-phosphate dehydrogenase) assays were carried out at day 2 post infection. The cytophatic effect of the virus was calculated by mean of the hexosaminidase assay, and expressed in terms of cell survival as percentage of vital staining (i.e. total living cells) in treated versus mock cultures. The cytolytic action of the virus was instead measured by the G6PD assay, and estimated as percentage of fully lysed cells (i.e. total enzymatic activity) in infected versus mock populations. Data from the hexosaminidase measurements are shown for multiplicities of infection from 0.625 to 10 pfu/cell (Fig. 19A) while the most representative differences on cell permissiveness to H-1PV are further displayed in parallel with the G6PD data at 5 pfu/cell (Fig. 19B and C).

The experiments confirmed the fact that normal untransfected REFs were poorly sensitive to H-1PV-induced killing. GFP-transfected control REFs did not show an increased susceptibility to H-1PV, excluding a side effect of the transfection procedure on the phenotype acquired by the selected cells (data not shown). In agreement with the literature (Franza et al., 1986; Serrano et al., 1997), *ras* overexpression alone drove cells to senescence and did not induce changes in REFs behaviour toward H-1PV infection. Surprisingly, single oncogenes of the same class played selective roles. On the one hand, immortalization through *p53* inactivation was not sufficient to boost cell sensitivity to H-1PV, and *SV40-largeT* overexpression rendered REFs more prone to the killing but the overall effect was not statistically significant. On the other hand, *c-myc* overexpression alone was enough to sensitize REFs to the virus cytotoxic action (Fig. 19B).

The nature of oncogene cooperation affected the outcome of cell permissiveness to the virus. Remarkably, full cell transformation was not necessary for the acquisition of permissiveness to the virus, since cells immortalized by the activation of multiple oncogenes were powerfully killed. *ras* activation increased cells sensitivity when expressed in cooperation with

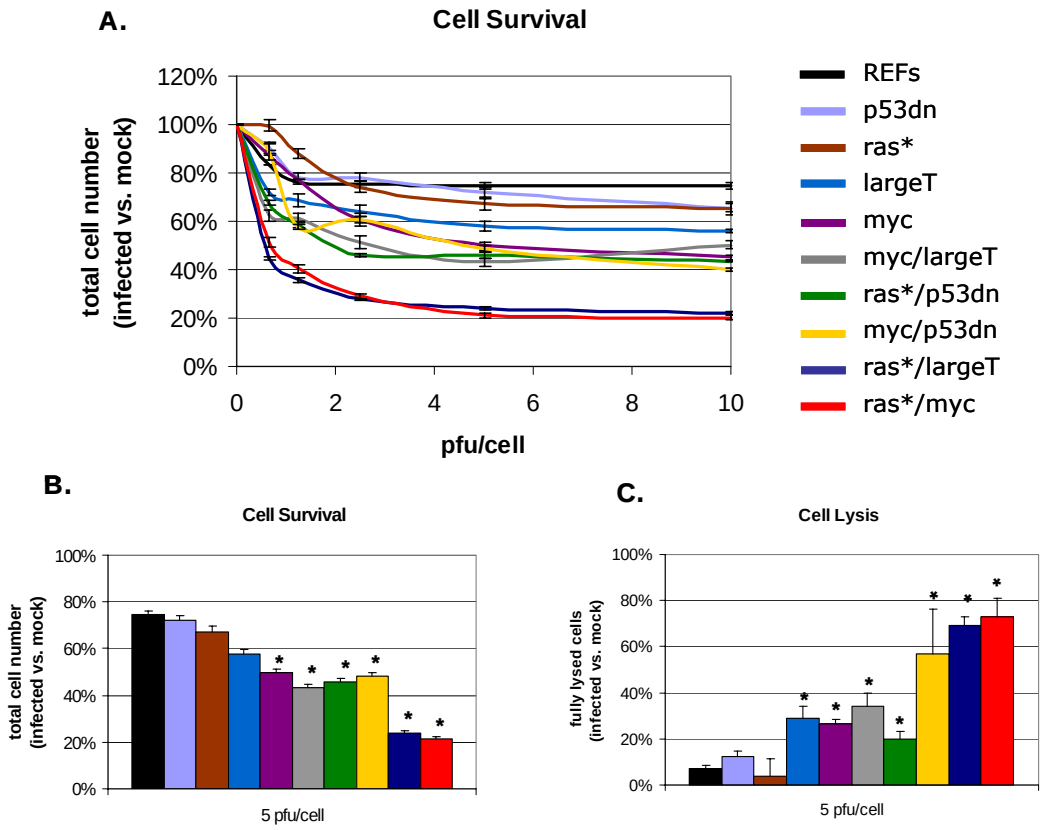


Figure 19. *H-1PV-induced killing of REFs transfectants*. Cell were analyzed by means of the hexosaminidase assay 48 h after infection with parvovirus H-1 at multiplicities of infection from 0.625 to 10 pfu/cell (A) or 5 pfu/cell (B). Cell survival is expressed as percentage of vital staining (i.e. total living cells) in infected versus mock cultures. (C) Cell lysis was detected by measuring the release of G6PD into the medium 48 h after H-1PV infection (5 pfu/cell). Data are expressed as total G6PD activity in mock versus infected cultures, normalized with respect to a fully lysed positive control. Statistically significant differences ($p<0.05$) are indicated by a star (*). Average values and standard deviation bars from three independent experiments, each in triplicate.

c-myc and *SV40 large T* immortalizing oncogenes, but not with *p53dn*. Indeed, *ras*/myc* and *ras*/largeT* REFs were characterized by the most enhanced transformed phenotype, and were sensitive to H-1PV already after infection at 0.625 pfu/cell. On the contrary, *ras*/p53dn* transformed REFs were almost as susceptible as *myc*, *myc/p53dn* and *myc/largeT* immortalized cells (Fig. 19A).

Interestingly, H-1PV acquisition of an oncolytic capacity responsible for active cell killing was dependent on *c-myc* and/or *SV40-largeT* overexpression. After infection at 5 pfu/cell, the inhibition of cell growth correlated almost completely with the ability of H-1PV to induce lysis in *largeT*, *myc*, *myc/largeT*, *myc/p53dn*, *ras*/largeT* and *ras*/myc* transfected REFs. In contrast, G6PD release accounted for only 20% fully lysed cells in

the *ras*/p53dn* transfectants versus a 60% decrease in cell survival. Hence, the nature of cell sensitivity to the virus (necrosis vs. active cell lysis) was also associated to the activation of selective oncogenes. The amount of G6PD activity measured in the supernatant of infected normal, *p53dn* and *ras** transfected REFs was estimated as basal release resulting from experimental handling (Fig. 19C).

In order to exclude the possibility that the higher sensitivity of certain transfectants was due to multiple cycles of infection, hexosaminidase and G6PD assays were further performed on cells infected for 48 h with H-1PV (0.625 to 10 pfu/cell), and incubated with neutralizing antibodies raised against H-1PV capsid proteins, diluted 1:200 in the culture medium 5 h post infection (data not shown). The concentration of neutralizing antibodies was chosen according to a preliminary optimization experiment, as to be the minimal quantity sufficient to block viral infection, and exclude a second round of infection occurring during 2 days incubation. The outcome of the experiments was not different from that already described, thus confirming that the observed effects of different oncogenes on REFs permissiveness to H-1PV-induced killing were consequence of a single viral infection.

In conclusion, even if the lytic action of the virus was supported also in cells where *SV40-largeT* was overexpressed alone, only *c-myc* was capable of significantly sensitizing rat embryo fibroblasts to parvovirus H-1-dependent cytotoxicity.

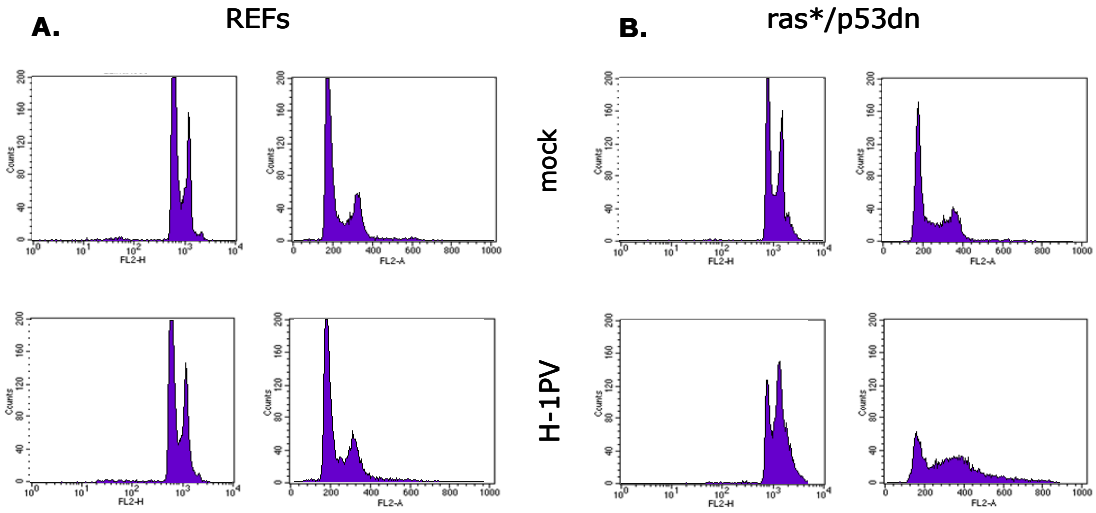
4.11. Oncogenes differently impinge on parvovirus H-1 capacity of inducing cell cycle arrest in infected rat embryo fibroblasts

Parvoviral infections provoke cell cycle perturbations, since both autonomous parvovirus minute virus of mice and human parvovirus B19 have been show to induce a multistep cell cycle arrest in G_1-, S-, and G_2-phase after infection of permissive cells (Morita et al., 2003; Op De Beeck et al., 1995). In general, S- and G_2-phase arrest has been well documented, the former confirmed to be p53-dependent, the latter to be both p53- and p21-dependent (Op De Beeck and Caillet-Fauquet, 1997). More recently, G_1-phase arrest has been observed to be mediated by the viral protein NS1

(Op De Beeck et al., 2001). It was therefore decided to analyze whether H-1PV triggered a DNA damage checkpoint by activating a p53-dependent pathway.

Cell cycle distribution was studied in transfected REFs to investigate if different oncogenes could play a role in the way cells would eventually undergo a virus-mediated phase arrest. After 24 h infection with H-1PV (5 pfu/cell), cells were harvested and stained with propidium iodide (20 μg/ml) in order to determine their DNA contents by flow cytometry. Neither infected normal REFs nor both *p53dn* and *ras** transfectants did show substantial perturbation in the cell cycle distribution with respect to the mock populations (Fig. 5A and C). *SV40-largeT* overexpression instead induced cell arrest at the G_2/M phase transition, and proved to have a higher impact in the double transfectants than *myc* and *ras** activation (Fig. 5C). Besides, *c-myc* overexpression mediated cell arrest at the G_0/G_1 phase transition, while the fully transformed *ras*/myc* and *ras*/p53dn* transfected REFs displayed a more classical behavior, undergoing respectively S- and S-G_2/M-phase arrest (Fig. 5B and C).

A 4% increase in the G_2/M portion of the *myc/p53dn* infected population was noted. This increment was observed in the *p53dn* and *ras** REFs as well, but in the *myc/p53dn* transfectants corresponded to a doubling of the G_2-phase subpopulation. Moreover, the percentage of the G_1 mock population was higher in these transfectants than in the other cells, suggesting that more cells might have been resting in the G_0/G_1-phase (Fig. 20C, underlined). Since the low genetic complexity of parvoviruses renders them highly dependent on cellular factors that are expressed during the S-phase of the cell cycle (Cotmore and Tattersall, 1987; Deleu et al., 1998), the percentage of *myc/p53dn* REFs in S-phase was compared with the total portion of infected cells in order to estimate the importance of the G_2-phase increase. If the two percentages had been almost equal, all infected cells would have been in S-phase and eventually undergone a G_2-phase arrest. Because H-1PV infected the 63% of *myc/p53dn* transfected cells (Fig. 21B), a fraction 2.5 times bigger than the S-phase subpopulation (Fig. 20C, underlined), it was possible to conclude that the cell arrest at the G_2/M phase transition was in this context not significant.



C.

Cell populations	G0/G1-phase (%)	S-phase (%)	G2/M-phase (%)
REFs	57,15 ± 2,6	28,34 ± 2,7	14,51 ± 4,8
REFs + H-1PV	56,03 ± 3,2	29,08 ± 5,2	14,89 ± 5,6
p53dn	60,84 ± 5,7	27,65 ± 3,2	11,51 ± 5,4
p53dn + H-1PV	54,87 ± 2,5	30,91 ± 4,3	14,22 ± 3,8
ras*	48,19 ± 4,4	22,63 ± 2,6	29,18 ± 1,7
ras* + H-1PV	44,85 ± 3,8	21,59 ± 2,3	33,56 ± 3,5
largeT	53,67 ± 2,4	26,91 ± 3,3	19,42 ± 1,9
largeT + H-1PV	35,34 ± 3,1	26,77 ± 3,4	37,89 ± 1,8
myc	55,54 ± 2,3	31,35 ± 1,1	13,11 ± 1,7
myc + H-1PV	79,49 ± 2,9	12,63 ± 2,7	7,87 ± 1,4
myc/largeT	44,18 ± 2,2	31,26 ± 1,9	24,56 ± 2,5
myc/largeT + H-1PV	18,49 ± 1,5	7,79 ± 3,9	73,72 ± 2,3
ras*/p53dn	48,01 ± 4,2	39,89 ± 1,6	12,1 ± 2,1
ras*/p53dn + H-1PV	23,24 ± 3,4	52,45 ± 2,5	24,31 ± 3,1
myc/p53dn	70,53 ± 0,9	24,94 ± 2,2	4,53 ± 1,3
myc/p53dn + H-1PV	78,82 ± 1,5	12,69 ± 1,7	8,49 ± 1,1
ras*/largeT	68,64 ± 3,2	22,14 ± 1,9	9,22 ± 2,3
ras*/largeT + H-1PV	52,01 ± 2,7	18,51 ± 1,7	29,48 ± 2,6
ras*/myc	57,61 ± 1,9	36,89 ± 3,2	5,5 ± 1,5
ras*/myc + H-1PV	46,98 ± 2,5	51,96 ± 2,6	1,06 ± 3,1

Figure 20. *H-1PV-induced cell cycle arrest*. Propidium iodide staining was performed on REFs transfectants 24 h after infection with parvovirus H-1 (5 pfu/cell). Flow cytometry panels for normal (A) and *ras*/p53* transfected (B) REFs are shown [FL2-H and FL2-A channels]. Data for all transfectants (C) are reported as average values ± standard deviations from three independent experiments. *myc/p53* REFs are underlined, for explanations refer to text.

Taken together, the data revealed that all REFs transfectants were proliferating prior to H-1PV infection; hence, the percentage of cells in S-phase could not explain the differences in permissiveness to parvovirus H-1. Moreover, virus-induced cell death was not associated with a particular perturbation of the cell cycle and therefore occurred independently of p53.

4.12. REFs immortalization by single oncogene transfection differently affects parvovirus H-1 life cycle

It was at this point important to investigate if oncogenes or tumorsuppressor expression differently affected H-1PV life cycle. REFs transfectants were therefore assessed for the expression of the viral non structural protein NS1 and the replication of the viral genome.

First of all, it was considered whether the reduced cytopathic effect observed in certain REFs transfectants correlated with a lower number of infected cells. FITC-conjugated monoclonal antibodies against NS1 were used to evaluate viral protein expression by flow cytometry, 24 h after infection of normal and transfected REFs with H-1PV (5 pfu/cell). Data were processed with the CellQuest™ software from BD Bioscience (Fig. 21A and B). In the transfectants permissive to H-1PV, the portions of NS1-expressing cells correlated with the percentages of the infected subpopulations, as deducted by the survival curves (Fig. 21B, compared to Fig. 19B). NS1 was found to be expressed in 30-35% of normal and *p53dn* transfected REFs as well, although these cells proved to be insensitive to the virus, supporting the hypothesis that NS1 cytotoxic action is enhanced by cell transformation (Mousset et al., 1994). Most interestingly, NS1 expression was almost absent in *ras** transfected REFs, being detected only in 10% of the cell population (Fig. 21B). This data suggested that *ras* activation alone induced changes in REFs genetic expression profile that interfered with the early steps of viral infection, while in combination with other oncogenes it triggered viral protein expression.

A second aim was to study oncogenes effect on H-1PV genome replication, to evaluate viral DNA conversion into a monomeric replicative form after host cell entry, and viral single-stranded DNA production later

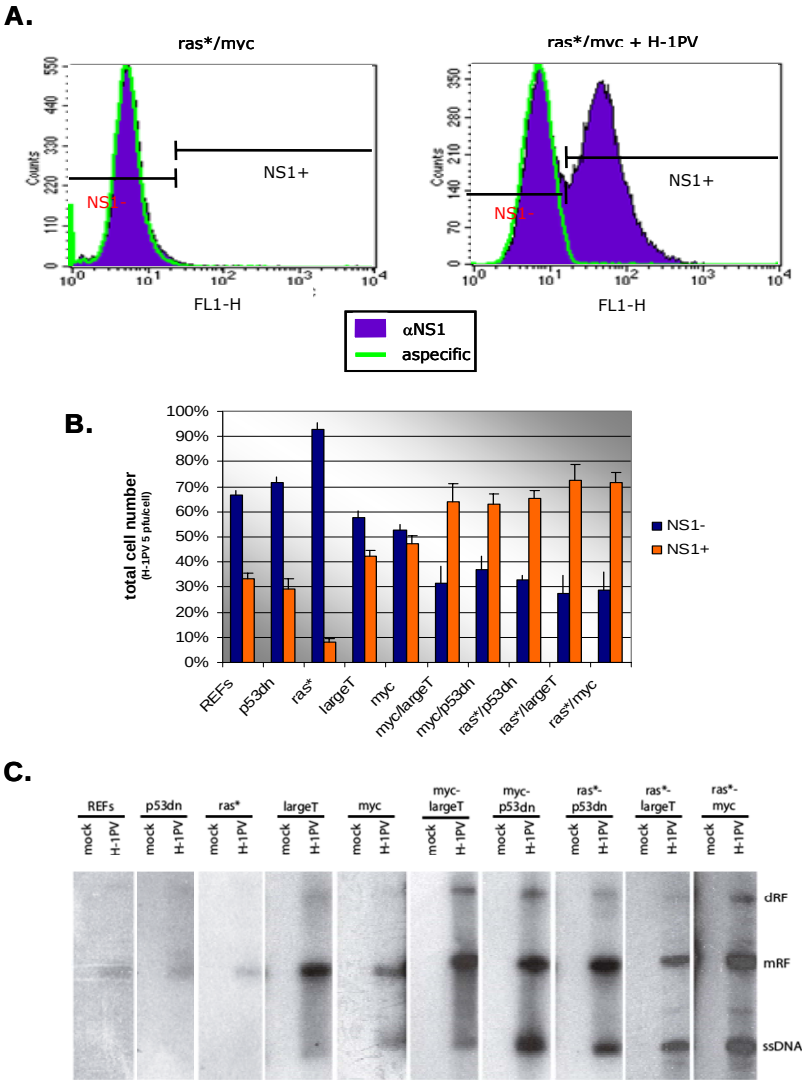


Figure 21. *H-1PV genome replication and protein expression*. FITC-NS1 staining was performed on normal and transfected REFs 24 h after infection with parvovirus H-1 (5 pfu/cell). Flow cytometry panels for *ras*/myc* REFs (A) are shown [FL1-H channel]. Data for all transfectants (B) are reported as average values ± standard deviations from three independent experiments. (C) Southern blotting analysis of H-1PV replication in REFs transfectants, 24 h after mock treatment or infection (5 pfu/cell). dRF = dimer replicative form; mRF = monomer replicative form; SS = single-stranded DNA.

after infection. Low-molecular-weight DNA was extracted from cell pellets 24 hours after H-1PV (5 pfu/cell) or mock treatment, and analysed by Southern blot performed using an NS1 gene-specific DNA probe. The monomeric replicative form of viral DNA (mRF) was detected in all transfectants, though the respective bands were weak for normal and *p53dn* transfected REFs, and barely visible for *ras** transfected cells (Fig. 21C). After infection, the input single-stranded DNA of H-1PV (ssDNA) is first converted into a monomeric form in a process mediated by several host S-phase factors that does not

rely on viral proteins (Cotmore and Tattersall, 1995). In the *largeT* REFs and in all the transfectants sensitive to H-1PV, a marked mRF band plus a ssDNA and a dimeric replicative form (dRF) bands were observed, even if unsteady in intensity (Fig. 21C). These data were in accordance with the observations on NS1 expression (Fig. 21B), since the elongation mechanism resulting in the formation of double stranded monomeric and multimeric intermediates as well as the encapsidation process of the viral progeny have been demonstrated to be NS1 dependent (Cotmore and Tattersall, 1995). The synthesis of progeny ssDNA appeared nonetheless to be more pronounced in the fully transformed and in the *myc/p53dn* REFs.

In summary, immortalization through single oncogenes expression had different effects on the capacity of REFs to support full viral infection. H-1PV was not able to replicate in cells where *p53* was inactivated, in agreement with the lack of cell permissiveness previously discussed, while it could produce a progeny ssDNA in cells sensitized by *c-myc* or *SV40-largeT* overexpression, alone or in combination with *ras* activation. Furthermore, the quality of oncogene cooperation seemed to play a role since ssDNA synthesis was comparable between the single transfectans and the double immortalizing *myc/largeT* REFs while it became massive in the double immortalizing *myc/p53dn* as in fully transformed cells.

4.13. Apoptosis is triggered after parvovirus H-1 infection of ras*/p53dn transfected REFs

Once evaluated the effect of genetic alterations on REFs permissiveness to parvovirus H-1-induced cytosuppression and active killing, it was decided to investigate the death pathways activated after infection of these cells to study if and how the different oncogenes were influencing the intracellular signaling controlling programmed cell death.

To determine whether parvovirus-induced death was of apoptotic type, the induction of caspase 3 activity was monitored 24 and 48 h post infection of REFs transfectants with H-1PV (5 pfu/cell) incubating cell lysates with a synthetic substrate and measuring the fluorescence intensity of the cleaved product (Fig. 22A). Infected populations were compared with

untreated control samples and a 4–5 fold increase in caspase 3 activity levels was considered a hallmark of strong apoptosis induction. This threshold was established upon the observations obtained in a preliminary experiment in which apoptotic cell death was detected in normal REFs 48 h post treatment with cisplatin (2.5 µg/ml) according to several parameters: activation of caspases 3 and 9, DNA ladder formation after genomic DNA extraction, presence of apoptotic bodies after Hoechst staining of infected cell (data not shown).

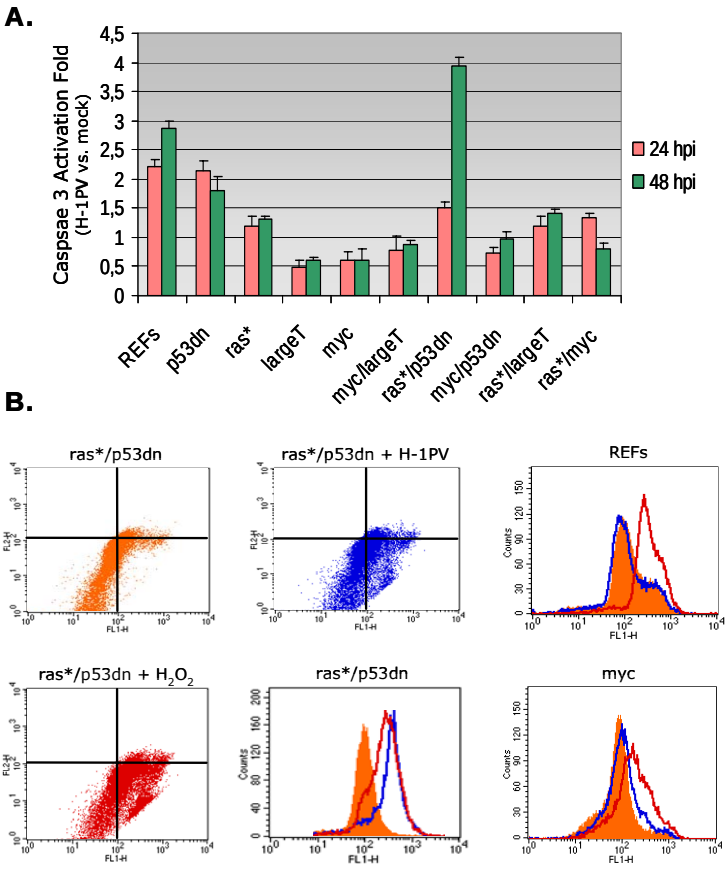


Figure 22. *Apoptosis is triggered in ras*/p53dn transfected REFs after H-1PV infection.* (A) Caspase 3 activity was monitored at 24 and 48 h after H-1PV infection (5 pfu/cell) of normal and transfected REFS. Average values and standard deviation bars from three independent experiments. (B) ΔΨm permeabilization was analyzed by JC-9 staining of REFs transfectants 24 h post infection with parvovirus H-1 (5 pfu/cell - blue) or 30 min after treatment with H_2O_2 (5 mM - red). Flow cytometry panels for normal and *myc* REFs [FL1-H channel] versus *ras*/p53* transfectants [FL1-H or FL1-H vs. FL2-H channels].

Apart for a marginal activation of caspase 3 measured in normal REFs between 24 and 48 h after H-1PV infection, signs of ongoing apoptosis were not identified in any of the other transfectants, with the exception of *ras*/p53dn* REFs. In these cells, caspase 3 activation fold did not notably increased at 24 h post infection with H-1PV but showed a significant boost at 48 h post infection, leading to the hypothesis that *ras*/p53dn* transfectants underwent apoptosis at a late time point after infection.

To confirm this assumption, it was tested whether the virus was triggering the activation of caspase 3 via the intrinsic pathway, thus inducing an alteration of the mitochondrial membrane potential. Mitochondrial membrane depolarization assays were performed by flow cytometry 24 h after infection of REFs transfectants with parvovirus H-1 (5 pfu/cell), measuring the potential-dependent accumulation of JC-9 dye. Positive control cells treated with H_2O_2 5 mM were included; data for normal, *myc* and *ras*/p53* transfected REFs are shown (Fig. 22B). The appearance of a distinct sub-population with increased FL1-H values, an indication of the occurrence of $\Delta\Psi$m alteration, was recorded in the *ras*/p53* transfectants after stimulation with H_2O_2 or viral infection. Moreover, the two peaks almost overlapped, suggesting that the degree of mitochondrial membrane depolarization in the infected cells was rather massive, comparable with the positive control situation where H_2O_2 high concentration was used to induce a strong effect on the disruption of $\Delta\Psi$m. On the contrary, in all the other REFs transfectants the FL1-H peak of the infected population overlapped with that of the mock population and only the H_2O_2-treated cells showed an increased value.

In conclusion, in the ras*/p53dn transfected REFs a permeabilization of outer mitochondrial membrane occurred at 24 h post infection, which was followed by caspase 3 activation at 48 h post infection. Independently of the effect of the different oncogenes towards the acquisition of sensitiveness to the virus, apoptosis was not found to be triggered after parvovirus H-1 infection in any of the other REFs transfectants. Therefore, the virus jeopardized the survival of these cells through another death pathway.

4.14. Acidic compartments swelling and leakage after H-1PV infection of permissive REFs

Given H-1PV-induced activation of non-apoptotic pathways in human glioma cells, it was decided to investigate the accumulation of acidic vesicles upon viral infection in the REFs system, to verify whether this novel death mechanism occurred in other cell type or was limited to brain tumor cells. REFs transfectants were infected with H-1PV (5 pfu/cell) and acridine orange staining was analyzed by flow cytometry (Fig. 23A) and fluorescence microscopy (Fig. 23B) at 24 and 48 h post infection. Neither acridine orange shifts of fluorescence nor changes in the morphology of dye-positive compartments were recorded in H-1PV-resistant primary REFs (Fig. 23A and B), or *p53dn* and *ras** transfected cells (data not shown). On the contrary, already at 24 h post infection a double peak was detected in the FL3-H profile of *largeT* transfected REFs and all the transfectants sensitive to H-1PV (Fig. 23A; displayed only *myc*, *myc*/*p53dn* and *ras*/largeT* transfected cells) with the exception of *ras*/p53dn*-REFs. A double peak corresponded to both increase in number and/or size of acidic vesicles and leakage of acidic compartments, indicating that following H-1PV infection the accumulation of acidic vacuoles was occurring faster in the REFs system than in the glioma NCH82 cells (Fig. 8B). In fact, a 48 hours infection led to the leakage of acidic compartments in the whole infected cell population (Fig. 23A; shown for *ras*/largeT* transfectants). Remarkably, at this time point a left shift in the acridine orange fluorescence was observed also in the *ras*/p53dn* REFs, a possible indication of membrane blebbing in the last stage of virus-induced apoptosis (Fig. 22A and 23A).

It became at this point evident that REFs transfected with *SV40 largeT antigen* only, though showing a highly variable and not statistically significant response to H-1PV infection, behaved at a molecular level like all the other transfectants sensitive to H-1PV-induced killing. Upon parvovirus H-1 infection of these cells, acidic compartments initially increased in number and accumulated in infected cells, finally leaking and releasing their content in the cytosol. Moreover, the mock population basal amount of acidic vesicles was progressively higher in the single oncogene immortalized-, in the double oncogene immortalized- and in the transformed-REFs as compared to

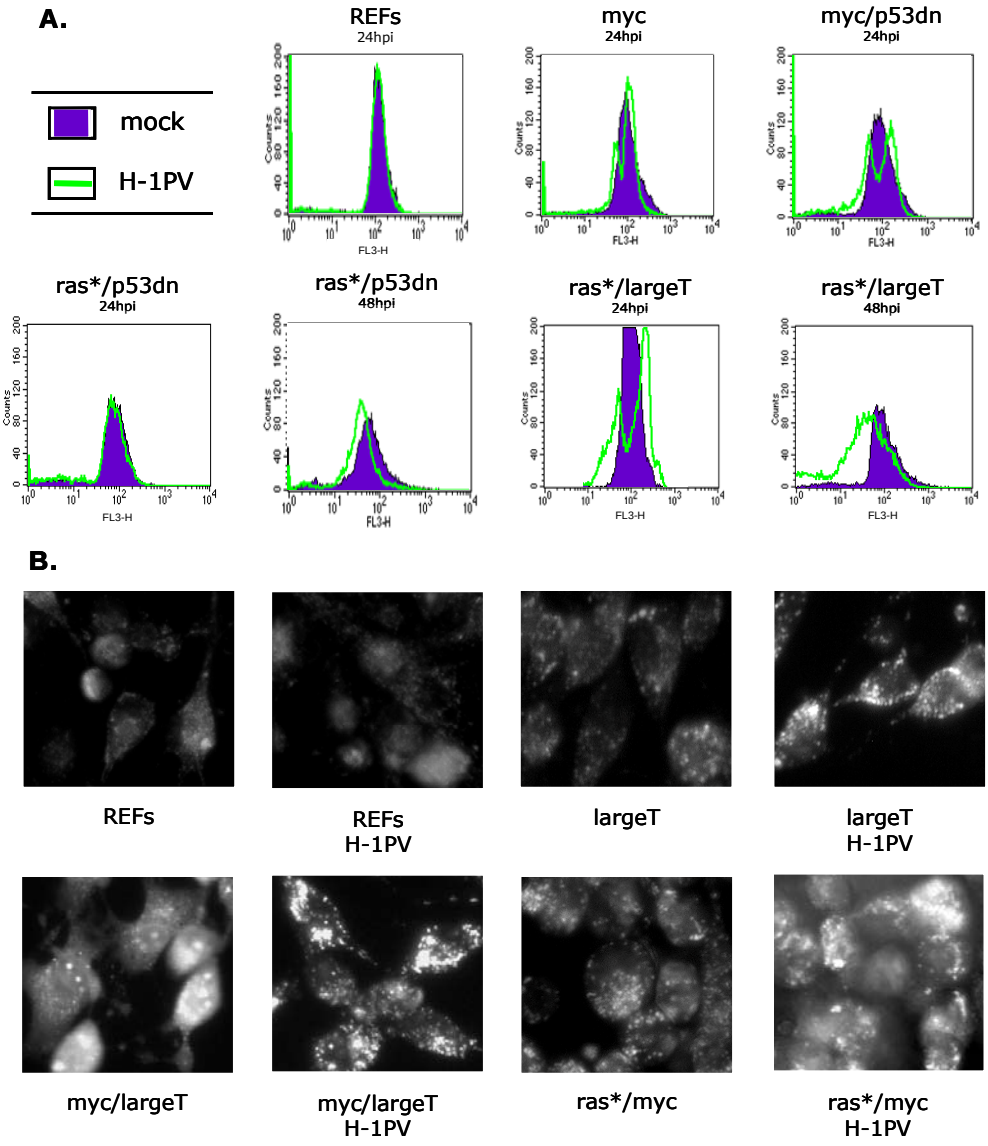


Figure 23. *Accumulation of acidic vescicles in the cytosol of REFs transfectants sensitive to H-1PV infection*. Acridine orange staining of normal and *largeT*, *myc*, *myc/p53dn*, *myc/largeT*, *ras*/p53dn*, *ras*/largeT* and *ras*/myc* transfected REFs infected or not with H-1PV (5 pfu/cell). (A) Flow cytometry analysis at 24 and 48 h post infection; FL3-H channel. (B) Fluorescence microscopy at 24 h post infection; red filter, magnification 800x.

primary cells (Fig. 23B; displayed only *largeT*, *myc/largeT* and *ras*/myc* REFs). The different oncogenes therefore appeared to influence this virus-induced mechanism, in terms of rendering the cells less or more prone to its activation by modulating their acidic vesicles physiological levels. This observation pointed to the identification of the acidic compartments with autophagy-like vesicles and to the hypothesis that H-1PV was triggering a non-apoptotic cell death through the activation of autophagy.

4.15. Autophagy is triggered upon parvovirus H-1 infection of permissive REFs

To verify whether REFs adapted to changes in the cellular metabolism induced by oncogene-driven immortalization and/or transformation through the activation of autophagic degradative pathways (Cuervo, 2004), parvovirus H-1-induced activation of autophagy was further investigated in the REFs system. Primary cells and transfectants permissive to the virus were infected with H-1PV (5 pfu/cell) for 24 h and the expression of Beclin1 and LC3 I/II isoforms was analyzed by Western blot.

When compared to the normal phenotype, Beclin1 basal expression was slightly increased in all REFs transfectants tested, with the exception of *ras*/p53dn*-transfected cells. Nevertheless, considering the different cell populations one by one, no change in Beclin1 expression was observed in mock-treated versus infected cells (Fig. 24, 2nd lane). It was therefore concluded that oncogene-driven immortalization or transformation did not impinge on the expression of this pro-autophagic factor, which was not influenced by parvovirus H-1 infection too.

LC3-I expression turned out to be affected by the induction of an immortalized or transformed phenotype in rat embryo fibroblasts, since it was stronger in all transfectants tested compared to normal REFs (Fig. 24, 4th lane). In agreement with the data collected so far, the appearance of the LC3-II band was observed 24 h after infection with H-1PV (5 pfu/cell) in *largeT* and *myc* single-transfected REFs, in the double oncogenes immortalized- and in transformed-cells, but not in the *ras*/p53dn* transfectants. Besides, when analyzing *myc* transfected versus *largeT* transfected cells, and *ras*/myc* versus *ras*/largeT* REFs, the conversion of LC3-I to LC3-II seemed to be more pronounced in *myc* overexpressing cells. Since the formation of the autophagosome and its fusion with lysosomes is a rather fast and dynamic event, the basal activation of autophagy in the mock populations was most likely behind the threshold for the detection of a second band corresponding to the LC3-II isoform. This non-apoptotic programmed cell death pathway was then triggered after parvovirus H-1 infection in cells not predisposed to the activation of apoptosis. Moreover, a

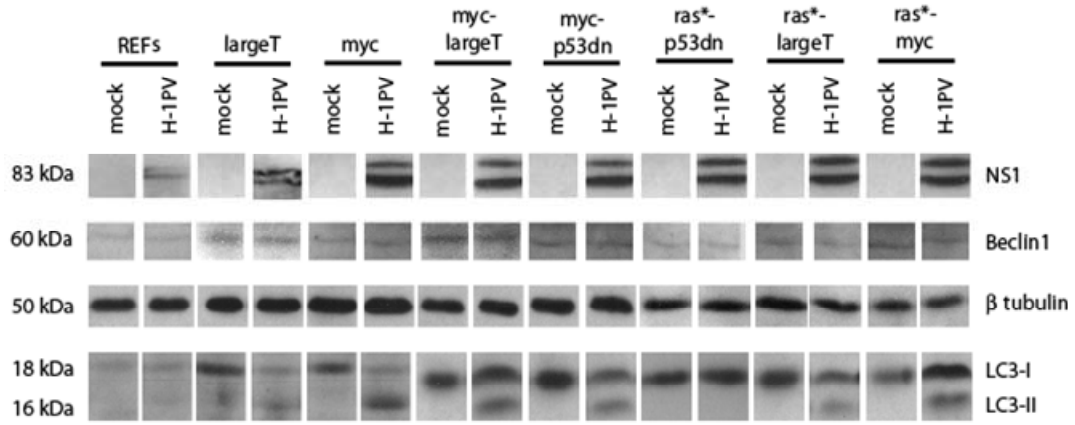


Figure 24. *Autophagy activation following parvovirus H-1 infection of REF transfectants.* Western blot analysis for the expression of NS1, Beclin1, LC3 I and II isoforms in cells infected for 24 h with H-1PV (5 pfu/cell) or mock-treated. β tubulin was used as a standard for protein load matching.

barely visible band for the LC3-II isoform was noticed in infected normal REFs as well, indicating that autophagy and not apoptosis was the preferential killing mechanism promoted by the virus.

In summary, immortalization and transformation modulated the basal levels of the autophagic process in REFs transfectants. Autophagy was boosted upon parvovirus H-1 infection in REFs overexpressing c-myc and/or SV40 large T antigen.

4.16. Parvovirus H-1 infection of sensitive REFs induces cathepsin B cytosolic activation and cystatin B down-regultation

With the exception of *ras*/p53dn* transfected cells, parvovirus H-1 appeared to trigger in permissive REFs transfectants a non-apoptotic mechanism similar to that observed in NCH82 glioma cells. To corroborate this hypothesis, cathepsin B intracellular activity was analyzed 24 h after infection with H-1PV (5 pfu/cell). An average 2-fold increase in cathepsin B cytosolic activity was detected in infected REFs overexpressing *c-myc* and/or *SV40 large T antigen* (Fig. 25A). The cytosolic versus lysosomal cathepsin B ratio was instead not affected by H-1PV infection of normal, *p53dn*, and *ras** transfected REFs, in agreement with the previous data. In contrast to what observed in human gliomas (Fig. 7D), apoptosis initiation in *ras*/p53dn* REFs seemed interestingly to occur independently of cathepsin B activation, as no

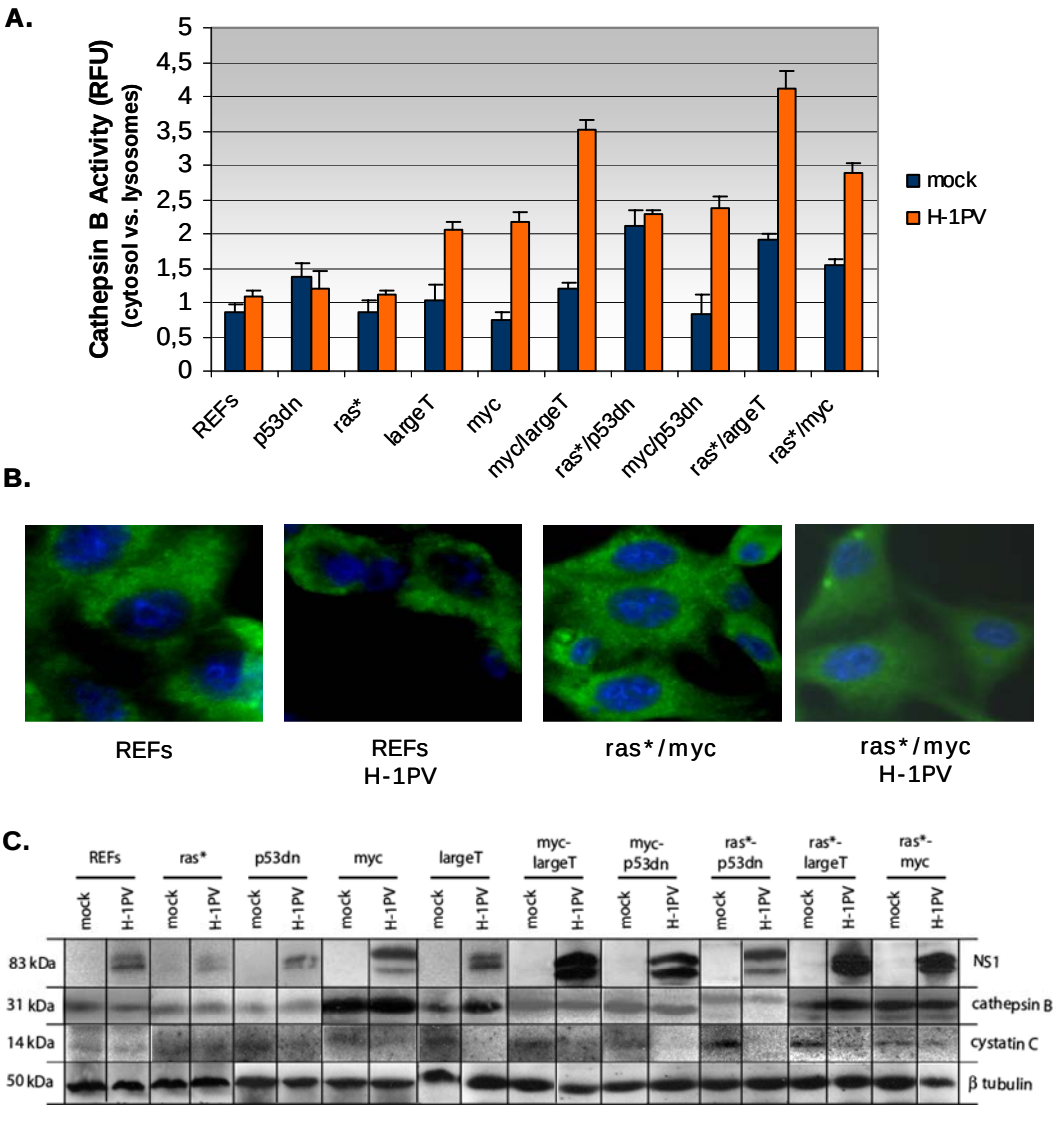


Figure 25. *Cathepsin B cytosolic activation and cystatin B down-regulation upon H-1PV infection in REFs transfectants.* Cells were infected for 24 h with H-1PV (5 pfu/cell). (A) Cathepsin B activity, expressed as relative amount in cytosol versus lysosomes. RFU, relative fluorescence units; average values and standard deviation bars from three independent experiments, each in triplicate. (B) Immunofluorescence assay for cathepsin B localization in normal and *ras*/myc* REFs; an anti-rabbit FITC-conjugated secondary antibodies were used; nuclei were stained with DAPI. (C) Western blot analysis for the expression of cathepsin B and its inhibitor cystatin B; the parvoviral NS1 protein served as a marker for H-1PV infection while β tubulin was used as a standard for protein load matching.

difference in the cytosolic versus lysosomal amount of this protease was detected (Fig. 25A). H-1PV-induced cytosolic activation of cathepsin B was additionally substantiated in *ras*/myc* transfectants versus normal REFs by immunofluorescence analysis (Fig. 25B). Furthermore, cathepsin B and cystatin B levels were measured by Western blotting in the REFs transfectants infected for 24 h with H-1PV (5 pfu/cell), to evaluate whether parvovirus H-1 could regulate cathepsin activity through the modulation of

its inhibitor (Fig. 25C). The expression of cystatin B was clearly down-regulated in infected versus mock-treated cells. Surprisingly, H-1PV-dependent cystatin B down-regulation was observed even in primary REFs and cells that were insensitive to virus-induced killing. Cathepsin B levels resulted to be in average almost unchanged, slightly reduced in normal and *ras*/p53dn* REFs, increased in infected *p53dn*, *myc* and *ras*/largeT* transfectants.

In summary, cathepsin B cytosolic activation occurred in all REFs sensitive to H-1PV with the exclusion of *ras*/p53dn* transfected cells. On the contrary, the reduction in the expression levels of the cathepsins inhibitor cystatin B was a general effect of parvovirus H-1 infection, occurring even in cells that were not killed by the virus.

4.17. Autophagy inhibition rescues permissive REFs transfectants from H-1PV-mediated cell killing

To substantiate the previous findings on the assumed roles of cytosolic cathepsin B and autophagic vesicles in H-1PV-dependent cell killing, the capacity of autophagy and cathepsins inhibitors to prevent parvovirus H-1-induced cell death was finally analyzed in the REFs study system.

All transfectants were infected for 48 h with H-1PV (5 pfu/cell) and co-incubated with 3-MA (500 μM), E64d (1 μM), Ca-074 Me (10 μM), or DEVD-CHO (20 μM); cell survival was quantified by mean of the hexosaminidase assay (Fig. 26A). Supporting the evidence on autophagy activation upon H-1PV infection, all transfectants sensitive to virus-induced cell killing were protected by 3-MA treatment, with the exception of *ras*/p53dn* transfected REFs. On the contrary, the latter was the only cell population to be rescued from death by incubation with the caspase 3 inhibitor DEVD-CHO. Cathepsin B inhibitor Ca-074 Me was almost as effective as 3-MA in *myc/p53dn* REFs while exerted a partial protection in *largeT*, *myc*, and *ras*/largeT* transfectants. Finally, E64d was able to moderately block H-1PV cytotoxicity in *myc* and *ras*/largeT* transfected REFs.

Taken together, these data indicated that in general it was possible to prevent virus-promoted cell killing by chemically blocking the initiation event

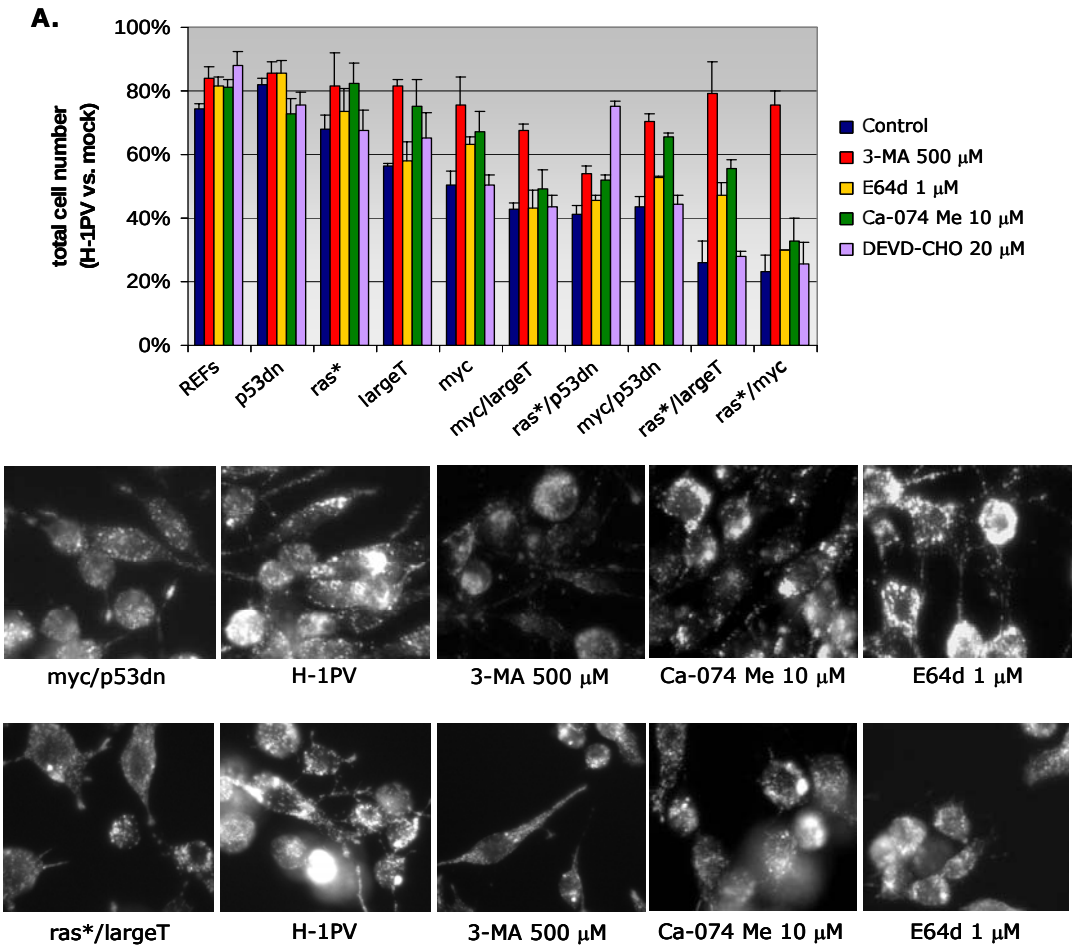


Figure 26. *Effect of autophagy and cathepsins inhibitors on cell rescue from H-1PV-induced killing.* REFs transfectants were incubated with 3-MA, E64d, Ca-074 Me and DEVD-CHO at the indicated concentrations, 5 h post infection with parvovirus H-1 (5 pfu/cell). (A) Hexosaminidase assay performed 48 h after infection of the cells. (B) Acridine orange staining 24 h after treatment of *myc/p53dn* and *ras*/largeT* REFs. Fluorescence microscopy, red filter, magnification 800x.

of the autophagic process. Oncogenes combined or independent overexpression differently affected REFs responsiveness to cathepsins inhibitors Ca-074 Me and E64d, but it was not possible to assign an oncogene a key role or determine a specific effect in response to the acquisition of a particular phenotype. Nevertheless, cathepsins inhibitors' capacity of rescuing REFs transfectants from cell death was reduced, with a few exceptions, in respect to the effect of these compounds in NCH82 glioma cells, and to 3-MA overall action.

Above observations pointed towards the initiation of autophagic cell death as early step during viral infection. It was therefore supposed that cathepsins inhibitors Ca-074 Me and E64d would not have prevented the formation of autophagic vesicles in REFs transfectants infected with

parvovirus H-1. To verify this, acridine orange staining was performed on *myc/p53dn* and *ras*/largeT* trasfected REFs at 24 h post infection with H-1PV (5 pfu/cell) and co-culture with 3-MA (500 μM), E64d (1 μM), or Ca-074 Me (10 μM) (Fig. 26B). As already reported, an increased amount of acidic vesicles was observed in the cytosol of infected versus mock-treated cells. As expected, incubation with 3-MA markedly reduced the number of acridine-positive compartments while both Ca-074 Me and E64d did not inhibit their accumulation.

5 Discussion

5.1. Autophagy activation in NCH82 human glioma cells infected with parvovirus H-1

The action of successful anticancer drugs depends largely on their ability to trigger cell death, in particular apoptosis, in tumor cells. Their efficacy is often impaired by death escape mechanisms developed by cancer cells to evade anti-tumorigenic signals (Hanahan and Weinberg, 2000). Drugs inducing apoptosis fall into two classes according to their ability to activate either the extrinsic receptor-dependent apoptotic pathway, as do TRAIL and TNFα (Lavrik et al., 2005), or the intrinsic pathway, as do cisplatin and other DNA-damaging agents (Danial and Korsmeyer, 2004). Parvovirus H-1 is able to kill human glioma cells by circumventing their resistance to inducers of both the intrinsic and extrinsic apoptotic pathways (Di Piazza et al., 2007). H-1PV activates a type of death that resembles autophagy but shows exclusive features. This uniqueness enables H-1PV to kill glioma cells even though autophagy, like apoptosis, is usually down-regulated in human tumors including high-grade gliomas (Gozuacik and Kimchi, 2004). Investigation of the interaction of H-1PV with gliomas can thus help to unravel of novel facets of cell death mechanisms and to develop new therapeutics likely to be effective against tumors that resist conventional anticancer treatments.

H-1PV-induced glioma cell death differs from apoptosis, since organelle membrane integrity is maintained and the killing effect does not depend on caspase 3 activation. Besides, infected cells exhibit several hallmarks of autophagy: increased acidic vesicle content, structural identification of acidic compartments with autophagosomes and autolysosomes, increased conversion of LC3-I to II isoform, Beclin1 overexpression. Autophagy is an evolutionarily conserved and highly regulated homeostatic process that degrades cytoplasmic components and damaged or surplus organelles for protein removal or turnover (Levine and

Klionsky, 2004). Autophagy is believed to play a fundamental but controversial role in cancer development, having been involved in tumor cells killing but also protection from unfavourable conditions (Kondo et al., 2005). Surprisingly, H-1PV can still kill glioma cells that are deficient in autophagic processes. In particular, NCH82 cells lack the PTEN phosphatase (Di Piazza et al., 2007), which triggers autophagy by interfering with the class I PI3-kinase / Akt survival pathway (Arico et al., 2001). Moreover, Bcl-2 is suggested to play a major role in the control of autophagy, being able to either sensitize cells to autophagy (Shimizu et al., 2004), inhibit autophagy (Boya et al., 2005), or permit autophagy to develop by inhibiting apoptosis (Degenhardt et al., 2006). Interestingly, Bcl-2 overexpression does not hamper the induction of autophagic death in H-1PV-infected glioma cells, even though it does prevent H-1PV from causing permeabilization of the outer mitochondrial membrane and hence from triggering caspase 3 activation. Parvovirus H-1 is thus unable to interfere with the anti-apoptotic function of Bcl-2, but it bypasses its protective effect against autophagy. Consequently, H-1PV can kill glioma cells under conditions where caspase 3 is not activated. In apoptosis-competent glioma cells, H-1PV infection leads to the activation of effector caspases. In apoptosis-resistant glioma cells, the activation of effector caspases may reinforce the cytotoxic action of the virus but is not necessary for cell killing through autophagy.

A possibility to explain how parvovirus H-1 triggers a Bcl-2-independent non-apoptotic death is that cell infection by H-1PV induces a starvation-like status leading to induction of autophagy. The formation of autophagic vesicles is reported to be activated in response to stress conditions like nutrient starvation or viral infection, the initiating event being the phosphorylation of translation factor eIF2α by the RNA-dependent protein kinase (PKR) (Talloczy et al., 2002). Although it is unknown whether H-1PV can activate PKR, it has been reported that cells resistant to the virus do not express TCTP, a factor involved in tumor cell control of translation (Tuynder et al., 2004). Starvation-associated autophagy begins with the isolation of double-membrane-bound structures inside an intact cell (Kihara et al., 2001). This process is initiated by a dynamic multiprotein complex between class III PI3K, Beclin1, UVRAG and Bcl-2 (Liang et al., 2006) that can be suppressed by 3-MA, an inhibitor of class III PI3K (Petiot et al.,

2000). 3-MA blocks the formation of acidic vesicles upon H-1PV infection increasing cell survival and rescuing glioma cells from virus-induced death. Autophagy induction by H-1PV might be mediated by Beclin1, since the expression of this pro-autophagic factor is increased after parvovirus H-1 infection of glioma cells while on the opposite the low levels of Beclin1 in tumor cells correlate with reduced sensitivity towards starvation-induced autophagy (Liang et al., 1999). Against this hypothesis, Beclin1 can be sequestrated by Bcl-2 (Pattingre et al., 2005), which is shown not to interfere with parvovirus H-1 cytotoxicity. Nevertheless, the formation of autophagic vacuoles occurs also upon parvovirus H-1 infection of NCH82 glioma cells overexpressing Bcl-2 (data not shown). Despite the fact that a direct investigation of the effect of parvovirus H-1 on the interaction between Beclin1 and Bcl-2 is missing, it is logical to suppose that Beclin1 is involved in the autophagic death mechanism triggered by parvovirus H-1 after infection of glioma cells. To further confirm this assumption, it would be interesting to evaluate whether Beclin1 knockdown by a siRNA approach is blocking parvovirus H-1-induced cell death by impeding the onset of autophagy. The signaling pathway composed of PI3-kinase/Akt/mammalian target of rapamycin (mTOR) plays a central role in the regulation of cell proliferation, growth, differentiation and survival (Oldham and Hafen, 2003; Vogt, 2001). Rapamycin specifically inhibit the function of mTOR by blocking the phosphorylation of downstream molecules like p70S6 kinase (p70S6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) (Huang and Houghton, 2003; Sawyers, 2003), thus controlling the activation of autophagy. Indeed, treatment of human glioma cell with mTOR inhibitor, alone or in synergistic combination with PI3-kinase/Akt inhibitors (Takeuchi et al., 2005) or mTOR siRNA (Iwamaru et al., 2006), induces autophagic cell death. In NCH82 glioma cell, starvation-like autophagy is triggered after treatment with either H-1PV or rapamycin. Moreover, preliminary experiments indicate that parvovirus H-1 infection correlates with a decreased phosphorylation level of p70S6K but not of 4EBP1 (data not shown) as recently reported to occur upon rapamycin treatment of established glioma cell lines (Iwamaru et al., 2006). Altogether, H-1PV appears to trigger autophagy in infected glioma cells inducing a starvation-

like status leading to the negative regulation of the mTOR pathway and to Beclin1-promoted formation of autophagosomes.

Interestingly, oncolytic myxoma virus has been shown to kill glioblastoma-derived cultures (Lun et al., 2005), and tumor cell sensitiveness to the virus has been recently demonstrated to be dependent on the interaction between the Akt kinase and the viral M-T5 protein, which is required for viral replication (Wang et al., 2006). Myxoma virus-induced autophagy has not been investigated, but a recent publication reports on the possibility to extend the host range of this virus to non-permissive tumor cells by rapamycin co-treatment (Stanford et al., 2007). Other pathogens besides parvovirus H-1 have been shown to activate autophagy. This process may be a defense mechanism by which cells digest infectious agents (Kirkegaard et al., 2004; Liang et al., 1998) or generate an immune response (Lee et al., 2007; Paludan et al., 2005), the mechanism being hijacked by some viruses to the benefit of their life cycle (Belov et al., 2007; Jackson et al., 2005; Wileman, 2006). Remarkably, human parvovirus B19 kills erythroid progenitor cells by triggering apoptosis, but mitochondrial autophagy is activated in a erythroid cell line resistant to the virus (Nakashima et al., 2006). Moreover, a conditionally replicating adenovirus has been observed to induce glioma *in vitro* suppression and *in vivo* regression by inducing mTOR inhibition-dependent autophagy (Ito et al., 2006). It might be speculated that in H-1PV-infected glioma cells, autophagy inhibition allowing uncontrolled proliferation is counteracted by autophagy induction in response to the pathogen. Whether autophagy contributes to the multiplication or propagation of nuclear-replicating parvoviruses remains a matter of debate.

5.2. Cathepsins and cystatins contribution to the autophagic death triggered by parvovirus H-1 in NCH82 glioma cells

Another unique feature of H-1PV-induced glioma cell killing is its requirement both for the formation of acidic vesicles and for activation of cathepsins in the cytosol. Lysosomes have been linked to cell death in multiple ways, as they are direct components of the autophagy machinery

(Mizushima et al., 2002) but can also promote programmed cell death execution through lysosomal membrane permeabilization (LMP) and resulting release of lysosomal enzymes into the cytosol (Foghsgaard et al., 2001; Guicciardi et al., 2000). Chemically-induced inhibition of autophagy or lysosomal cathepsins blocks respectively the early and the late steps of viral infection and rescues glioma cell from death. In fact, autophagy still occurs when cytosolic cathepsins are inactivated while cathepsins are prevented to be activated in the cytosol of infected cells when autophagy is inhibited. Thus, in the sequence of events leading to cell death, parvovirus H-1 promotes the onset of autophagy, which in turn is involved in lysosomal membrane permeabilization and cathepsins release. Moreover, the inhibition of “free” cathepsin activity in apoptosis-competent NCH82 cells reduces H-1PV cytotoxic activity and prevents the virus from processing effector caspases. Yet cathepsin-dependent caspase activation is not essential to virus-induced glioma cell death, since Bcl-2 suppresses the former but not the latter. Further study is thus needed to identify the cellular targets of cytosolic cathepsins mediating H-1PV cytotoxicity.

Since cathepsins are normally confined to the lysosomes, their presence in an active form in the cytosol of infected cells implies that H-1PV causes their release by LMP. Interestingly, this relocation is specific, since the intracellular distribution of other lysosomal enzymes of similar molecular weight is not altered in infected cells. Furthermore, acidic vesicles containing lysosomal enzymes remain structurally intact until the cells eventually die. This means that cytosolic cathepsin activation takes place before the onset of detectable cell degeneration. H-1PV thus does not appear to induce an overall destabilization of lysosomal membranes. Permeabilization of vesicles may result from reactive oxygen species (ROS) production (Antunes et al., 2001) or from the activation of cellular phospholipases (Zhao et al., 2001). H-1PV has been shown to block ROS generation in HeLa cells (Ran et al., 1999), and inhibition of $cPLA_2$ by chemical agents has no effect on H-1PV-induced cell killing in NCH82 glioma cells (data not shown). Yet a domain displaying PLA_2 activity has been identified within the capsid protein VP1. Under acidic conditions (e.g. in endosomes), the capsid structure is modified, resulting in exposure of this domain and enabling the virus to exit from the acidic vesicles (Farr et al., 2005; Suikkanen et al., 2003; Zadori et al.,

2001). Should neosynthesized VP proteins or progeny particles transit through autophagic vesicles, VP1 might imaginably affect the stability of vesicle membranes and, as a result, alter the trafficking of cathepsins between the cytosol and the organelles. This hypothesis is consistent with the detection of viral proteins in the vesicles of H-1PV-infected glioma cells, particularly in the Lamp2-positive fraction (Di Piazza et al., 2007). Acidic vesicles accumulating in the cytoplasm of H-1PV-infected glioma cells might interconnect with the “free”-cathepsin-dependent death pathway by providing a source of active enzyme.

The degree of LMP is proposed to determine the balance between apoptotic and necrotic death, with partial lysosomal permeabilization favoring the former and extensive lysosomal destabilization leading to the latter (Guicciardi et al., 2004). This study suggests that limited and very selective permeabilization of lysosomes might be enough to trigger a necrotic type of death, as observed in H-1PV-infected glioma cells. The dominance of necrosis in this system may be due, at least in part, to the presence of anti-apoptotic signals in glioma cells. Other cells, such as U937 cells derived from a myeloid leukemia, have indeed been shown to die of apoptosis upon H-1PV infection (Rayet et al., 1998). LMP is reported to be triggered by various inducers of p53- dependent and independent death, including TNF and pro-apoptotic drugs, in rodent fibroblasts (Foghsgaard et al., 2001), colon cancer cells (Erdal et al., 2005), and myeloid leukemic cells (Yuan et al., 2002). It is particularly noteworthy that H-1PV cytotoxicity does not appear to be associated with the induction of detectable TNF production, that U937 cell variants surviving H-1PV infection are also resistant to death induction by TNF (Rayet et al., 1998), and that TNF-induced killing of U937 cells depends on the expression of cathepsins (Deiss et al., 1996). Thus, cathepsin activation appears to be a key element in parvoviral cytotoxicity, although the type of ensuing cell death may vary according to other cellular determinants. TRAIL is also unlikely to mediate redistribution, since this ligand has recently been shown to promote cell death through caspase 8-induced cathepsin B activation (Nagaraj et al., 2006). Generally speaking, the cytosolic accumulation of cathepsins in H-1PV-infected glioma cells occurs under conditions where effector caspases are not activated. It can be distinguished from LMP caused by the above-mentioned agents. Inhibitors of

NAD-consuming enzymes have also been shown to affect the balance between apoptosis and necrosis after cell infection with H-1PV (Ran et al., 1999). This may be due to the role of lysosomes in parvovirus-induced cell death, since the NAD^+/NADH system is involved in electron transport and acidification in these organelles (Gille and Nohl, 2000).

Another interesting finding of the present study is that induction of glioma cell death by H-1PV correlates not only with cytosol enrichment in cathepsins, but also with reduced levels of the cathepsin inhibitors cystatin B and cystatin C. This reduction presumably contributes to the striking increase in cathepsin activity in the cytosol of infected cells, and is necessary for their killing. This points to a role for cystatin-family proteins in the survival of cancer cells. In keeping with this, cystatin C and cystatin B are overexpressed in gliomas (Zhang et al., 2003), accounting for the resistance of these tumors to LMP-inducing agents. It is noteworthy that highly malignant gliomas show lower cystatin C levels than astrocytomas (Nakabayashi et al., 2005), suggesting that the oncolytic activity of H-1PV may be even stronger against high-grade than low-grade gliomas. Successful H-1PV-induced glioma cell killing thus requires both relocation of cathepsins and their relief from cystatin inhibition. Nevertheless, autophagy still occurs when NCH82 cells are prevented from H-1PV cytotoxic action by cystatin B overexpression, pointing to a central role for autophagy in the initiation of parvovirus-induced cell death. Then, it cannot be excluded that parvovirus H-1 infection triggers the formation of autophagic vesicles and the modulation of cystatins, cathepsins lysosomal release being a consequence of the autophago-lysosomal trafficking and cathepsins cytosolic activation occurring because of cystatins decreased expression.

5.3. Parvovirus H-1 as therapeutic tool for glioma treatment

The concept of oncolytic virotherapy implies the use of viruses to specifically infect and kill tumor cells (Kirn et al., 2001). The current tendency in this field is to test replication-competent viruses naturally selective for cancerous cells and non cytophatic toward healthy tissues (Parato et al., 2005). A comparison of parvovirus H-1 versus other oncolytic

viruses' cytotoxic potential against human gliomas will be helpful in the assessment of this virus as promising candidate for a virotherapy approach. An advanced study has evaluated the antitumor efficiency of nine unrelated viruses with oncolytic potential in two glioblastoma cell lines (Wollmann et al., 2005). Vesicular stomatitis virus (VSV) has displayed the greatest potential, being able to target different glioma phenotypes, to release progeny from a small number of cells, to rapidly spread throughout the culture and efficiently kill the cells. On the opposite, Sindbis virus (SIN) and both the fibrotropic prototype and an immunosuppressive strain of MVM prefer specific glioblastoma genotypes, and only SIN exerts self-amplification of the antineoplastic effect. In this respect, parvovirus H-1 successfully suppresses several established and low passage human glioma cell lines displaying different mutation profiles. H-1PV progeny spreading capacity has not been tested *in vitro*, but when intracranially injected into glioma-bearing rats the virus obviously diffuses inside the tumoral mass and induces the regression of the neoplasm.

As already mentioned, a main limitation of anticancer treatments is represented by side effects produced on healthy tissues. SIN, the two MVM strains and passage-adapted VSV have shown not to significantly infect human fibroblasts, used as control, while VSV progeny has been on the contrary hypothesized to be implicated in latent cell death among control cells (Wollmann et al., 2005). Indeed, a more recent investigation has proposed to combine VSV infection with interferon pathway inducers in order to kill human glioma cells but preserve glial cells (Wollmann et al., 2007). A more appropriate *in vitro* control for human non-malignant brain tissue is provided for parvovirus H-1 studies. Previous (Herrero y Calle et al., 2004) and current (Di Piazza et al., 2007) data show that H-1PV infection does not jeopardize the survival of normal human astrocytes. In particular, infected normal astrocytes do not fill up with acidic vesicles and do not show any other sign of autophagy, even though they sustain detectable levels of H-1PV DNA replication and gene expression (Di Piazza et al., 2007). This oncospecificity of H-1PV cytotoxic effects may be due to various factors, as normal mouse and human astrocytes differ from glioma cells by their lower permissiveness to parvovirus multiplication (Abschuetz et al., 2006; Herrero y Calle et al., 2004), their lower level of cathepsin B, and their lack of

cystatin B down-regulation upon infection. Further work in this direction should evaluate parvovirus H-1 lack of affinity for neurons, since a possible therapeutic application of the virus must exclude the eventuality of damage to the central nervous system. Moreover, given the evidence of the existence of a brain cancer stem cell pool responsible for tumor development and maintenance (Singh et al., 2004), it would be important to scrutinize H-1PV sensitivity of neural versus cancer stem cells.

In conclusion, H-1PV has developed an efficient strategy for killing human glioma cells. It targets lysosomal proteases from two sides: accumulation of autophagic vesicles and cathepsins functional cytosolic activation. Cathepsins are highly expressed in high-grade tumors and participate in invasion by malignant cells (Mohanam and Jas, 2001; Rempel et al., 1994). A strategy taking advantage of this overexpression to induce killing of tumor cells seems particularly attractive. The evidence presented here suggests that parvoviruses are especially relevant to the treatment of gliomas, through their ability to efficiently hijack the lysosomal/cathepsin pathway and kill tumor cells, whether or not they are resistant to conventional death inducers.

5.4. Genetic determinants of rat embryo fibroblasts sensitiveness to parvovirus H-1-induced killing

Proliferating transformed cells are efficiently killed by parvoviruses because they provide an environment suitable to viral multiplication and spreading. In particular, cell transformation contributes to the oncotropic and oncolytic activities of parvoviruses by inducing changes in the structure, regulation and/or activity of the viral non-structural protein NS1, which is considered the major effector of virus-mediated cytotoxicity (Caillet-Fauquet et al., 1990; Li and Rhode, 1990; Moffatt et al., 1998; Mousset and Rommelaere, 1982). Several studies have suggested that cell immortalization could in certain contexts be sufficient to sensitize cells to parvovirus-induced cell killing (Salomè et al., 1990). Fisher rat fibroblasts have been shown in example to be naturally resistant to parvovirus minute virus of mice (MVM) but to exhibit a progressive increased susceptibility to

infection when transfected with immortalizing or transforming oncogenes (Legrand et al., 1992).

The present study focuses on parvovirus H-1 because of its potential as therapeutic tool for an alternative approach in the treatment of gliomas (Di Piazza et al., 2007; Herrero y Calle et al., 2004). Cell sensitization to H-1PV infection has been studied in rat embryo fibroblasts since they constitute a simple model for oncogene cooperation in malignant transformation. Immortalized or transformed phenotypes have been induced in these cells by using, in single overexpression or in different combination, *c-myc*, *SV40 large T* antigen and activated *Ha-ras* oncogenes, and a dominant negative mutant form of the anti-oncogene *p53*. These genes have been chosen because they are frequently mutated in the majority of human cancers (Nesbit et al., 1999; Sahai and Marshall, 2002; Vousden and Lu, 2002), but also in the perspective of applying the knowledge grasped from REFs experiments on the study of human and rat astrocyte transformation in correlation to acquisition of permissiveness to H-1PV. In fact, Ras is the downstream main effector of the signalling network activated by receptor tyrosine kinases (RTKs) whose genes are frequently overexpressed/amplified in gliomas (Tysnes and Mahesparan, 2001). Additionally, Ras triggers the Akt/PKB survival pathway that is negatively regulated by PTEN, a phosphatase whose loss of function is often detected in human gliomas (Zhu and Parada, 2002). The inactivation of p53 has been reported in both primary and secondary gliomas, directly through locus mutation or indirectly due to Mdm2 overexpression or loss of the INK4a/ARF locus (Zhu and Parada, 2002). Mutation occurring to *c-myc* expression are rare in high-grade adult brain tumors (Maher et al., 2001), but there is a tight connection between control of *c-myc* functions and loss of $p19^{ARF}$ (Qi et al., 2004; Zindy et al., 1998). Finally, a truncated form of *SV40 large T* antigen has been already used to study the inactivation of pRb proteins in the development of high-grade astrocytomas (Biernat et al., 1997b; Xiao et al., 2002a).

Evidence is here given that *c-myc* overexpression is sufficient to sensitize rat embryo fibroblasts to parvovirus H-1-induced cell lysis. Indeed, c-Myc oncoprotein enhances immortalized rat fibroblasts susceptibility to the killing effect of parvoviruses (Salomè et al., 1990), and it has been proposed to mediate apoptotic cell death induction after H-1PV infection of human

promonocytic U937 cells (Rayet et al., 1998), whose virus-resisting clones show a significant decrease in the accumulation levels of c-Myc (Lopez-Guerrero et al., 1997). On the opposite, p53 inactivation or *Ha-ras* overexpression are not sufficient to render REFs permissive to viral infection. Therefore, differences in the modes of action of single oncogenes seem to ascribe for distinct responses in terms of cell sensitivity to H-1PV.

Viral genome amplification has been considered as a critical step that can be modulated by malignant transformation (Rommelaere and Cornelis, 2001). In agreement with this statement, the present work underlines that the quality of the immortalized/transformed status of the cell has a different effect on parvovirus H-1 protein expression and capacity to fulfil its life cycle. Specific oncogenes have been shown to directly enhance parvovirus genome replication: NS1 synthesis is driven by the early parvoviral promoter P4, which contains several binging sites, conserved between MVM and H-1PV, for transcription factors activated by Ras through the MAPK signalling pathway (Fuks et al., 1996; Perros et al., 1995), by c-Myc (Plaza et al., 1991) and SV40 large T (Gu et al., 1999; Gu et al., 1995). It is therefore not surprising that REFs transfected with *c-myc* or *SV40 large T antigen* alone are able to sustain H-1PV genome replication. This event is then rendered progressively more pronounced in cells restraining the combined double oncogene expression, and in transfectants transformed by these oncogenes in cooperation with *ras**. Indeed, increased viral genome amplification and proteins translation has been already documented in association with parvovirus infection of transformed cells (Cornelis et al., 1988; Cornelis et al., 1990). Human lung fibroblasts transformed with SV40 and infected with MVM support a 10-fold-higher level of DNA replication than their untransformed progenitors (Cornelis et al., 1988), and processing of viral multimeric replicative intermediates has been correlated with SV40-induced malignancy (Kuntz-Simon et al., 1999). Finally, p53 exerts a direct or p21-mediated inhibition of cell growth (Lundberg et al., 2000), and the blockage of parvovirus infection in primary cells may be at least partly resulted from p53-induced cell cycle arrest (Op De Beeck et al., 2001). Despite of this argumentation, p53 inactivation alone is not promoting viral replication, but it could contribute to it in cells overexpressing *c-myc*, *ras** or *SV40 large T antigen* by stimulating cell proliferation.

The data presented in the current investigation are partially in contrast with an analogous report on rat embryo fibroblasts sensitiveness to parvovirus H-1 (Telerman et al., 1993). In that study stable transformed REFs subpopulations were generated by *c-myc* overexpression and *Ha-ras* activation plus p53 concomitant inhibition with a dominant negative mutant or an inactive form carrying a point mutation. A vector coding for a short form of p53 incapable of abrogating wild-type p53 DNA binding activity was used as control. Since only this last sub-population resisted to H-1PV infection, the conclusion was that there is a close relationship between wild-type p53 function and resistance to parvovirus H-1-induced killing. Besides, *c-myc* alone was failing in sensitizing *ras*-transformed REFs to viral infection. The possibility of disturbances on the intracellular signalling network and metabolism due to triple-oncogene transformation has not been considered. However, rat embryo fibroblasts have shown a highly variable behaviour in response to the introduction of genetic alterations. In example, primary REFs cultures resist specific oncogene-induced transformation (Inoue et al., 1995), while REF52 cells are immortalized fibroblasts but require co-expression of a cooperating oncogene for transformation by *ras*, thus acting similarly to primary cells (Franza et al., 1986). Hence, it is possible that the transfected short form of p53, despite not inactivating wild-type p53 functions, induced an aspecific cell reaction that affected the outcome of REFs sensitivity to viral infection. Moreover, in that work cells were infected with H-1PV at an MOI of 100 for 7 days, while the transfectants here analysed have been treated with an amount of virus ranging from MOI 0.625 to 10 for a period of 2 days, even excluding a second round of replication. Altogether, it should be concluded that the experimental setting used in this study has allowed a more precise evaluation of the molecular events conferring permissiveness to parvovirus H-1.

Altogether, the acquisition of cell permissiveness to the virus seems to correlate with the extent of single oncogene efficacy in evading mechanisms controlling cell division and proliferation, and preventing the activation of programmed cell death pathways. In general, p53 acts as a transcription factor: its cellular levels increase following DNA damage and result in cell cycle arrest followed by either DNA repair or, if the damage is irremediable, apoptosis (Vousden and Lu, 2002). The regulation of cell cycle progression

by p53 mainly occurs by affecting the phosphorylation status of retinoblastoma proteins (pRb_f) through the induction of growth arrest effectors, principally $p21^{cip1}$ (Johnson et al., 1993) but also $p15^{INK4b}$ and $p16^{INK4a}$ (Lundberg et al., 2000). The perturbation on cell cycle control introduced by inactivating p53 with a dominant negative mutant can be further enhanced by overexpressing *SV40 large T* antigen. In addition to binding and blocking p53 (Ali and DeCaprio, 2001), SV40 large T can sequestrate pRb proteins independently of their phosphorylation status (Whyte et al., 1989), thus promoting E2F transcription factor-mediated gene expression, which trans-actives among others *c-myc* (Hiebert et al., 1989). c-Myc is a multifunctional transcription factor regulating the expression of a broad number of genes involved in cell proliferation, differentiation and growth (Henriksson and Luscher, 1996), and even apoptosis (Evan et al., 1992). Previous reports have proposed that the mechanisms for *c-myc*-stimulated apoptosis are distinct from *c-myc*-induced hyperproliferation pathways (Conzen et al., 2000; Qi et al., 2004; Xiao et al., 1998). On the one hand, c-Myc directly stabilizes p53 and triggers p53-dependent transcription (Hermeking and Eick, 1994; Wagner et al., 1994). On the other hand, c-Myc can induce p53 through activation of the tumor suppressor $p19^{ARF}$, which in turn can inhibit *c-myc* expression and its transactivation of key target genes by acting independently from p53 (Qi et al., 2004; Zindy et al., 1998). Indeed, cells transfected with *c-myc* are initially sensitive to apoptosis, but may overcome cell arrest crisis and acquire immortalized properties upon loss of $p19^{ARF}$ (Zindy et al., 1998). Furthermore, c-Myc can commit mammalian somatic cells to DNA replication by regulating the cyclin E-CDK2 complex independently and in parallel to the pRb/E2F pathway (Santoni-Rugiu et al., 2000), thus overcoming $p16^{INK4a}$-induced cell cycle arrest (Alevizopoulos et al., 1997).

In summary, c-Myc mediates on the one hand the disruption of molecular mechanisms controlling cell growth and proliferation that involve also the alteration of p53 and pRb signalling pathways. Nevertheless, this overall synergistic action seems not to account for the acquisition of cell sensitivity to parvovirus H-1, as the present study demonstrate that the virus is not inducing significant cell cycle perturbations and normal, *p53dn* or *ras** REFs proliferate in culture despite being insensitive to viral infection,

thus excluding an effect of the cell cycle. On the other hand, *c-myc* overexpression selects for additional not completely determined genetic changes that can predispose cells to the activation of the apoptotic signalling pathway. In particular, *c-myc* sensitizes immortalized cells to death by destabilizing mitochondrial integrity; this process can be activated in response to TNF or TRAIL (Klefstrom et al., 2002), is mediated by pro-apoptotic members of the Bcl-2 family such as Bak and Bax (Juin et al., 2002), and is inhibited by anti-apoptotic members of the same family like Bcl-X_L or Bcl-2 (Finch et al., 2006; Iaccarino et al., 2003). In contrast, *ras** overexpression promotes cell survival through the PI3K/Akt pathway, which antagonizes apoptosis initiation (Giehl, 2005). A consideration of the consequence of *ras* activation on the predisposition to sensitization provided by *p53dn* and *c-myc* overexpression strengthens this assumption. *ras* oncogene transducted alone has been demonstrated to push rat embryo fibroblasts to senescence, partially by inducing $p16^{INK4a}$ expression (Serrano et al., 1997). It is therefore not surprising that *ras**-transfected REFs do not support viral infection. On the other side, cells are highly sensitive to H-1PV-induced killing when transformation is achieved by *ras* and *c-myc* concomitant activation, while ras*/*p53dn*-transfected REFs are less susceptible to parvovirus H-1, not more than REFs transfectants sensitized by *c-myc* single overexpression. Therefore, *c-myc*-overexpressing cells, apart from being immortalized, appear also to be more prone to the activation of programmed cell death pathways in response to external stimuli.

5.5. Oncogenes impact on the activation of apoptotic or alternative death pathways upon REFs infection with parvovirus H-1

Parvoviruses have been reported to promote the initiation of apoptosis (Moehler et al., 2001; Rayet et al., 1998), necrosis (Ran et al., 1999) and autophagy (Nakashima et al., 2006). Upon infection of rat embryo fibroblasts overexpressing *c-myc* and/or *SV40 large T* antigen, the virus promotes the activation of a non-apoptotic cell death characterized by the appearance of autophagic vesicles, the cytosolic activation of lysosomal cathepsin B and the

down-regulation of cathepsins inhibitors. In REFs transformed by *ras* overexpression and p53 inactivation, H-1PV induces apoptosis via mitochondrial outer membrane permeabilization and caspase 3 cleavage. Interestingly, virus-mediated cystatin B modulation occurs also in these cells, thus appearing to be a general direct effect of parvovirus H-1 infection. On the contrary, it is not possible to identify a virus-intrinsic killing mechanism activated in response to the acquisition of a precise phenotype. As already mentioned, the hypothesis is that single oncogenes differently impinge on cells predisposition to the activation of an apoptotic or autophagic programmed death pathway to counteract viral infection.

In the cell populations analyzed, the basal content of acidic vesicles is higher in *c-myc* and/or *SV40 large T* immortalized and transformed REFs as compared to primary cells. The state of tumoral cell differentiation is certainly a factor that may influence the autophagic response (Houri et al., 1995; Houri et al., 1993). In this perspective, *c-myc* overexpression induces autophagy in established rat fibroblasts that retain primary embryonic features, and sensitizes these cells to a stronger activation of autophagy under serum starvation conditions (Tsuneoka et al., 2003). More recently, a shorter isoform of the tumor suppressor $p19^{ARF}$ (smARF) has been shown to induce autophagic cell death upon mitochondrial translocation and p53 or Bcl-2/Bax independent partial dissipation of the outer membrane potential without cytochrome *c* release (Reef and Kimchi, 2006). It has to be noted that smARF is rapidly degraded by the proteasome, but accumulates after inappropriate proliferative signals generated by *SV40 large T*, *c-myc* and *E2F-1* oncogenes (Reef et al., 2006) that have also been demonstrated to induce $p19^{ARF}$ longer isoform (Bates et al., 1998; Zindy et al., 1998). Controversial is instead the role of Ras in the regulation of the autophagic process. It has been initially shown that autophagy is triggered by *ras* overexpression in human glioma and gastric cancer cell lines (Chi et al., 1999) while more recently Ras has been identified as a negative regulator for nutrient deprivation-induced autophagy in NIH3T3 cells (Furuta et al., 2004) and in yeast (Budovskaya et al., 2004; Schmelzle et al., 2004) by acting respectively through the class I PI3-kinase and the PKA signalling pathways. In addition, Ras downstream signalling controls autophagy activation in cancer cells response to amino acids and nutrient starvation by differently

influencing respectively the ERK or the Akt pathway (Pattingre et al., 2003). It is therefore possible that *ras* exerts a synergizing effect increasing the tendency of *c-myc* or *SV40 large T* overexpressing REFs to activate autophagy as degradative pathway. On the contrary, transformed *ras*/p53dn* REFs seem to be over-protected against p53-dependent apoptosis to achieve uncontrolled cell proliferation, due to p53 inactivation and Ras-mediated anti-apoptotic effect. However, it has not to be excluded that they cannot antagonize p53-independent apoptotic reaction to external stimuli like H-1PV infection, since *ras* has been demonstrated to activate $p19^{ARF}$ (Groth et al., 2000), which can promote the induction of apoptosis in a p53-independent manner (Lowe and Sherr, 2003). Indeed, REF52 cells stably transformed by *ras* oncogene have a limited ability to proliferate in response to mitogenic signals and require the continuous expression of a collaborating oncogene, such as SV40 large T, to be maintained in culture (Ragozzino et al., 1991). It thus seems that primary cells try to counteract transformation by inducing alternative mechanisms to restore disrupted pathways controlling cell cycle progression and programmed cell death.

Lysosomal proteases, cathepsins, may play a role in immortalized and/or transformed REFs proneness to the activation of an apoptotic or autophagic programmed death pathway after parvovirus H-1 infection. In particular, cathepsins B and L translocate from lysosomes to the cytosol in reaction to a large array of death-inducing stimuli and trigger different types of death, from classic apoptosis (Boya et al., 2003; Foghsgaard et al., 2001) to necrosis (Kagedal et al., 2001), depending on the extent of the lysosomal membrane permeabilization and the amount of cathepsins released to the cytosol (Guicciardi et al., 2004). Moreover, cathepsin B seems to act as molecular switch between apoptosis and autophagy, the former being favoured by cathepsins chemical inhibition (Lamparska-Przybysz et al., 2005). Most interestingly, immortalization and transformation have been demonstrated to sensitize murine embryonic fibroblasts (MEFs) to cathepsin B and L dependent cell death (Fehrenbacher et al., 2004). While primary MEFs are not susceptible to tumor necrosis factor (TNF)-mediated killing, cathepsins-promoted and caspase 3-independent apoptosis is triggered after TNF incubation of MEFs spontaneously immortalized through either p53 mutation or $p19^{ARF}$ loss. *ras**-induced but not *SV40 large T*-driven

transformation of immortalized MEFs increases cathepsins expression and provides cells with additional sensitization to TNF treatment (Fehrenbacher et al., 2004). Remarkably, TNF-mediated killing of transformed MEFs is reported to activate a cathepsins-dependent programmed cell death (Fehrenbacher et al., 2004; Gyrd-Hansen et al., 2004), allowing the conclusion that a non-apoptotic process is promoted in this context. Hence, oncogene selective activation could influence cell sensitization to an autophagic death promoted by cathepsins via either mitochondrial permeabilization (Jäättelä, 2004) or lysosomal leakage leading to intracellular acidification and stabilization of pro-autophagic members of the Bcl-2 family (Kubasiak et al., 2002). However, various genetic backgrounds could differently impinge on lysosomal death pathways at the level of lysosomal membranes sensitivity to permeabilization-inducing events, grade of lysosomal membrane permeabilization, type of activated downstream effector mechanisms (Fehrenbacher and Jäättelä, 2005).

6 Conclusions

The present work studied the capacity of parvovirus H-1 to kill human glioma cells and identified a new viral cytopathic pathway and a novel regulatory mechanism of virus-induced cell death. H-1PV triggered a non-apoptotic type of death characterized by both the accumulation of autophagic vesicles and the activation of lysosomal cathepsins in the cytosol of infected cells. On the basis of the presented experimental data, a model for H-1PV-mediated cell killing is proposed (Fig. 27). In addition, the present study tested the effect of genetic alterations on cell permissiveness to parvovirus H-1-induced killing. To this purpose, rat embryo fibroblasts were immortalized or transformed by overexpressing *c-myc*, activated *Ha-ras*, and *SV40 large T antigen* oncogenes, or a dominant negative mutant form of *p53*. A second model describing how different oncogenes affect H-1PV-mediated cell killing is depicted (Fig. 28).

Parvovirus H-1 infection triggers an autophagic and cathepsins-dependent cell death in glioma cells. In cultured NCH82 cells cathepsins are confined within lysosomes and are neutralized by cystatins, their physiological inhibitors, in case of an eventual release into the cytosol. Autophagy is activated as default mechanism involved in nutrient recycling and protein turnover. Upon parvovirus H-1 infection, autophagosomes formation is stimulated along the conventional pathway depending on Beclin1, but independently of Bcl-2. The cytosol of infected cell is rapidly filled in with acidic vesicles, as result of the dynamic fusion of autophagosomes with lysosomes. By 24 h after infection, acidic compartments start to leak thus releasing their content into the cytosol. Since parvovirus H-1 down-regulates cystatins expression, cytosolic cathepsins are no more counterbalanced and exert their proteolytic activity digesting the cell from inside. Whether cathepsins lysosomal release is a selective process or an event due to a more general permeabilization of the lysosomal membrane is still a matter of debate. Anyway, cathepsins cytosolic

activation takes place after and as consequence of H-1PV induction of autophagy. Besides, cathepsin B promotes the onset of apoptosis by mediating mitochondrial membrane depolarization and downstream activation of caspase 3. Cell lysis is accomplished in 80% of the cultured NCH82 cell population by 48 h after parvovirus H-1 infection.

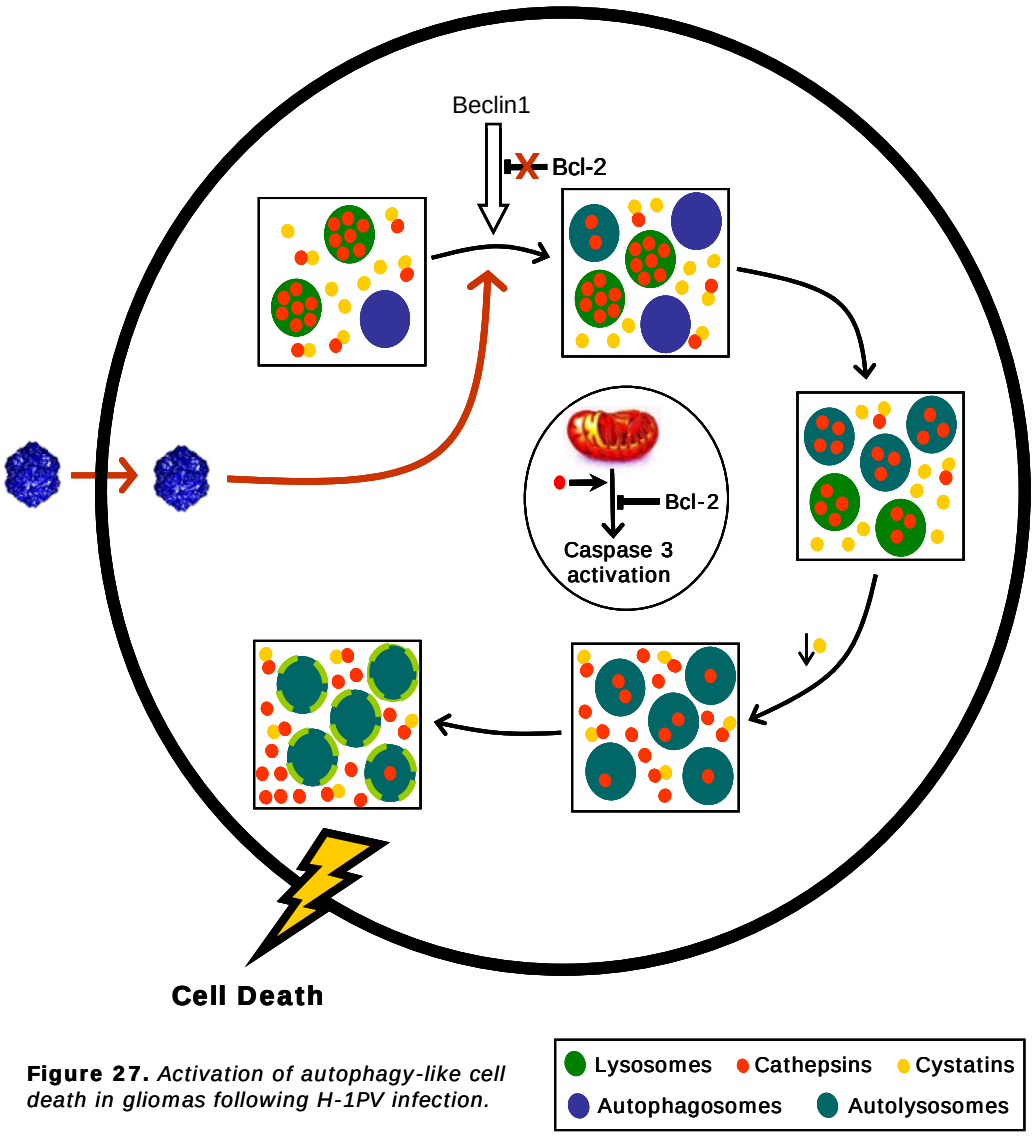


Figure 27. *Activation of autophagy-like cell death in gliomas following H-1PV infection.*

Oncogene selective sensitization of rat embryo fibroblasts to parvovirus H-1-induced killing. Primary untransfected REFs and *ras**-senescent cells are insensitive to H-1PV infection. Immortalization because of *p53* inactivation is not sufficient to render cells permissive to the virus but,

remarkably, *c-myc* overexpression alone in enough to sensitize REFs to H-1PV cytolytic action. Immortalization through double oncogene expression and cell full transformation enhance furthermore the cytotoxic power of the virus. As in the glioma model, parvovirus H-1 infection triggers a non-apoptotic death pathway in REFs overexpressing *c-myc* and/or *SV40 large T* antigen, alone or in combination with another oncogene. This process is distinguished by acidic compartments multiplication, accumulation and final disruption. Cathepsin B is therefore active in the cytosol of infected cell and initiates a proteolytic process that leads to cell rupture. Surprisingly, H-1PV promotes the activation of a classical apoptotic pathway in REFs transformed

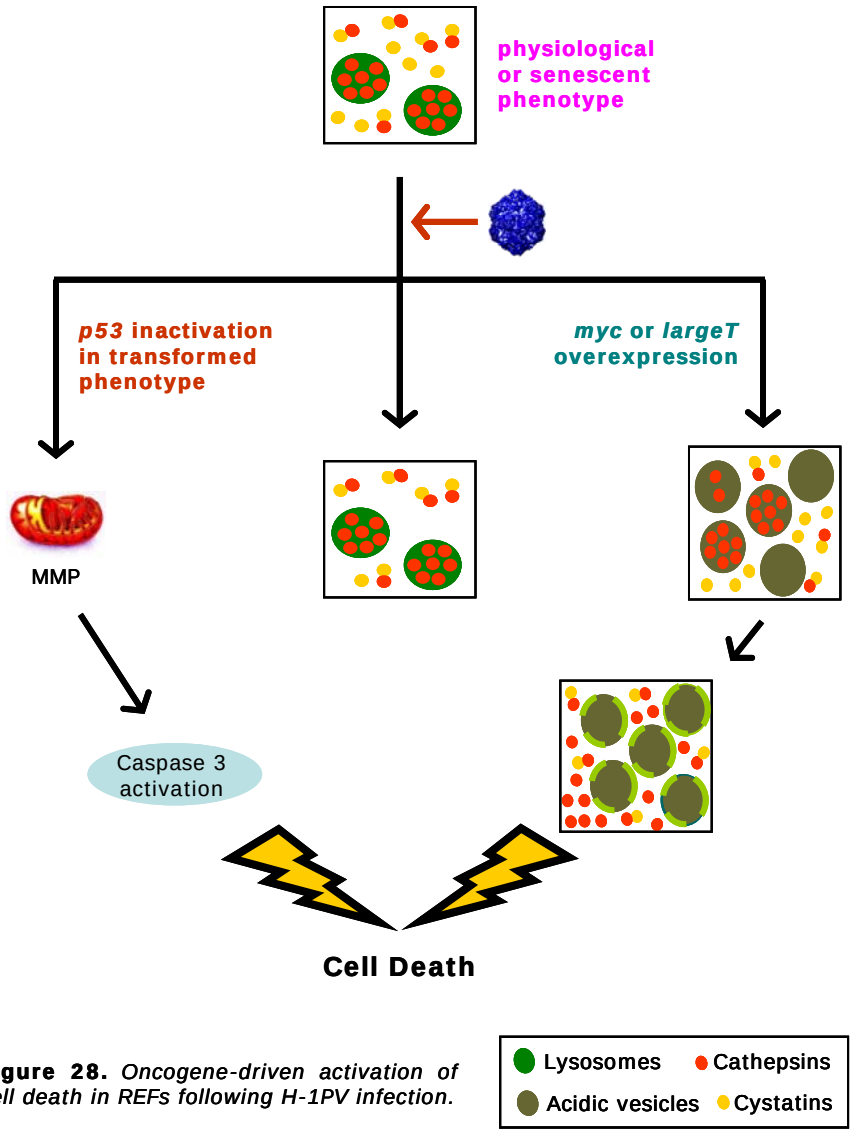


Figure 28. *Oncogene-driven activation of cell death in REFs following H-1PV infection.*

by *p53* inactivation in combination with a second oncogene overexpression. Particular is the case of cells transfected with *SV40 largeT antigen* only. At a molecular level, these cells are susceptible for H-1PV activation of autophagic and cathepsin B-dependent cell death. At a cellular level the overall behaviour of the cultured population toward H-1PV infection is highly variable and therefore the sensitivity of these cells to the virus is not statistically significant.

The cellular and molecular disturbances triggered by parvovirus H-1 in tumor cells shed new light on the interplay between apoptosis and alternative death pathways in tumor development, an emerging topic with interesting potentials for novel therapeutic applications. Considering the abilities of this virus to distinguish neoplastic (sensitive) from normal glial cells (resistant), and to overcome the resistance developed by gliomas and many other tumors to death inducers, the present work opens up future prospects for the use of H-1PV in cancer virotherapy.

7 References

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