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“Caprine Herpesvirus 1 (CpHV-1) a potential candidate for oncolytic virotherapy”

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A me stessa, alla mia forza, alla mia caparbietà, ai miei sacrifici.
A chi mi ha sempre incoraggiato e sostenuto in ogni mia scelta.
A Lella, mia fedele compagna di viaggio.

‘Scopri chi sei e non temere di esserlo’
Mahatma Gandhi

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Ringraziamenti

AAV	Helper-dependent viruses
AD	Adenovirus
AKT	Protein-kinase B
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BHV-1	Bovine herpesvirus type 1
BHV-4	Bovine herpesvirus type 4
CAR	Chimeric antigen receptor
CELO	Chicken embryo lethal orphan
CNS	Central nervous system
CpHV-1	Caprine herpesvirus type 1
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAMPs	Damage-associated molecular pattern molecole
DC	Dendritic cell
EBV	Epstein–Barr virus
FDA	Food and drug administration
GBM	Glioblastoma multiforme
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCC	Hepatocellular carcinoma
HMGB1	Hig mobility group box 1
HN	Hemagglutinin neuraminidase
hNIS	Human sodium iodide symporter
HSPs	Heat shock proteins
HSV	Herpesvirus
IC	Intracranial

ICD	Immunological cell death
IFN	Interferon
IV	Intravenous
MHC	Major histocompatibility complex
MoMLV	Moloney murine leukemia virus
MV	Measles virus
NDV	Newcastle disease virus
NK	Non killer
OVs	Oncolytic viruses
OVT	Oncolytic virotherapy
PAMPs	Pathogen-associated molecular pattern
PD-L1	Programmed death-ligand 1
PKR	Protein kinase R
PV	Parvovirus
RB	Retinoblastoma
ROS	Reactive oxygen species
RV	Reovirus
SCID	Sever combined immundeficient mouse
SLAM	Signalling lymphocyte-activating molecule
TAAs	Tumor-associated antigens
TGFß	Transforming growth factor
TK	Timydine kinase
TLRs	Tool like receptors
TME	Tumor microenviroment
TNF-α	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand

TREG	Regulatory T cells
VAC	Vaccinia virus
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VSV	Vescicular stomatitis virus
WT	Wild type

1 Immunogenic cell death (ICD)

2 Immunologic barriers to successful oncolytic virotherapy

3 Graphic representation of herpetic virion

4 Apoptotic morphologycal changes

5 Schematic representation of apoptotic activation

6 Structure of the members of the Bcl-2 family members. TM: transmembrane domain (Martin & Vuori, 2004).

7 Mechanism of action of pro-apoptotic proteins belonging to the Bcl-2 family (Desagher & Martinou, 2000)

8 Apoptosis induction by p53. (www.rndsystems.com/cb_detail_objectname_SP04_p53.aspx)

9 Schematic representation of autophagy (http://physiologyonline.org)

10 Regulatory mechanisms of autophagy. (http://www.biochemsoctrans.org).

11 Morphological representation of necrosis. (https://www.studyblue.com).

12 Activation of necroptosis process. (www.cell.com).

13 Permissiveness of immortalized human cancer cell lines to Caprine Herpesvirus 1 (CpHV-1).

14 Permissiveness of immortalized human cancer cell lines to Caprine Herpesvirus 1 (CpHV-1).

15 Dose–response curve of human cancer cell lines infected with different MOI of CpHV-1 and observed at different time on cell viability

16 Time course of virus production and cell death in Human cancer cell lines infected with CpHV-1.

17 CpHV-1 induce apoptosis in human cancer cell lines. Replicate cultures of human cancer cell lines were mock infected or infected with CpHV-1 at MOI 2.5.

18 Caspases activation is induced in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines cells infected with CpHV-1 at 12h pi

19 Caspases activation is induced in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines cells infected with CpHV-1 at 24h pi

20 Caspases activation is induced in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines cells infected with CpHV-1 at 48h pi.

21 Autophagy activation in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines cells infected with CpHV-1.

Abstract

Oncolytic virotherapy is a promising avenue of cancer therapy. It is a therapeutic approach to cancer treatment that utilizes native or genetically modified human viruses that selectively replicate in tumor cells or nonhuman viruses that are naturally oncotropic and preferentially replicate in tumor cells, displaying minimal adverse effects in normal healthy cells. To date, a wide variety of viruses have been evaluated for their oncolytic potential, including DNA viruses as well as RNA viruses. CpHV-1 is a species-specific herpesvirus closely related to Bovine herpesvirus type 1 (BHV-1). Previuos works have demonstrated that CpHV 1 is able to induce apoptosis in goat peripheral blood mononuclear cells, moreover we have investigated on the pro apoptotic potential of CpHV 1 in Madin Darby bovine kidney cells, evaluating apoptotic profiles like chromatin condensation and DNA laddering. Recently, the intracellular pathway by which CpHV 1 is able to induce apoptosis has been characterized in more details, by analyzing the gene expression response during the apoptotic phase of CpHV 1 infection in a murine neuroblastoma cell line (Neuro 2a). Thus, the aim of the present research was to investigate the ability of CpHV-1 to replicate, cause cell death and affect cellular viability in a panel of human cancer cells lines. Human breast adenocarcinoma (MDA-MB-468), Human cervical adenocarcinoma (HeLa), Human osteosarcoma (U2OS), Human prostatic adenocarcinoma (PC3), Human lung carcinoma (A549) and Chronic Myelogenous Leukemia (K562) cell lines were used. Madin Darby bovine kidney (MDBK) cell line was used as control. In a first series of experiment we have analyzed the effect of CpHV-1 infection on cell viability by means of the MTT assay at different time post infection (p.i.)

and several multiplicity of infection (MOI). All cell lines, except K562 cells, showed a marked cytopatic effect (CPE), demonstrating an oncolytic potential of CpHV-1 in tested human cancer cells. The reduction of cells viability was associated with significant levels of viral production as assayed by TCID50 after 24 h p.i. for MDBK, MDA-MD468, A549, U2OS, and after 48 h p.i. for PC3, HeLa and K562 cell lines. Then, we investigated virus induced cytotoxicity, viability, and apoptosis within a single assay well, by using the ApoTox-Glo Triplex assay. Analysis of virus-infected cells revealed activation of caspase-3, a marker of apoptosis at 24 h post-infection. Analysis of virus-infected, cells by western blot assay, revealed activation of caspase-3 and cleaved caspase 3 at 24 h p. i. in MDBK, PC3, MDA-MD-468 and U2OS cell lines. Our findings demonstrate the effect of CpHV-1 infection in neoplastic cell lines in terms of caspase activation and apoptosis modulation, thus CpHV-1 could be a novel candidate oncolytic viru

Capitolo 1

Oncolytic virotherapy

1. Oncolytic virotherapy

Neoplastic diseases represent one of the most common causes of death among humans and pets. In 2006, the World Health Organization reported cancer as the second cause of death in developed countries and the third cause in the worldwide (Lopez et al.; 2006). Numerous risk factors responsible for cancer development have been characterized, such as smoking, obesity, unhealthy diet, chronic infection or exposure to natural or chemical carcinogens. Some inherited genetic factors can also predispose to cancer development. Altough treatments for the neoplastic disease have significantly improved, conventional therapeutic options often remain insufficient and unsatisfactory; therefore, new and innovative strategies and approaches are higly needed. Oncolytic viruses (OVs) are viral strains that can infected and kill malignant cell (oncolysis), leaving surrounding healthy cells unharmed. In addition to direct cytotoxic activity, OVs enage and amplify host immune responses, leading to the distruction of residual malignant cells and establishment of lasting antitumor immunity. Oncolysis can be either a natural property of the virus (naturally occurring OVs, for example reovirus) or a consequence of manipulation of the viral genome (genetically engineered OVs, for example adenovirus) and renders oncolytic virotherapy (OVT) a potential therapeutic modality in cancer treatment. OVT has been studied for the last century; initial interest in the use of viruses to treat cancer dates to observations made in the early 1900s of tumor regression in the context of natural viral infection. One of the first case report of a drammatic regression of cervical cancer in a patient receiving the Pasteur-Roux live

attenuated rabies vaccine, was presented in 1910 (Sinkovics et al.; 2008). In 1940s, human studies with different types of viruses were launched (Hoster et al.; 1949). The era of modern oncolytic virotherapy started in the early 1990s, when a genetically modified, live-attenuated, thymidine kinase (TK)-negative herpes simplex virus (HSV) strain was locally injected into human glioma kenograft models, showing positive results (Martuza et al.; 1991). Recent advances in genetic engineering technology spurred a renewed interest in OVT, to enhance the safety and efficacy of oncolytic vectors. Today, IMLYGIC™ (T-VEC/Talimogene Laherparepvec), a genetically engineered HSV, is the first OV approved by the FDA for use in the United States and the European Union for patients with locally advanced or non-resectable melanoma (FDA, 2015; EMA, 2015). Numerous clinical trials have demonstrated sinergy between OVT and other standard and emerging anticancer therapy (Khuri et al.; 2000; Karapangiotou et al.; 2012). Combination treatment, particularly with immune-modulating therapies, remains a promising field of research. Activation of the host immune system is a crucial component of OV-mediated tumor destruction; optimization of viral replication and propagation as well as the generation of anticancer immunity remain a significant challenge facing OVT. With a better understanding of the complex immunological interactions between OVs, tumor cells and the host immune system, the next generation of OVs will be poised to exploit the full immunotherapeutic potential of OVT.

1.1 Oncolytic viruses: brief history of oncolytic viruses

Oncolytic viruses show the natural propensity to infect malignant cells. Cancer cells are resisted to apoptosis and growth suppression, evade immune-mediated destruction and proliferate indefinitely; similar characteristics are also referable to viral replication (Choi et al.; 2016). Moreover, many tumors develop defects in cellular antiviral response pathways, like type I interferon (IFN) signaling, rendering these cells more susceptible to viral infection (Nguyen et al.; 2008). OVs have the capacity to selectively replicate in cancer cells, causing antitumor effects through a variety of mechanisms, including direct lysis of infected cells and immune-mediated destruction of both infected and non-infected cells (Ito et al.; 2006; Boozari et al.; 2010). Although viruses have been used as therapeutic agents in the form of vaccines since the late 1700s (Pasteur, 1885; Willis et al.; 1997), their potential application as cancer therapy had not been explored until the early 1900s (Bierman et al.; 1953). In the most often-cited report, G. Dock decribes the case of a 42-years old woman suffering from leukemia who showed remission after infection with presumed influenza (Dock; 1904). In another case, a four-years old boy with leukemia showed a remarkable remission after acquiring chickenpox (Bierman et al.; 1953), but after a one-month remission, his leukemia relapsed and progressed rapidly to death. Several early landmark human clinical trials demonstrated both the potential of viral therapy as a cancer treatment, as well as its alarming side effects (Asada 1974; Southam et al.; 1952). Altough some patients experienced short-lived clinical remission of cancer, a notable proprortion either died from the side effects of the viral therapy like hepatitis or fatal neuroencephalitis, or had the brief remission

that were reversed by a strong anamnestic response by the patient's immune system, leading to continued cancer progression and death for the primary disease (Asada; 1974). These observations suggested that occurring viruses possessed an innate ability to kill cancer, but in order to harness the potential of oncolytic viral therapy, modifications would be requie to both improve cancer cell selectivity, safety and efficacy of virotherapy. Following a series of disappointing clinical trials, interest in OVs declined in the 1970-80s until the development of genetic engineering in the 1990s made possibile to improve selectivity and decrease toxicity. In another landmark study, Martuza et coll. reported that treatment with a thymidine kinase (TK)-mutated HSV-1 could reduce glioma in mice brains with low neurotoxicity (Martuza et al.; 1991).

ONYX -015 was the first virus to enter Phase I clinical trials in 1996 and the adenovirus mutant H101 became the world's first OV approvated for cancer treatment in 2005 (Garber et al.; 2006). Following a Phase III trial showing improved durable response rate for the intralesional treatment of melanoma, T-VEC became the first oncolytic virus approved by the FDA in October 2015 (Andtbacka et al.; 2015).

1.2 Mechanisms of tumor selectivity

1.2.1 Natural viral tropism for cancer cells and general mechanisms of oncolysis

OVs have been generally categorized into DNA and RNA viruses and further divided into double- and single-stranded. OVs demonstrate a natural ability to infect cancer cells selectively and more efficiently than normal cells. Cancer cells have several distinct hallmarks that separate

them from normal cells: sustained growth signals, insensitivity to anti-growth signals, evasion of apoptosis, increased angiogenesis, cell immortality and evasion/metastasis (Hanahan et al.; 2000; Hanahan et al.; 2011). Cells infected with viruses show many of the same properties as transformed cells, as viruses have developed various mechanisms to replicate within the host evading its immune system. Infact, some viruses naturally exploit the aberrant signaling pathways that sustain cancer growth to selectively infect and replicate within cancer cells as opposed to normal cells. For instance, constitutively active AKT (protein-kinase B) pathway signaling serves as a sustained growth and survival signal in many diferent types of cancer (Testa et al.; 2001). Wang et coll. demonstrated that the natural tropism of mixoma virus in cancer cells exploits the endogenous AKT activity via complex formation between AKT and M-T5, a myxoma viral protein (Wang et al.; 2006). OVs can enter within cells through fusion with the plasma membrane or through binding to receptors in their surface. Infact, another mechanism of action by which viruses may selectively bind to and infect cancer cells is the overexpression of selected surface receptors. In squamous cell carcinoma, higher expression of the cell surface adhesion molecule nectin-1 was correlated with the increase HSV-1 infection and citotoxicity compared to cells that had lower nectin-1 levels (Yu et al.; 2005). Measles virus has been shown to use the surface receptor CD46 for cellular entry, wich is overexpressed in a variety of human cancers, including hepatocellular carcinoma, colorectal cancer, ovarian cancer and breast cancer (Dorig et al.; 1993; Anderson et al.; 2004). Furthermore, OVs can also take advantage of a deficient anti-viral defense mechanism in cancer cells.

When normal cells are infected by viruses, release of interferons (IFNs) and activation of tool-like receptors (TLRs) by recognition of viral elements activate several downstream pathways, leading to protein kinase R (PKR) activation (Kaufman et al.; 2015). Phosphorylated PKR subsequently blocks protein synthesis and prevents viral replication in the cell. Cancer cells may have abnormal IFN pathways and/or abnormal PKR activity, making them more susceptible to viral infection.

An essential characteristic of an oncolytic virus is the ability to establish lytic cycle in malignant but not normal tissues, either by naturally exploiting inherent tumor weaknesses, such as RAS pathway activation (Strong et al.; 1998; Alain et al.; 2007) or by genetic modification. For example, knockdown of TK gene in HSV can lead to preferential killing of tumor cells, as TK-negative HSV can replicate only in dividing cells depending on their TK activity (Jamieson et al.; 1974; Field et al.; 1978; Martuza et al.; 1991). Thymidine kinase (TK), an important enzyme involved in viral DNA synthesis and repair (Whitley et al.; 2001) is highly expressed in activated cells in G1 phase in vitro (Gasparri et al.; 2009). OVs can establish a niche of continous viral replication within the tumor, to recruit uninfected cells nearby generating syncithia, to infect dividing and non-dividing cells, and to be stable in vivo, yet lack chromosomal integration and do not result in major disease (Verheije et al.; 2012). OVs, like reovirus (Prestwich et al.; 2008), HSV (Toda et al.; 1998; Toda et al.; 1999), or vaccinia virus (Thorne et al.; 2008), can induce tumor–specific adaptive immune responses and indirectly cause malignant cell death. Adenovirus (Diaconu et al.; 2012), Coxsackie B3 (Miyamoto et al.; 2012) and measles virus (Donnelly et al.; 2013) can lead to endoplasmatic

reticulum stress and cause immunologic cell death, a type of cell death that leads to release of danger-association molecular patterns, like adenosine triphosphate, calreticulin and high-mobility group box-1, which attract immune cells (Kepp et al.; 2001).

OVs can also selectively target tumor neo-vasculature. Vescicular stomatitis virus (VSV) can selectively infect endothelial cells and cause thrombosis in the tumor vessels (Breitbach et al.; 2011); HSV and vaccinia virus can also selectively damage tumor endothelium (Benencia et al.; 2005; Breitbach CJ, et al.; 2013). OVs can be also genetically engineered to express anti-angiogenetic factors, like vascular endothelial growth factor (VEGF) inhibitors (Zhang et al.; 2005; Gholami et al., 2014).

1.2.2. Mechanisms of action

The mechanisms of action of OVs are still incompletely understood; however, the overall antitumor effect induced by oncolytic viral treatment has as major components the local cell death of both virally-infected and non-infected cells and induction of the systemic immune response to virally-induced cell destruction within the tumor. OVs mediated tumor cell death via direct and indirect mechanisms, working as both direct cytotoxic agents and therapeutic cancer vaccines. These mechanisms are connected by the propensity of many OVs to induce immunogenic forms of tumor cell death, including immunogenic apoptosis, necrosis, pyroptosis and autophagic cell death, wich activate host immune responses (Boozari et al.; 2010; Myamoto et al.; 2012). Immunogenic cell death (ICD) is a type of cell death that leads to robust antitumor immune responses (Guo et al.; 2015). OVs infection of cancer cells induced cell death through the above

cited mechanisms, which are often dependent on either the virus type, the cancer cell type or a combination of both (Elankumaran et al.; 2006). OV-mediated cell death induces the release of cytokines, tumor-associated antigens (TAAs) and other danger signals, including damage-associated molecular pattern molecules (DAMPs) and pathogen–associated molecular pattern (PAMPs) molecules. The host immune response to these signals has been associated with local release of cytotoxic perforins and granzymes, that can kill adjacent non-virally infected tumor cells, the so called "immune-associated" bystander effect (Obeid et al.; 2007; Schietinger et al.; 2010). In addition, some OVs also target tumor vasculature, leading to death of uninfected tumor cells due to loss of the tumor blood supply (Breitbach et al.; 2007; Liu et al.; 2008). Except for apoptosis, the modalities of cell death listed above are highly immunogenic, leading to activation of both the innate and adaptive immune responses. ICD can induce antitumor immune response via dendritic cells (DC) activation. Direct oncolysis of OV-infected cancer cells leads to release of TAAs, that can be used to generate antigen-specific antitumor immunity (Bartlett et al.; 2013). Antigen presenting cells, such as DCs, are crucial mediators of innate and adaptive immunity, faciliting the generation of immune responses by releasing of cytokines and activating naive T cells. Recruited to sites of infection and inflammation, DCs capture viral and tumor antigens released during oncolysis and present them to naive T cells, thereby initiating the generation of antigen specific adaptive immune reponses that mediate destruction of residual and recurrent tumor cells (Prestwich et al.; 2008). In addition to releasing OV specific PAMPs, OV mediated cell death also

release DAMPs, including adenosine triphospate (ATP), calreticulin, heat shock proteins (HSPs) and hig mobility group box 1 (HMGB1) protein (Obeid et al.; 2007; Zitvogel et al.; 2010; Borde et al.; 2011; Tang et al.; 2012). The tumor microenvironment (TME) is often characterized by a state of immunodepression. Tumors overexpressed cytokines like interleukin-10 and transforming growth factor-ß (TGFß), which inhibit natural antitumor immune responses. Therefore, tumor derived cytokines and chemokines also include those promoting growth and vascularization like tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (Prestwich et al.; 2010). OV infection stimulates the release of cytokines (IL-1, IL-6, IL12, IL-18, IFNs AND TNF-α) and chemokines from infected dying cells, resident and infiltrating immune cells, altering the balance of pro- and anti-inflammatory factors within TME. Viral infection and resulting localized inflammation enhance the action of infiltrating immune cells and faciltate the generation of antitumor immunity (Guidotti et al.; 2001). The presence of TAAs, DAMPs, PAMPs and cytokines stimulate the antigen presenting cells (APC) maturation which primes T lymphocytes in the adaptive host immune response by cross presentation (Aymeric et al.; 2010; Bartlett et al., 2013; Guillerme et al.; 2013). Therefore, type 1 IFNs and DAMPs also directly stimulate natural killer (NK) cell response against cancer cells, so the innate immune system is also involved in OV mediated cell death (Kaufman et al.; 2015). OV mediated cell death also received a significant contribution from neutrophils (Di Carlo et al.; 2001; Zhang et al.; 2012). Neutrophils are the firts immune responders, being thus responsible for initiating an antimicrobal response at sites of infection (Drescher et al.; 2013). Once

activated, in addition to secreting the TNF-related apoptosis inducing ligand (TRAIL) and TNF-α, neutrophils also generate a large quantity of reactive oxygen species (ROS), which disseminated cell death (necrosis, necroptosis), contributing to further inflammation and to the oncolytic effect (Drescher et al.; 2013; Dey et al.; 2016).

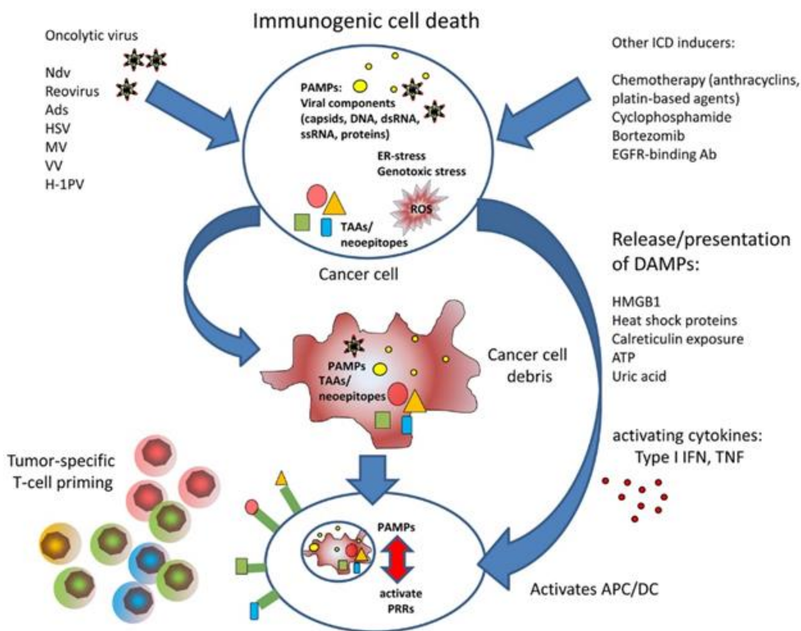


Figure 1. Immunogenic cell death (ICD). Oncolytic viruses induce ICD, wich activate host immune responses (https://www.frontiersin.org).

1.2.3 Immunological barriers to successful OVT

The multimodal immunogenic cell death mediated by viral infection and oncolysis activate innate and adaptive immune responses that contribute to killing tumor cells; however, host immune responses to viral infection can be detrimental to overall efficacy of OVT (Figure 1). Numerous preclinical studies have demonstrated in immunocompetent compared to immune-compromised hosts reduced viral replication, earlier clearance and decreased antitumor efficacy (Nemunatitis et al.; 2003; Parato et al.; 2005; Thorne et al.; 2006). The systemic antitumor response can result in clearance of OV by antibodies generated against viral PAMPs and/or cytotoxic T cells that recognize viral PAMPs (Kaufman et al.; 2015). NK cells have also been directly implicated in decreasing the efficacy of viral therapy via upregulation of natural citotoxicity receptors in virally-infected cells (Alvarez-Breckenridge et al.; 2012).

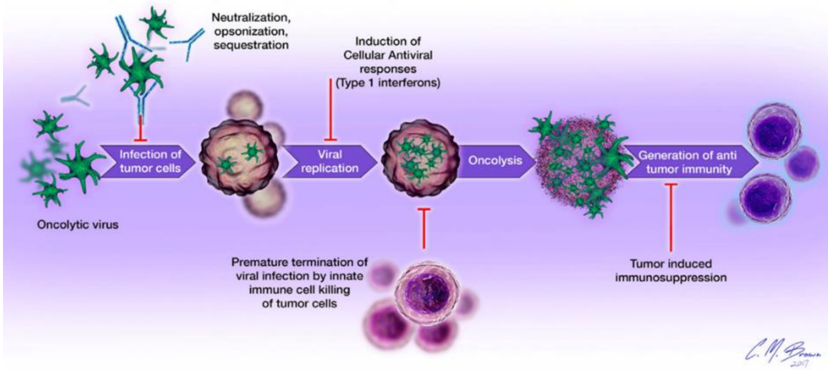


Figure 2. Immunologic barriers to successful oncolytic virotherapy: (1) the presence of neutralizing antibodies, complement proteins, and sequestration in organs such as the liver and spleen interfere with the oncolytic virus delivery to tumor sites (2) cellular antiviral responses, such as type I interferon signaling limits viral replication within tumor cells; (3) destruction of infected tumor cells by cells of the innate immune system (neutrophils, macrophages, NK cells) prematurely terminates viral infection; (4) tumor-induced immunosuppression (elaboration of immunosuppressive cytokines, accumulation of regulatory T cells, overexpression of negative checkpoint regulators of T cell function) inhibits the generation and effector functions of antigen-specific antitumor immune responses. (Filley et al.; 2017).

1.2.4 OVs and innate immunity

Viral infection triggers the production of antiviral proteins, synthesis of cytokines and recruitment of immune cells to the site of infection. Viral replication is inhibited by type 1 IFNs that reprogram gene expression in infected and uninfected cells; IFNs also induce cell cycle arrest and apoptosis, upregulate major histocompatibility synthesis and drive the development and proliferation of memory T cells (Guidotti et al.; 2001). Among the innate immune cells, APCs, neutrophils and NK cells are the first responders to viral infection (Guidotti et al.; 2001). In particular neutrophils react to viral pathogens by secreting reactive oxigen species and proteases, inducing necrotic cell death and localized inflammation

(Khuri et al.; 2000). In a heterotopic murine model of colon cancer, intratumoral neutrophlis accumulation in response to OV infection resulted in tumor vasculature destruction and widespread tumor cell apoptosis (Breitbach et al.; 2007). NK cells have been shown to be key effectors of OV-induced antitumor immune responses (Prestwich et al.; 2009; Miyamoto et al.; 2012;). They target cells lacking MHC molecules or displaying virally induced markers of cellular stress and induce cell death by releasing granzyme and perforin enzymes and activating apoptosis (Guidotti et al.; 2001; Thorne et al.; 2006). The agonist/antagonist relationship of the immune system and OV is not static but evolves with the phase of the infection and tumor destruction. In order to exert maximal therapeutic effects, OVs must persist long enough and induce sufficient oncolysis to stimulate the generation of long lasting adaptive antitumor immunity. Viruses are foreign pathogens and naturally stimulate host immune responses mediating their clearance. Destruction of infected tumor cells by innate immune cells and viral antigen-specific T cells can also terminate OV infection before full therapeutic effects have been achieved (Breitbach et al.; 2007). Transient suppression of host early immune responses could be a potential chance to improve OV delivery to tumor sites, prolong viral infection and enhance the overall therapeutic efficacy of OVT. Inhibiting early intratumoral immune cell infiltration with low dose chemotherapy or TGF-β treatment has been shown to enhance viral replication, decrease clearance and improve antitumor effects in several murine models of glioma (Fulci et al.; 2006; Kurozumi et al.; 2007). Pretreatment with immunosoppressive chemotherapeutics like cyclophosphamide has been shown to improve viral delivery, promote

replication and enhance oncolytic effect of HSV-based OVs in murine models of glioma by depleting antiviral antibodies and impairing complement function (Fulci et al.; 2006; Ikeda et al.; 2000 a, b).

1.3 OVs and clinical trials

Several ongoing clinical trials are testing the safety and efficacy of OV agents alone or in combination with chemo- and/or radiotherapy. OVs have been generally categorized into DNA and RNA viruses and further divided into double- and single-stranded.

1.3.1. DNA Viruses

1.3.1.1. Herpesviruses

Vectors based on herpes simplex virus (HSV)-1 are, together with the adenoviruses, furthest along in their development and testing for virotherapy. The first replication-competent vector for cancer therapy (malignant glioma) based on HSV-1 F strain was described in 1991 (Martuza et al.; 1991). This vector was deleted in the TK gene but was shown to be oncolytic to human glioma xenografts in nude mice. Although TK-negative HSV vectors are attenuated in normal cells, the deletion of this gene eliminates the possibility to use commercial anti-herpetic drugs to control the infection.

The most HSV vectors in therapy use today are deleted for the main neurovirulence gene γ34.5, which severely restricts their ability to replicate in the adult central nervous system and to form latency. These mutated

vectors have been the first HSV vehicles to enter clinical trials (Markert et al.; 2000). However, in order to avoid the generation of wild-type (WT) HSV, the so called second generation vectors have been deleted in several genes. The γ34.5 gene deletion reduced replication efficiency; therefore, modifications restoring the efficency have been introduced in the following generations. The third-generation vector G47Δ has been deleted for the ICP47 gene and shows increased replication efficacy and oncolysis in animal models compared to the parental G207 vector (Todo et al.; 2001). To date, five replication-competent HSV-1 vectors have entered clinical trials: the first generation vector 1716, a recombinant based on HSV-1 strain 17 and deleted of γ34.5 gene; NV1020 (formerly R7020), a chimeric second generation recombinant of HSV-1 and HSV-2 with deletions in the UL24 and UL 56 genes and in one copy of the γ34.5 gene; the second generation vector G207 deleted in ICP6 gene (ribonucleotide reductase) and both copies of the γ34.5 gene; OncoVEX (GM-CSF), a recombinant vector deleted in the γ34.5 gene but engineered to express higher levels of US11 wich compensates for the reduced replication efficiency; the HF10 vector, a multiple mutation recombinant HF strain of HSV-1 (Varghese et al.; 2002; Nakao et al.; 2002). In addition to the modified vectors described above, HSV variants with full replication efficiency and less cytotoxic than clinical isolates have been generated by serial passage on human cancer cells in cell culture (Taneja et al.; 2001). Furthermore, the oncolytic capacity of many HSV vectors has been augmented by various inserts. One of this strategy has been to produce cytokines and chemokines (e.g.IL-12 or GM-CSF) from the vectors to stimulate immune responses (Epstein et al.; 2005). Many vectors exist

derived from other members of the herpesviridae family. Notably, since also herpes simplex type 2 is a human pathogen, interest in this virus has resulted in its development into an oncolytic vector (Fu et al.; 2006). The vector FusOn-H2, was created by deletion of the ICP10 ribonucleotide reductase gene, corresponding to the ICP6 gene in HSV-1, which eliminates the tumorigenic potential of the vector and confers syncytia-forming properties. Indeed, when compared to the others oncolytic vectors based on HSV-1, FusOn-H2 demonstrated more efficacy even after intravenous (i.v.) administration in a nude mouse model of human breast cancer. Making use of the natural tropism of Epstein–Barr virus (EBV), a recombinant non-oncolytic vector based on the prototype strain B95.8 was recently created for targeting of B-cells which are otherwise notoriously difficult to transduce (Hellebrand et al.; 2006). This vector, expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), was able to induce maturation of DCs in vitro and to induce specific T-cell activation, and is thus being considered for vaccination against B-cell lymphomas. Furthermore, it was recently shown that replication- competent bovine herpesvirus 4 was oncolytic for several cancer cell lines in culture and destroyed human A549 lung carcinoma xenografts in nude mice (Gillet et al.; 2005). The apoptosis was shown to be dependent on the expression of viral genes. In addition to conventional HSV vectors, vehicles based on saimiri virus, the prototype of the c-2 subfamily of herpesviruses, represent a promise as a new virotherapeutic vector. These viruses can persist non-lytically in T-cells, and when engineered to express TK, they have shown significant anti-tumoral efficacy in animal models of leukemia. Non-oncolytic amplicon vectors based on the roseoloviruses human herpesvirus

6A and 6B and 7 are being developed for tumor vaccination (Frenkel et al.; 2006). HHV-6 infects lymphocytes and macrophages via the CD46 receptor and HHV-7 shows strict CD4 T-cell tropism. Finally, vectors based on equine herpesvirus 1 have recently been constructed, but these have not yet been tested for oncolytic cancer therapy. The main advantages of herpesvirus vectors are their capacity to carry large transgenes, safety issues such as neurotoxicity at high virus doses, the possible oncogenicity, but there is the risk of recombination with or activation of endogenous herpesviruses.

1.3.1.2 Vaccinia viruses

In early Phase I studies, ITu JX-594, a genetically engineered TK-mutant/GM-CSF expressing VAC, demonstrated promising results in patients with melanoma (Mastrangelo et al.; 1999) and hepatocellular carcinoma (HCC) (Park et al.; 2008). A study group of 10 patients with melanoma received ITu injections of JX-594 (Hwang et al.; 2011). In another Phase I trial, JX-594 was directly injected into HCC; the injection was well tolerated and associated with viral replication, decreased tumor perfusion and tumor necrosis (Heo et al.; 2011). The treatment was followed by therapy with sorafenib; the sequential therapy regimen was well tolerated and associated with decreased tumor perfusion, with tumor necrosis up to 100%. JX-594 has also demonstrated systemic delivery potential. The results of a Phase I dose-escalation trial of IV JX-594 in patients with metastatic tumors showed selective activity, replication and expression of transgene products in cancer tissue in a dose-related trend (Breitbach et al.; 2011). JX-594 was tested also in a Phase I dose-

escalation trial in patients with metastatic, refractory colorectal cancer (Lee et al.; 2013). A Phase II study comparing intravenous and intratumoral Jx-594 injections in patients with advanced HCC have progressed on sorafenib, is underway (Heo et al.; 2013).

1.3.1.3 Adenoviruses

Adenoviruses were isolated in 1953 from human adenoid tissue samples in culture undergoing ‘‘spontaneous’’ regression, and were dubbed adenoidal–pharyngeal–conjunctival viruses based on their capacity to induce disease symptoms in experimentally infected humans (Rowe et al.; 1953). Since then adenoviruses have become the most widely used and most extensively studied viruses for oncolytic therapy. In China, oncolytic adenovirus mutants in combination with chemotherapy have been accepted as a standard treatment form for refractory nasopharyngeal cancer. The immediate-early genes E1 are expressed promptly upon adenovirus entry into a cell. In particular, two genes of this group have been the targets of modification in order to create tumor-specific viruses: E1A and E1B (E1B55K). Normally the products of these genes act in concert to force the host cell to enter S phase, a prerequisite for the rest of the viral replication process. Deletion of E1A will render the virus susceptible to the antiviral mechanisms of the retinoblastoma (Rb) protein, specifically by blocking the G1 to S transition. Deletion of E1B, on the other hand, allows p53 to induce apoptosis in infected cells, aborting replication and spread of the virus. Therefore, productive replication of adenoviral E1-deletion mutants can only take place in cells deficient in Rb and p53. Most gliomas fulfill these requirements and thus become ample targets for selective oncolysis

by adenoviral E1-deletion mutants (Jiang et al.; 2006). An example of an E1B-negative adenovirus (dl1520) is the Onyx-015 vector, which is frequently used in both experimental and clinical gene therapy (Rogulski et al.; 2000; Kirn et al.; 2001; Nemunaitis et al.; 2001; Geoerger et al.; 2002; Reid, 2002; Aghi et al.; 2005;). In a Phase II study of 37 patients with head and neck cancer, ONYX-015 was combined with chemotherapy. It was approved for human use in China in 2006 (Garber et al.; 2006) but its clinical development in the United States and Europe has been stopped since 2000 due to lack of efficacy. E1A-deletion mutants, such as D24 (dl922–947), have shown superior oncolytic efficacy compared to E1B mutants both in vitro and in vivo (Fueyo et al.; 2000; Heise et al.; 2000). To further enhance the oncolytic efficacy of E1A mutants they can be engineered to express functional p53, such as the commercial vector ADVEXINTM (Introgen Therapeutics Inc.), which enhances late stage replication in p53-deficient tumor cells by inducing apoptosis and facilitating the release of new virions (Merritt et al.; 2001; Van Beusechem et al.; 2002). This strategy is being implemented in several clinical trials (Vecil et al.; 2003).

Therefore, several retargeting strategies have been developed. For example, the incorporation of tumor-targeting peptides into the fiber protein of adenoviruses has been shown to increase both the specificity and oncolytic efficacy in glioma cells (Yoshida et al.; 1998; Shinoura et al.;1999). Similarly, placement of an RGD-motif into the fiber knob of a replication competent adenoviral vector dramatically enhanced its oncolytic efficacy in A549 human lung carcinoma xenografts in nude mice (Suzuki et al.; 2001). The currently employed adenoviruses in clinical

trials are all based on human adenovirus serotype 5, although they employ different modes of tumor selectivity. Several other adenoviral vectors have been investigated and developed. For example, although not specifically developed for human use, canine adenoviral vectors have been used in mouse models for cancer with promising results (Hemminki et al.; 2003). Further, vectors based on both porcine and bovine adenoviruses are available, showing efficient transduction of many human cell lines (Bangari et al.; 2005). The ovine adenovirus type 7 vectors OAdV623 and OAdV220 have been tested in animal models of cancer with good results (Voeks et al.; 2002; Wang et al.;2004). Finally, a recombinant vector based on the avian adenovirus chicken embryo lethal orphan (CELO) virus and expressing TK displayed (in combination with relevant prodrugs) modest oncolytic capacity in several human cancer cell lines in vitro and prolongation of animal survival in an immunocompetent mouse model for melanoma (Shashkova et al.; 2005). As with most non-human adenoviruses, CELO does not replicate productively in human cells and is therefore replication-deficient. It is possible to utilize these non-human adenoviruses in translational research and in the future, they may be applied in clinical settings in humans.

1.3.1.4 Parvoviruses

Parvoviruses are non-enveloped single-stranded DNA viruses. The family includes the helper-dependent viruses (AAV), wich require molecular functions supplied in trans via co-infection with herpes- or adenoviruses, and the autonomous parvoviruses that can replicate with the help of cellular factors. Only autonomous viruses are oncolytic agents, although

both display oncosuppressive effects. The mechanisms of action of these viruses are different; for example, they can inhibit the transforming capacity of other oncogenic viruses (Mousset et al.; 1982), and have been shown to suppress the proliferation of some cancer cell lines by inducing cell cycle arrest and terminal differentiation (Bantel-Schaal et al.; 1995). However, the non-structural proteins of parvoviruses can cause epigenetic modifications in cancer cells and revert them to a benign phenotype (Iseki et al.; 2005). PV B19 can induce apoptosis in erythroid cells through non-structural proteins (NS1)-induced caspase 3 activation (Morey et al.; 1993; Bauder et al.; 2000). ParvOryx01 is a first in humans, Phase I/IIa, dose-escalation study of H-1PV given locally and through systemic administration in patients with recurrent glioma (Geletneky et al.; 2014). Thus, antitumor effect by these viruses may involve both oncolysis and tumor suppression/reversion (Cornelis et al.; 2004). Several members of the dependent viruses have been used for cancer therapy (Li et al.; 2005). Some data reported that the tumor-trasduction efficacy of five different AVV strain was compared, and it was demonstrated that serotype 2 was the most efficient killer of solid tumor cells (Hacker et al.; 2005). Dupressoir et coll. (Dupressoir et al.; 1989) demonstrated the oncolytic capacity of the autonomous rodent parvovirus H-1 in a mouse model of mammary cancer. However, this study underlines an important safety aspect, since the virus used did not cause tissue damage and its replication in non-tranformed cells was considerably attenuated. Dupont et coll. (Dupont et al.; 2000) showed the oncolytic effect of a recombinant tiny virus of mice in a panel of cancer cells. Finally, parvovirus types unable to infect human cells can be used to target human cancer cells by capsid

retargeting. For example, a modified feline panleukopenia virus is a currently being tested as a cancer targeting tool (Maxwell et al.; 2001).

1.3.2 RNA viruses

1.3.2.1 Reoviruses

Reoviruses are very common in the human respiratory and gastrointestinal tracts (up to 100% seropositivity in the adult population, not associated with any disease). These viruses are oncolytic and show high tumor-specificity upon remote administration, while they are attenuated in healthy tissue. Reoviruses preferentially target cancer cells based on their higher rates of cell division, which differs from that of normal cells (Gong et al.; 2014). For example, marked inhibition of tumor growth and prolonged survival of immunocompetent mice were observed upon multiple intravenous (i.v.) injections in a model of lung-metastasizing mammary cancer. The oncolytic efficacy was reduced in pre-immunized animals, indicating that pre-existing immunity represents an obstacle, but combining treatment with immunosuppression agent overcame the restriction (Hirasawa et al.; 2003). In another study, reoviruses administered intracranially (i.c.) were able to eradicate breast cancer metastases in the central nervous system (CNS) of nude mice (Yang et al.; 2004). The replication of reoviruses is inhibited in normal cells by the cellular anti-viral defense mechanisms, which is activated by recognition of the viral genome by the enzyme protein kinase R (PKR); the main advantage of these viruses is their well-established safety profile in man. An unmodified, non-pathogenic, type 3 Dearing reovirus strain (Reolysin)

has been extensively evaluated in preclinical models and clinical studies. This RV has a dual mechanism of action including the selective lysis of tumor cells and induction of an anti-tumor immunity. The selective permissiveness of cancer cells to reovirus replication and lysis is dependent from endogenous and exogenous factors. The formers are defective PKR signalling, RAS activation and/or mutations of RAS-effector proteins that downregulate the IFN-induced antiviral response and dysfunctional or deleted tumor suppressor-genes (e.g., p53 and ATM) (Yoshida et al.; 1998; Glasgow et al.; 2004). Exogenous factors include cellular stress deriving from chemo- and/or radiotherapy and reovirus modulation of interferon signalling (Nakayama et al.; 2006; Stoff-Khalili et al.; 2006). These factors allow for viral replication, oncolysis and cancer cell death; the presence of infected cell and the release of viral- and tumor-associated antigens after tumor cell death induce the innate and adaptive immune responses (Prestwich et al.; 2008; Hall et al.; 2012; Adair et al.; 2013). Therapy using Reolysin has also demonstrated synergy and/or additive effects with standard chemotherapies (Wadler et al.; 2004; Sei et al.; 2009) and immunosuppressant agents (Hirasawa et al.; 2003). Combination therapy can overcome pre-existing immunity to RV without affecting metastatic tumor regression. Reolysin is one of the best-studied OV and several Phase I and II trials have been completed. Several patients have been received RV treatment, single or in combination with radiotherapy or chemotherapy. It was administered with paclitaxel and carboplatin in patients with non-small cell lung cancer (NSCLC) (Villalona-Calero et al.; 2016). REO 017 was a single arm Phase II study of Reolysin with gemcitabine in chemotherapy-naïve patients with

advanced pancreatic adenocarcinoma (Mahalingam et al.; 2015); biopsies after treatment revealed virus localization in malignant cells, caspase-3 activation and increased programmed death ligand-1 (PD-L1) expression in malignant cells (Mahalingam et al.; 2015 a, b).

1.3.2.2 Paramyxoviruses

This vast group of viruses includes prototypic members such as measles virus, mumps virus and the Newcastle disease virus (NDV); all of wich have been extensively used in cancer therapy. The specificity of these viruses to cancer cells likely stems from the inability of tumor cells to respond to type I interferons and to mount an anti-viral defense (Krishnamurthy et al.; 2006). However, these viruses are classified as being lentogenic (avirulent in poultry and not capable of producing infectious progeny in most tissues) and mesogenic (moderately virulent in chickens).

NDV is not pathogenic in humans and has been extensively studied as an oncolytic agent in several different human tumor cell lines and tumor models (Reichard et al.; 1992; Lorence et al.; 1994). In 1965 NDV was used as an anti-tumor agent in humans in a clinical trial led by Dr's Cassel and Garrett who used the live attenuated 73-T strain to treat a patient with cervical carcinoma (Cassel et al.; 1965). Over the years, the 73-T strain has also been used in humans in the form of oncolysates to target melanoma (Cassel et al.; 1977; Cassel et al.; 1992). NDV binds cells via hemagglutinin neuraminidase (HN) protein (Park et al.; 2003). NDV has been used both as an oncolysate and live virus in several clinical trials.

PV701 and MTH68/H are live attenuated oncolytic viral strains of NDV, wich have the capacity to selectively replicate in lysate tumor cells and to stimulate immune responses (Fournier et al.; 2012). PV701 has shown to replicate in tumor cells, resulting in lysis of different tumor types (Lorence et al.; 2003; Freeman et al.; 2006). In another study, a purified poultry vaccine dubbed MTH-68/H based on live attenuated NDV was administered i.v. to a total of 14 patients with high-grade glioblastoma multiforme (Csatary et al.; 2004). The PV701 strain and the other oncolytic NDV strains MTH-68/H and 73-T are considered mesogenic; the 73-T strain is perhaps the most potently oncolytic of the NDV strains studied to date, and re-emerged in experimental cancer targeting studies (Sinkovics et al.; 2000; Phuangsab et al.; 2001). In addition, several other strains, including italien and the non-oncolyticn lentogenic Ulster strain, have been used to target cancer cells both in vitro and in vivo with high specificity (Schirrmacher et al.; 2001). Based on results from the numerous animal experiments and clinical trials, NDV is an extremely safe oncolytic agent. Mumps virus (genus rubulavirus) was also among the first paramyxoviruses to be tested in humans. There are different strains used to treat several patients with different malignancies; Urabe strain was used as an oncolysate to treat a variety of cancers (Neagoe et al.; 1986). One of the characterized mumps viruses, the live attenuated vaccine strain S79, shows promise as an oncolytic veichle based on its selective infection of cancer cells in vitro and significant tumor inhibition in nude mice (Yan et al.; 2005). Another rubulavirus, simian virus 5, has been engineered for cancer targeting (Yan et al.; 2005).

MV enters within cells through interaction of its H protein cellular CD46 (membrane cofactor protein) and signalling lymphocyte-activating molecule (SLAM) (Tatsuo et al.; 2000; Dorig et al.; 1993). The wild type MV enters within cells more effectively through SLAM; however, the MV vaccine strains enter more effectively via the CD46 receptors (Yanagi et al.; 2003). MV vaccine affects only cells with high density of CD46, that overexpressed on tumor cells (Fishelson et al.; 2003) and does not affect normal cells (Anderson et al.; 2004). MV kills tumor cells by inducing cell to cell fusion through F protein, formation of syncithia and subsequent apoptotic cell death (Anderson et al.; 2004; Galanis et al.; 2001). MVs have been used in several preclinical studies in animal models, including both solid tumors and hematologic malignancies; treated animal tumors demonstrate cytopathic effect with syncithia formation followed by apoptotic cell death of MV-infected tumor cells (Zhang et al.; 2012). Peng et coll. (Peng et al.; 2002) demonstrated the feasibility of using a recombinant measles vector to preferentially infect and destroy humal ephitelial ovarian cancer cells in vivo via CD46. Moreover, a recombinant vector based on the Edmonston virus and engineered to express carcinoembryonic antigen caused marked regression of U87 glioma xenografts in nude mice (Phoung et al.; 2003); this vector entered a Phase I clinical trial to treat glioblastoma multiforme.

1.3.2.3 Orthomyxoviruses

Influenza viruses were used for experimental cancer therapy already in the 1950s (Wagner et al.; 1954) and later as oncolysates in clinical trials (Freedman et al.; 1988). Vaccination against influenza showed to provide

protection against certain types of cancer later in life (Kolmel et al.; 2005). The NS1 protein of influenza virus inhibits the activity ok PKR in target cells, which in turn permits viral replication. PKR is also inhibited by Ras in many tumor cell types, which thereby constitute preferential targets for infection by NS1-deleted influenza virus. Recently, Bergmann et coll. (Bergmann et al.; 2001) reported the utilization of a recombinant NS1 deletion mutant of influenza virus A strain PR8 that efficiently lysed Ras-expressing cells, both in vitro and in a subcutaneous tumor model in sever combined immundeficient mouse (SCID); as expected, normal cells were shown to be resistant, constituting an important safety aspect.

1.3.2.4 Coronaviruses

Coronaviruses are common respiratory pathogens of mammals and birds. Although human coronaviruses await conversion into cancer targeting vectors, non-human coronaviruses, including feline coronavirus and murine hepatitis coronavirus A59, display significant oncolytic activity in human cancer cells in vitro (Wurdinger et al.; 2005; Verheije et al.; 2006). Viral tropism is determined only by the expression of the proper viral receptors on the cell surface; once inside the cells non-human coronaviruses are capable to initiate a productive replication.

1.3.2.5 Picornaviruses

Several members of these RNA viruses are being developed into tools for virotherapy. Poliovirus infects a wide type of human cancer cell lines and primary explants (Ansard et al.; 2001). By attenuation of a neurovirulent poliovirus strain, Gromeier et coll. (Gromeier et al.; 2000) obtained a

highly efficacious recombinant virus, PV1 (PVS)-RIPO, which displayed significant tumor tropism and oncolytic potential in subcutaneous and i.c. human astrocytoma xenografts in nude mice. In another study, this virus caused complete tumor regression in a small number of athymic rats harboring i.c. human glioma xenografts upon intrathecal administration. In addition to the recombinant vectors, a live attenuated strain of poliovirus 1 showed promise as an oncolytic agent (Toyoda et al.; 2004). The RIPO vector has entered a phase I clinical trial to treat malignant glioma. Coxsackievirus was recognized as an anti-neoplastic agent in the 1950s and was tested for oncolytic efficacy in animal models (Suskind et al.;1957). To date, oncolytic vectors based on coxsackievirus A21 are available (Shafren et al.; 2004; Au et al.; 2005), and phase I studies in targeting melanoma with this virus have been completed (results not yet published) (Parato KA, et al.; 2005). Another oncolytic picornavirus tested in the 1950s, bovine enterovirus, has gained renewed attention as a potential oncolytic agent (Smyth et al.; 2002; Taylor et al.; 1971). This virus shows oncolytic potential towards a variety of human cancer cell types, including some of lymphoid origin. Moreover, echovirus type 1 recently showed to dispaly a strong tropism for human ovarian cancer cells (Shafren et al.; 2005). Finally, live attenuated Seneca Valley virus SVV-001 (genus unassigned) entered phase I studies to treat carcinomas of neuroendocrine origin.

1.3.2.6 Retroviruses

Several members of this virus family were extensively used in different gene therapy applications. The most frequently used retrovirus to target

cancer is the Moloney murine leukemia virus (MoMLV). Due to the lack of active nuclear transport of the viral genome, all gammaretroviruses, including MoMLV, are unable to transduce nondividing cells, which can be considered an important safety aspect. Complete transduction of human U87 glioma xenografts in nude mice was reported following a single i.c. administration of a replication- competent MoMLV vector (Tai et al.; 2005). Retroviruses have been used in clinical trials to treat cancer, but data from these studies suggest that the dissemination of the vectors in solid tumors needs to be improved to reach clinical efficacy (Ram et al.; 1997; Colombo et al.; 2005).

Some vectors based on lentiviruses were also used to target cancer in pre-clinical experiments. For example, using equal amounts of infectious virus, VSV-G pseudotyped lentiviral vectors were shown to transduce a wide variety of tumor cells almost 10 times as efficiently as vectors based on adenovirus serotype 5 (Pellinen et al.; 2004). Like their retroviral counterparts, lentiviruses are not oncolytic. However, they have the capacity to transduce both dividing and non-dividing cells, which may be advantageous under certain circumstances. In contrast to both gammaretroviruses and lentiviruses, the spumavirus foamy virus shows intrinsic oncolytic capacity. Some data reported that replication-competent vectors based on the prototype strain of this virus were able to control subcutaneous U87 tumors in nude mice for up to 25 weeks (Heinkelein et al.; 2005).

1.4 Combination therapies

OV infection generates an antitumor immune response, that represents an indirect mechanism of malignant cell death for both infected- and non-infected cells (Zamarine et al.; 2014). The immune response can represent an obstacle to the success of OV therapy; the tumor-infiltrating lymphocytes can suppress viral replication and finally eradicate the virus. Furthermore, pre-existing antibodies can also bind administered OV and clear it from the circulation, minimizing viral penetration. Combination strategies of OV with chemotherapeutic agents can potentially overcome those obstacles. Chemotherapy and/or radioteraphy represent the standard of care for many malignancies, but clinical data show that combining OVs with the traditional sistemic therapies can increase the response respect to either therapy alone. Preclinical and clinical studies have demonstrated significantly enhanced antitumor immune and clinical responses in patients receiving combination therapies (Khuri et al.; 2000; Galanis et al.; 2005; Karapangiotou et al.; 2012). Chemotherapy complements virotherapy through a variety of known and unknown mechanism, including the direct killing of tumor cells, enhancement of tumor cell immunogenicity and suppression of antiviral immune responses (Choi et al.; 2016). The first human clinical trial evaluated was ONYX-015 (d11520); it was a genetically modified adenovirus in combination with cisplatin and 5-fluouracil in 37 patients with recurrent squamous cell head and neck cancer. This clinical trial showed 65% response rate in patients receiving the treatment, compared to just 14% response rate for virus alone (Khuri et al.; 2000). The mechanism of this synergistic effect between OVs and chemotherapy is not completely understood; some chemotherapy agents

may upregulate cell surface receptors that viruses use to enter and infect tumor cells. For example, MAP/ERK kinase (MEK) inhibitors have been shown to upregulate chimeric antigen receptor (CAR) expression, which enables enhanced adenovirus entry (Zurakowski et al.; 2007). However, chemotherapy agents can also enhance OV function by affecting the immune response to infection (Nguyen et al.; 2014). Paclitaxel upregulates MHC class 1 molecules expression, leading to enhanced antigen presentation and immune system cross-priming (Kaneno et al.; 2011). Cyclophosphamide showed in preclinical animal models to improve reovirus access to the tumor and preserve neutralizing antibody levels sufficient for prevention of severe toxicity (Qiao et al.; 2008). Doxorubicin downregulates programmed death-ligand 1 (PD-L1), which plays a role in immune suppression (Ghebeh et al.; 2010). OV combination therapy with radiation also showed to improve antitumor response in preclinical models by enhancing apoptosis in the combination therapy (Rogulski et al.; 2000; Dai et al.; 2014). Gemcitabine appears to impact negatively late phases of reovirus replication; however, the next effect is synergistic as it accelerates antitumor immunity generation most likely by decreasing immunosuppressive cells within the tumor microenvironment (Gujiar et al.; 2008). In a Phase II study of Reolysin in combination with gemcitabine, combination treatment did not prevent viral entry in malignant cells and subsequent apoptosis (Mahalingam et al.; 2015). Antibody response to Reolysin also appears to be attenuated with this combination strategy (Lolkema et al.; 2011). Intratumoral HSV-1 (G207) in combination with radiotherapy was used successfully in clinical trials for patients with recurrent glioblastoma multiforme (GBM), with six out of

nine patients demonstrating stable disease or partial response (Markert et al.; 2014). Some OVs are being engineered with therapeutic genes that enhance local radioactive particle delivery, in particular radioactive iodine. Both Vaccinia virus (VAC) and Measles virus (MV) have OV strains developed carrying the human sodium iodide symporter (hNIS), which allows entry of radioactive iodine into virus-infected cells to produce further tumor destruction through local radiation exposure (Dingli et al.; 2004; Gholami et al.; 2011).

The most promising combination therapy with OVs is with T cell checkpoint inhibitors. Recent discoveries in cancer immunotherapies showed that induction of T cell response alone is not sufficient for sustained antitumor effect; they point out that suppression of T cell inhibitory mechanism by blockade of T cell checkpoint factors, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programed death 1 (PD-1), can be useful in light of the immunosuppressive nature of advanced tumors (Dias et al.; 2012). There are preclinical data for synergy between OVs and immune checkpoint inhibition. In melanoma xenografts, the combination of Reolysin and anti- PD1 antibody significantly prolonged mice survival compared to either agent alone (Rajani et al.; 2016). There was evidence of enhanced antitumor cytotoxic T cell and natural killer (NK) cell activity with the combination therapy. Suppression of antitumor immunity by regulatory T cells (Treg) in Reolysin alone treated mice was ameliorated by anti-PD1 therapy. In an immunotherapy-resistant lung adenocarcinoma animal model, treatment with oncolytic adenovirus plus anti-PD-1 antibody, significantly increased antitumor immune responses to multiple neoantigens and decreased tumor growth, suggesting reversal of

anti-PD-1 resistance with oncolytic virotherapy (Woller et al.; 2015). Recent clinical trials are investigating the efficacy of OV combinations with ipilimumab, a monoclonal antibody against CTLA-4, and with pembrolizumab, a monoclonal antibody against PD-1.

NDV combined with immune checkpoint inhibition in immunogenic and non-immunogenic tumor animal models led to increased antitumor immunity and efficacy compared to either agent alone (Zamarin et al.; 2014). Synergy of oncolytic VSV with anti-PD1 antibody therapy was also demonstrated in glioma models (Cockle et al.; 2016). Oncolytic measles virus and adenovirus vectors have been engineered to express monoclonal antibodies against CTLA-4 and PD-1 with promising results; they showed to have improved antitumor activity compared to control virus (Dias et al.; 2012; Engeland et al.; 2014).

Small molecule inhibitors can potentially improve OV penetration and activity. Combination strategies of OV with agents targeting the VEGF/VEGFR pathway are based on the potential selective targeting of the tumor neovasculature. VEGFR TKIs can also have off-target effects on antiviral defense mechanisms (Jha et al.; 2011; Jha et al.; 2013). Reolysin showed synergy with VEGFR TKIs *in vitro* and *in vivo* in NSCLC models, with decrease in tumor growth and increase in antitumor immunity (Liu et al.; 2015).

1.5 T-Vec: the first FDA-approved OV

Talimogene laherparepvec (T-VEC) is an oncolytic herpes simplex virus type 1 (HSV-1) that was recently approved for the treatment of inoperable

malignant melanoma. Administered intratumorally into one or more accessible (usually cutaneous) lesions every 2 weeks for up to 18 months, the virus produced durable systemic responses in 16% of treated patients (Andtbacka et al.; 2015) and has since shown extraordinarily promising activity in the same disease indication when combined with ipilumumab or pembrolizumab checkpoint antibody therapy (Long et al.; 2016; Puzanov et al.; 2016). Efforts are now underway to explore the potential of T-VEC therapy across a spectrum of different cancers.

T-VEC (or JS1/ICP34.5_/ICP47_/GM-CSF (granulocyte macrophage colony-stimulating factor), the virus that was to become known as T-VEC) was originally described in 2003 (Liu et al.; 2003). The thinking behind the design of this virus was that its lab-adapted, g-34.5-deleted predecessors had been over-attenuated. Thus, T-VEC was derived from a fresh pathogenic virus isolate obtained from the cold sore of a lab worker. Although it was initially attenuated by disrupting both copies of the g-34.5 gene, the attenuation was partially reversed by engineering US11, whose product also blocks the shutoff of host cell protein synthesis, to be expressed at an earlier stage in the virus infection cycle. In addition to these de-attenuating modifications, the virus was engineered to more effectively boost the antitumor immune response, by achieving with the deletion of the ICP47 gene, whose product suppresses antigen presentation by the infected cell, and by inserting two copies of the GM-CSF gene into the virus to activate and promote the differentiation of locally resident antigen-presenting cells (APCs) in the infected tumor. T-VEC was rapidly advanced to the clinic and shown to be active in malignant melanoma, shrinking injected tumors and sometimes leading to the regression of

distant metastatic lesions (Kaufman et al.; 2010). The phase 3 T-VEC registration trial was launched in May 2009, 2 years before FDA approvals were granted for the anti-CTLA4 antibody ipilumumab and the B-raf inhibitor vemurafenib. Thus, the control randomization arm was subcutaneous GM-CSF, which has very little antimelanoma activity. Between May 2009 and July 2011, 436 patients with unresectable (stage III or IV) melanoma were randomly assigned to intralesional T-VEC or subcutaneous GM-CSF administered every 2 weeks. The durable response rate (responses lasting at least 6 months) was 16.3% in the T-VEC arm and 2.1% in the GM-CSF arm, and T-VEC was associated with a longer overall survival of 23.3 months versus 18.9 months with GM-CSF.5 Based on these positive findings, a biologics license application was filed, and U.S. marketing approval was granted in October 2015, with European and Australian approvals granted shortly thereafter.

Capitolo 2

Herpesviridae family

2. Herpesviridae family

Herpesviruses are a large family of dsDNA viruses whose virions are encased within a lipid bilayer envelope. They have a broad host range, infecting almost all vertebrates and some invertebrates (bivalves). The family includes about 200 viruses isolated by different hosts, such as molluscs, fish, amphibians, reptiles, birds and mammals (Roizman & Knipe; 2001). The name Herpesvirus, from the Greek erpein, means "hiding" and refers to the the ability of these viruses to induce latent infections that, after the depletion of clinical phase following primary infection, reactivated occasionally, as a result of different stimuli like a decrease in the immune response cell-mediated. Latency is a strategy implemented by the herpesvirus with the purpose of surviving in nature; also in the presence of an effective immune response, the virus has the capacity to persist within the cells in the form of episomes or extra chromosomal plasmids and it is able to reactivate in particular conditions like immunosuppression, fever and/or stress.

The family is divided into three subfamilies: alphaherpesviruses, betaherpesviruses and gammaherpesviruses.

-Alphaherpesvirinae, includes viruses characterized from large host spectrum, rapid replicative cycle, rapid dissemination in cell cultures with formation of syncythia and citoplasmic inclusions, remarkable necrotizing activity and finally capacity to establish latent infections in sensory ganglia. This subfamily includes the Simplexvirus genus and the Varicellovirus genus, which carpine herpesvirus type 1 (CpHV-1) belongs.

-Betaherpesvirinae, which belong Cytomegalovirus, Muromegalovirus e Roseolovirus genus. They induce latent infections in endothelial, epithelial

and lymphoid cells; they are characterized from close host spectrum, slow replicative cycle and in vitro they grow only on fibroblastoid cells, with the formation of cytoplasmic inclusions and giant cells (cytomegaly).

-*Gammaherpesvirinae,* wich includes Lymphocryptovirus e Rhadinovirus. They have guest host spectrum limited to the family or order of belonging to the natural host; they replicate in lymphoblastoid cells and, as they have specific tropism to T and B lymphocytes, they induced latent infection in the lymphoid tissues. Herpesviruses are among the largest and most complex kown animal viruses. Mature virion has a size ranging from 150 to 200 nm. In viral particle, the viral genome is enclosed from the nucleocapside and associated to several nucleoproteins to form the nucleoid. The protein capside consists of cable capsomeres that determine an icosaedric structure of the diameter of about 100 nm; there is a layer of amorphous protein material named "tegument" outside the nucleocapside. Finally, in the portion more external, there is the envelope consisting of lipids, polyamides and various glycoproteins such as gH, gC, gE, gG, gI, gB and gD, which act as a site to adsorb the virus to the cells. The Herpesvirus genome consists of double linear DNA filament of about 150 Kbp, capable of coding for necessary proteins to viral replication. Replication of DNA occurs in the nucleus, where the genome becomes circular (Garber et al., 1993; McVoy & Adler; 1994) and there is the formation of concatenamers (Roizman & Knipe; 2001), cut into specific sites to form molecules homogeneous (Deiss & Frenkell; 1986). For the presence of inverted and repeated sequences in the genome, these viruses are classified into six classes, named with the alphabet letters ranging from A to F. In particular, class D includes Caprine Herpesvirus 1, Varicella-

Zoster Virus, Bovine Herpesvirus 1 and Equine Herpesvirus 1. The class D genome consists of covalently combined sequences, called L (long) and S (short), each of which consists of unique sequences (UL and US, respectively). The latter is lined by inverted repeats defined IR (internal repeat) and TR (terminal repeat). The two UL and US components can change orientation and form four populations of DNA molecules that differ only in the order of these sequences (isomers). The mechanism of action of replicative cycle has been studied in the Herpes Simplex Virus (HSV), under the subfamily Alphaherpesvirinae (White & Fenner; 1994). The infection begins with the adsorption of the virus to the host cell by the recognition of eparan sulfate, present in proteoglycans, by the viral glycoprotein C (gC). Proteins of the tegument release into the cytoplasm while the nucleocapside are transported along the cytoskeleton to the nuclear pores through which the DNA is released to reach the nucleus where it circulates. The expression of viral genes is finely regulated by a mechanism a waterfall; it is necessary the presence of three classes of mRNAs, synthesized by the cellular RNA polymerase II. Two tegument proteins, respectively VHS (Virion Host Shutoff) and α -TIF (a-gene Trans Inducer Factor), play an important role in the early stages of viral replication. The first is responsible for the cytoplasm disruption of polyribosomes resulting in blocking of cellular protein synthesis; the second activates the transcription of Immediate Early gene (IE) in α-mRNA. After the translation, five of the six proteins are transferred in the nucleus to regulate the expression of Early (E) or ß. The sixth protein blocks the presentation of antigenic peptides on the cell surface to escape from the response immune. Proteins ß are enzymes necessary to increase

the synthesis of nucleotides (thymidine kinase and ribonucleotide reductase) and also for the replication of the viral genome (DNA polymerase, primacy-helicase, topoisomerase). These events stimulate the expression of genes Late (L) or γ and thus the synthesis of L proteins, structural components of virion. Some are assembled to form the capside and tegument while others form an area on the nuclear membrane from which the capside will acquire the envelope. Mature virions are released for exocytosis.

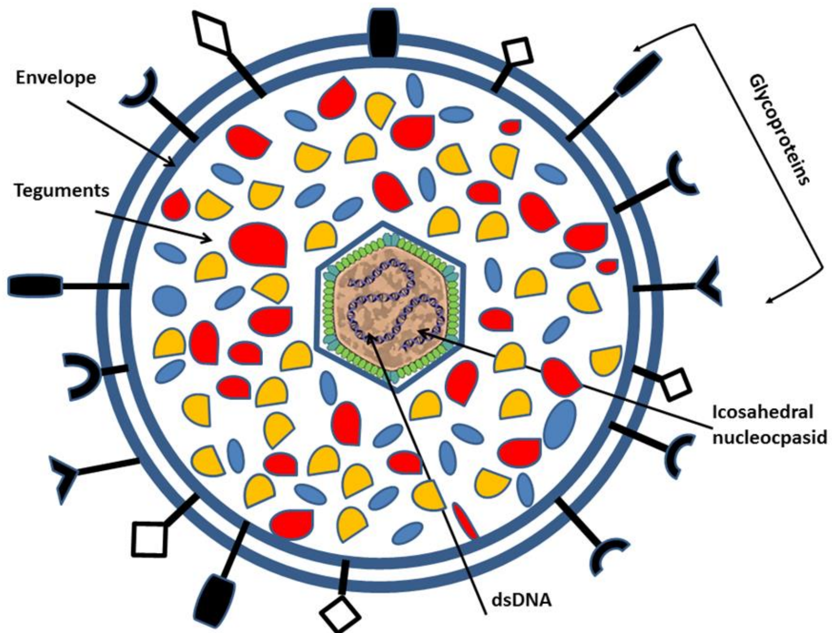


Figure 3. Graphic representation of herpetic virion. Mature virion has a size ranging from 150 to 200 nm; the protein capside of cable capsomeres that determine an icosaedric structure; there is an amorphous protein material layer named "tegument" outside the nucleocapside. (www.glycopedia.eu).

2.1 Bovine Herpesvirus 1 (BHV-1)

Bovine herpesvirus 1 (BoHV-1) is an Alphaherpesvirus, known to cause several diseases worldwide in cattle, including rhinotracheitis, vaginitis, balanoposthitis, abortion, conjunctivitis, and enteritis. It is spread horizontally through sexual contact, artificial insemination, and aerosol transmission and it may also be transmitted vertically across the placenta. BoHV-1 can cause both clinical and subclinical infections, depending on the virulence of the strain. Like other herpesviruses, BoHV-1 causes a lifelong latent infection and sporadic viral shedding. The sciatic nerve and trigeminal nerve are the sites of latency. BoHV-1 enters the animal through the mucous membrane in the respiratory tract or genital tracts. BoHV-1 has many mechanisms to evade the host immune systems involved in both innate immunity and adaptive immunity. The virus degrades interferon regulatory factor 3 (IRF3), effectively halting transcription of interferon type 1 (Muylkens et al.; 2007). BoHV-1 is also able to evade adaptive immune cells by inducing apoptosis in CD4+ cells, which assist in activating T cells when antigens are present (Muylkens et al.; 2007). This downregulates the number of immune cells that recognize the virus, allowing the virus to evade detection and elimination. After primary infection of BoHV-1, the latent infection is quite often found in the trigeminal ganglion of the cow, although on occasion infection can enter the central nervous system (Smits et al.; 2000). These latent infections can possibly reactivate, with or without clinical symptoms, under conditions of stress or by experimental methods (Smits et al.; 2000). Infected animals will be continuous shedders throughout their lifetime when the virus reactivates; therefore, successfully propagating the disease. Aside from

cattle, studies experimentally infecting animals showed that goats and buffalo can act as reservoirs for BoHV-1, as well as red deer, sheep, swine, and reindeer (Muylkens et al.; 2007). Shedding begins from the nasal mucosa as soon as infection occurs, and the virus has replicated in the upper respiratory tract. During replication in the respiratory tract ephitelial cells will undergo apoptosis (Nandi et al.; 2009). The necrosis will result in an entry site for secondary infections that may result in shipping fever. Clinical signs are represented from rinotracheitis, fever, serous to mucopurulent nasal discharge, coughing, sneezing, difficulty breathing, conjunctivitis and loss of appetite. Ulcers are common in the mouth and nose. Clinical symptoms include infectious pustular vulvovaginitis in cows and infectious balanoposthitis in bulls, fever, depression, loss of appetite, painful urination, a swollen vulva with pustules, ulcers, vesicles and erosions in cows, and pain on sexual contact in bulls. IBR can also cause abortion. Clinical signs and history are normally enough to make a preliminary diagnosis. To definitively diagnose the infection the virus should be identified in the tissues by virus isolation or PCR, or in bulk milk samples by ELISA. Vaccination is widely used both to protect cattle clinically in the case of infection and significantly reduce the shedding of the virus. Vaccination provides herd immunity, which lowers the likelihood of an animal coming into contact with an infected animal. Both inactivated and live attenuated vaccines are available. Immunity usually lasts approximately six months to one year. Marker vaccines are also available and recommended. Marker vaccines, also known as DIVA (differentiation of infected from vaccinated animals), have become popular to discriminate vaccinated animals from infected animals. A marker

vaccine uses either deletion mutants or a virion subunit, such as glycoprotein E (Bosch et al.; 1997).

2.1.1 BHV-1 Oncotropism

One of the main features of BHV-1 is the narrow spectrum of host compared to other herpesviruses, including HSV-1. Thus, BHV-1 is not capable of infecting human cells (Murata et al.; 1999), as confirmed by the absence of publications related to BHV-1 production or human seroconversion (Hammon et al.; 1963). This suggests a blockage of BHV-1 infection in human cells. Similarly, mice infected with wild type strains of BHV-1 show no clinical symptoms, while missing IFN-I and IFN-II receptors for RAG-2 gene deletion die a few days after infection (Abril et al.; 2004). As a result, IFN influences the host spectrum of the virus. From a study dating back to 1960, however, it emerges that BHV-1, determines a cytopathic effect in vitro on several transformed human cell lines, including HeLa, KB, HEp-2 lines and Had-1 (Hammon et al.; 1963). From a study to assess the susceptibility of different human tumor cell lines to BHV-1, it emerges that 72% of the cells are permissive to BHV-1 infection, resulting in a reduction in cell viability. Surprisingly, the reduction in viability occurred at a low multiplicity of infection (MOI) and was also detected in semi-permissive and non-permissive cellular cell lines (Cuddington et al.; 2014).

Rodrigues et coll. have demonstrated the ability of BHV-1 to replicate, to cause cytopathic effects and to compromise cell viability in normal, immortalized, and transformed cells of different histological origin (Rodrigues et al.; 2010). This capacity is strongly attenuated in normal

cells. However, it was demonstrated that BHV-1 infection of human cells fails to elicit IFN production at the mRNA or protein level and the ability of BHV-1 to kill immortalized cells does not correlate with defects in IFN pathways. Furthermore, although some cross reactivity between BHV-1 and HSV-1 exists, the majority of human antibody or serum tested samples failed to neutralize BHV-1 despite possessing HSV-1 neutralizing capacity. Thus, BHV-1 represent a novel candidate oncolytic virus with a distinct mechanism of tumor targeting.

2.2. Bovine Herpesvirus type 4 (BHV-4)

Bovine herpesvirus type 4 is a Gammaherspevirus, genus Rhadinovirus. Infection is normally sub-clinical but can cause reproductive disease in cattle such as endometritis, vulvovaginitis and also mastitis. Transmission is both vertical and horizontal. It can also be indirectly spread by fomites. Distribution is worldwide and the virus infects a range of ruminants, including bison, buffalo, sheep and goats. The disease may also be referred to as passenger virus and moyar virus. BHV-4 infection is often subclinical, with no observable clinical signs. However, the virus may cause abortion and retained foetal membranes, and if an infected fetus is born alive it may be weak. It can also cause mastitis in dairy cattle. It may be isolated from conjunctivitis and respiratory apparatus.

2.2.1 BHV-4 Oncotropism

BHV-4 is able to replicate in a broad range of host species both in vivo and in vitro (Egyed; 2000); it replicates and causes CPE in a large number of immortalized cell lines and primary cultures, but also in some cancer cell

lines (D’Onofrio et al.; 2002; Gillet et al.; 2004; Gillet et al.; 2005). Gillet et coll. have demonstrated that in vitro BoHV-4 infection induced apoptosis in A549 and OVCAR cell lines in a time- and dose-dependent manner. Furthermore, apoptosis was induced by the expression of an immediate-early or an early BoHV-4 gene, but did not require viral replication; in vivo experiments done with nude mice showed that BoHV-4 intratumoral injections reduced drastically the growth of preestablished A549 xenografts (Gillet et al.; 2005). Furthemore, Redaelli et al. have demonstrated that BHV-4 can infect mouse, rat and human glioma cell lines, primary cultures obtained from human glioblastoma in vitro and the ability of the virus to selectively infect gliomas induced in the rat brain in vivo (Redaelli et al.; 2010). These results suggest that BoHV-4 may have potential as a viro-oncoapoptotic agent for the oncolytic virotherapy and could be used to design new cancer therapeutic strategies.

2.3 Caprine Herpesvirus type 1 (CpHV-1)

CpHV-1 is an Alphaherpesvirus correlated with BoHV-1 which was isolated by 7-day-old kids with severe enteritis for the first time in 1974 in California (Saito et al.; 1974). A few years later, in 1979, it was found in a Swiss breeding farm (Mettler et al.;1979). The virus was identified not only in several European nations, including Italy (Roperto et al.; 2000), Greece (Koptopoulos et al.; 1988), Spain (Keuser et al.; 2004), Germany (Muluneh et al.; 1990) and Switzerland (Plebani et al.; 1983), but also in Australia (Grewal et al.; 1986; Piper et al.; 2008), Canada (Chenier et al.; 2004), California (Uzal et al.; 2004), New Zealand (Horner et al.; 1982). The prevalence of the disease is variable: in some countries, such as

Greece or in southern Italy, where goats represent an important economic source, is elevated (Koptopoulos et al.; 1988; Tempesta et al.; 1994; Guercio et al.; 1998). While, so far, no related data were reported on the identification of infection in Belgium and Great Britain. The pathogenesis of CpHV-1 infection in goats and BoHV-1 in the cattle is very similar; it is localized to respiratory or genital tract and subsequently the viremia associated to the mononuclear cells occurs, which results in systemic infection and abortion. The primary source of infection is represented by animals with acute infection or latent ones that can eliminate the virus through nasal, ocular and genital drain. The genital apparatus represents the entrance site of the virus and it is responsible for the persistence of infection in breeding (Tempesta et al.; 2000). CpHV-1 is responsible for two different forms of disease in the goat; it dipends from the age of animals. In adults, the infection may remain unapparent or appears with symptoms wich affect genital and respiratory apparatus, such as vulvovaginitis/ulcerative balanopostitis, dyspnoea, proliferative lesions on lips and hard palate (Horner et al.; 1982; Rosadio et al.; 1984; Williams et al.; 1997; Tempesta et al.; 1998). Abortion can also be manifested during the second half of the pregnancy, reproducible by experimental infection, through intranasal and intravenous inoculation of the virus (Tempesta et al.; 2004; Uzal et al.; 2004). In the kids of two weeks of life, however, CpHV-1 induces systemic disease characterized by high morbidity and mortality, rapid beginning and progressive slimming which often leads to death (Saito et al.; 1974; Mettler et al.; 1979; Van der Lugt & Randles; 1993; Buonavoglia et al.; 1996). From the anatomy-pathological point of view, CpHV-1 causes erosive, ulcerative and necrotic lesions on the

gastro-intestinal mucosa, especially in the large intestine; however, it is also responsible for pulmonary edema, hemorrhagic lesions on the bladder mucosa and hepatic necrosis. The istopathological examination underlines the loss of epithelial cells of the intestinal mucosa, while the lamina propria is infiltrated by mononuclear cells, especially macrophages containing intracellular eosinophilic inclusions; however, there is necrosis of lymphoid tissues (Roperto et al.; 2000). Like other Alphaherpesviruses, CpHV-1 is capable to interfere with the host immune responses (Hutchings et al.; 1990; Tempesta et al.; 1999; Hinkley et al.; 2000; Barcy & Corey; 2001; Pagnini et al.; 2005), and to establish latent infections susceptible to reactivation (Engels et al.; 1983). After infection and viral replication in the genital and respiratory apparatus, CpHV-1 spreads both in the extracellular spaces and from cell to cell. Through these mechanisms, it has the capacity to penetrate into the axons of the nervous cells and thus to reach the bodies of sensory neurons, in the regional ganglia. Generally, reactivation of the infection is observed during the reproductive season, while in experimental conditions it is difficult to obtain it (Buonavoglia et al.; 1996; Tempesta et al.; 2002); after the reactivation, the pathogenesis of the infection is similar to the primary infection, or rather the animals infected through nasal way eliminate the virus by both ways (nasal and vaginal), while vaginal infected ones eliminate the virus only by this way (Tempesta et al.; 2000). Viral excretion, after reactivation, is associated with relatively low antibody titres (Tempesta et al.; 1998) and it occurs through nasal, genital, rectal and ocular secretions; this evidence suggests the presence of several potential latency sites, such as sacral ganglia, where the presence of viral DNA was detected by PCR (Tempesta et al.;

1999; Tempesta et al.; 2002). The diagnosis of suspected infection can be done with clinical signs, wich are characteristic but not pathognomonic; the confirmed diagnosis can be done through direct or indirect methods. The direct method is the virus isolation on cell culture from nasal and genital secretions; it is evaluated the citophatic effect virus induced on a permissive cell line (Berrios et al.; 1975; Engels et al.; 1983). The indirect methods include serological techniques like serum neutralization and ELISA tests (Plebani et al.; 1983), although there could be cross-reactions caused by Alphaherpesvirus correlated (Nixon et al.; 1988; Lyaku et al.; 1992; Lyaku et al.; 1996). In order to avoid the cross-reactions, it is possible to resort to the molecular biology which allows the amplification, in positive samples, of a 414 bp fragment (Tempesta et al.; 1998), and real-time PCR, a quantitative analysis for the detection and quantification of viral DNA in goat (Elia et al.; 2008). Furthermore, another technique which allows the identification of isolated viruses and the discrimination from related microorganisms is Restriction Endonuclease Analysis (REA) (Rimstad et al.; 1992; Williams et al.; 1997; Pratelli et al.; 2000). At the current state of knowledge, prophylaxis is exclusively healthcare. There are no immunizing prescriptions, although several experimental studies demonstrate the possibility of protecting goats with inactivated vaccines (Tempesta et al.; 2001) and live attenuated etherologists (Thiry et al.; 2006).

Capitolo 3

Mechanisms of cell death

3. Mechanisms of cell death

3.1 Apoptosis (PCD I)

Apoptosis is a basic biological phenomenon with wide-ranging implications in tissue kinetics (Nagata; 2000). The term programmed cell death was introduced in 1964, proposing that cell death during development is not of accidential nature but follows a sequence of controlled steps leading to locally and temporally defined self-destruction (Lockshin et al.; 1964). Eventually, the term apoptosis was coined to describe the morphological processes leading to controlled cellular self-destruction and was first introduced in a publication by Kerr, Wyllie and Currie (Kerr et al.; 1972). The term Apoptosis is of greek origin, having the meaning "falling off or dropping off", in analogy to leaves falling off trees or petals dropping off flowers. Described in all multicellular eukaryotes (Ellis et al., 1991), apoptosis is an active biological process that is implicated in the early development such as during metamorphosis in insects and amphibians, organogenesis in virtually all multicellular organisms, and has a key role in morphogenesis (Vaux et al.; 1988), in sexual differentiation and epigenetic processes of self-organization of the immune and nervous system (Ameisen; 2002). Apoptosis also exerts a role opposite to mitosis in the maintenance of cell populations. Infact, balancing the life/death ratio of damaged cells in the adult, apoptosis allows tissue homeostasis (Orrenius et al.; 2003). As examples of this cellular altruism, apoptosis plays an active part in the removal of interdigital webs in fingers and toes and in the formation of T and B cell of the immune system by eliminating nonreactive or self-reactive cells. Apoptosis is also a protective mechanism, by directing lysis of virus-

infected cells, foreign cells or incipient neoplasm. It also plays a central role in the immune system. Under physiological conditions, immature lymphocytes that bind to autoantigens are eliminated by apoptosis. This is thought to protect against immune recognition of ‘self’. Defects in the deletion of these lymphocytes predispose to autoimmunity.

Apoptosis is therefore a regulated process, to which the cell participates actively, with a series of events morphologically and biochemically repeatable and well-defined (Figure 4). This suicidal pathway is characterized by stereotypical morphological changes: the cell shrinks, shows deformation and looses contact to its neighbouring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response. Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Saraste et al.; 2000). The oligosomal DNA fragments result in a distinct laddering pattern on an ethidium bromide-stained agarose gel that represents a hallmark of apoptosis (Wyllie et al.; 1980). As a result, cells shrink and condense into multiple small membrane-bound named ‘apoptotic bodies’, which display a particular

propensity as targets for phagocytes. These removes apoptotic cells without leaking the cytoplasmic contents into the intercellular space, minimizing tissue inflammation, avoiding damage to neighbouring cells, and efficiently degrading host (or viral) DNA. Apoptosis is in contrast to the necrotic mode of cell-death in which case the cells suffer a major insult, resulting in a loss of membrane integrity, swelling and disrupture of cells.

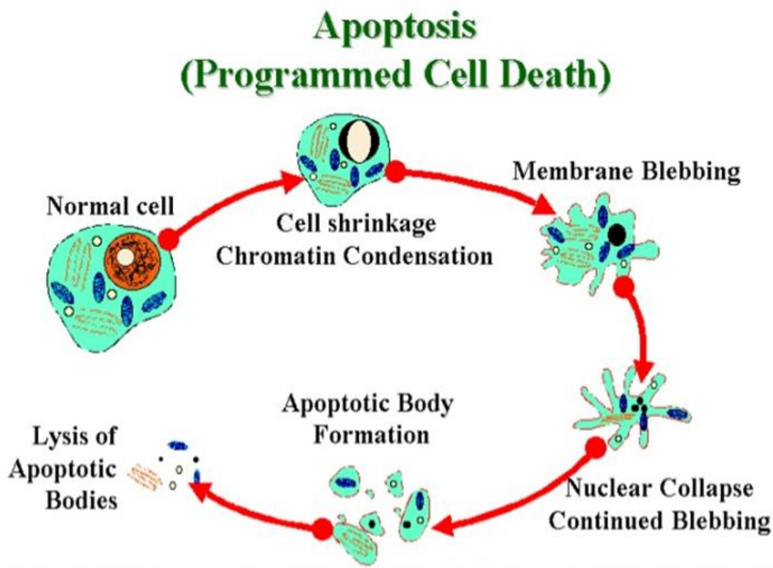


Figure 4. Apoptosis morphological changes. (1) Cell shrinks and chromatin condenses. (2) Plasma membrane bebbling. (3) Nucleus fragmentation. (4) Apoptotic bodies formation which contain cytosol, the condensed chromatin, and organelles. (5) The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue.

The components of the apoptotic signalling network are genetically encoded and ready to be activated by a death-inducing stimulus (Ishizaki et al.;1995; Weil et al.; 1996). Apoptosis can be triggered by various stimuli from outside or inside the cell, e.g. by ligation of cell surface receptors, by DNA damage as a cause of defects in DNA repair mechanisms, treatment with cytotoxic drugs or irradiation, by a lack of survival signals, contradictory cell cycle signalling or by developmental death signals. Death signals of such different origin nevertheless appear to eventually activate a common cell death machinery leading to the characteristic features of apoptotic cell death. Numerous environmental factors can activate cell suicide, and other factors can specifically antagonize apoptosis. Activators of apoptosis include tumour necrosis factor α (TNF α), Fas ligand (FasL), transforming growth factor ß (TGF ß), Bax (and other proapoptotic Bcl-2 family members), and glucocorticoids. In addition, aberrant oncogene expression (e.g. c-myc), or normal tumour suppressor gene function (such as p53) may trigger apoptosis under specific conditions. In many cases, simultaneous conflicting signals for growth stimulation and suppression trigger apoptosis. Most growth factors exert explicitly antiapoptotic signalling on their target cells. Cytokines regulate survival through their receptors, which trigger a cascade of intracellular signalling. Among the intracellular (noncytokine) factors which showed to suppress apoptosis, there are CD40 ligand, viral genes such as E1B from adenovirus, baculovirus p35, and antiapoptotic genes in the Bcl-2 family. Several DNAviruses have been demonstrated to encode factors which function to curtail the cellular apoptotic response (presumably a prerequisite for successful viral infection/ propagation). A

few apoptotic modulators, including FasL and TNF, induce apoptosis which is largely confined to the development and regulation of the immune system.

The apoptotic process can be divided in three phases:

-Induction: includes the sending of signals that will trigger apoptosis and primary transduction pathways triggered by them. The pro-apoptotic signals could be distincted in intrinsic and extrinsic, depending on whether they come from the outside environment or within the cell. The intrinsic signals interact with transmembrane receptors belonging to the superfamily of Tumor Necrosis Factor (TNF) receptors (Locksley et al.; 2001); the extrinsic signals instead, cause modifications of the mitochondria, such as the decrease in membrane potential and excessive production of radical species, with consequent opening of pores in the mitochondrial membrane (megachannel or mitochondral pores) and release in the cytoplasm of factors normally sequestered in the intermembrane space such as cytochrome C (cytC) or AIF (Apoptosis Inducing Factor) (Saelens et al.; 2004).

-Execution: during this phase, both extrinsic and intrinsic pathways converge in the activation of a class of proteases called caspase (cysteine dependent aspartate specific protease), characterized by the presence of a cysteine residue in its catalytic site and specificity for substrates containing an aspartate residue. The caspases are of central importance in the apoptotic signalling network which are activated in most cases of apoptotic cell death (Bratton et al.; 2000). So far, 7 different caspases have been identified in Drosophila, and 14 different members of the caspase-family were described in mammals, with caspase-11 and caspase–12 only

identified in the mouse (Denault et al.; 2002; Richardson et al.; 2002). Actually, strictly defined, cell death only can be classified to follow a classical apoptotic mode if execution of cell death is dependent on caspase activity (Leist et al.; 2001). In the cell, caspases are synthesized as inactive zymogens, the so-called procaspases; these proteins carry a prodomain at their N-terminus followed by a large and a small subunit, which sometimes are separated by a linker peptide. Upon maturation, the procaspases are proteolytically processed between the large and small subunit, resulting in a small and a large subunit. The proapoptotic caspases can be divided into the group of initiator caspases including procaspases-2, -8, -9 and –10, and into the group of executioner caspases including procaspases-3, -6, and –7. Whereas the executioner caspases possess only short prodomains, the initiator caspases possess long prodomains, containing death effector domains (DED) in the case of procaspases-8 and –10 or caspase recruitment domains (CARD) as in the case of procaspase-9 and procaspase-2. Via their prodomains, the initiator caspases are recruited and activated by death inducing signalling complexes either in response to the ligation of cell surface death receptors (extrinsic apoptosis pathways) or in response to inner signals originating by the cell (intrinsic apoptosis pathways). Furthermore, they are responsible for the activation of effective caspases, which guarantee the continuity of the process in its main catalytic events (Figure 5). In extrinsic apoptosis pathways, procaspase-8 is recruited by its DEDs to the death inducing signalling complex (DISC), a membrane receptor complex formed following to the ligation of a member of the tumor necrosis factor receptor (TNFR) family (Sartorius et al.; 2001). When bound to the DISC, several procaspase-8 molecules are

near to each other and therefore are assumed to activate each other by autoproteolysis (Denault et al.; 2002). Intrinsic apoptosis pathways involve procaspase-9 which is activated downstream of mitochondrial proapoptotic events; the release of cytochrome c from mitochondria induced the formation of the so called apotosome, a cytosolic death signalling protein (Salvesen et al.; 2002b). In this case there is the dimerization of procaspase-9 molecules at the Apoptotic-proteaseactivating-factor-1 (Apaf-1) scaffold that is responsible for caspase-9 activation (Denault et al.; 2002). Once the initiator caspases have been activated, they can proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed.

-Phagocytosis: the membrane of the apoptotic bodies exposes molecules such as phosphatidyl-serine, normally located in the cytosolic layer, on the surface (Bratton et al.; 2000), or calreticulin (Gardai et al.; 2005), which are recognized by specific receptors present on macrophages cell surface, marking the beginning of phagocytosis.

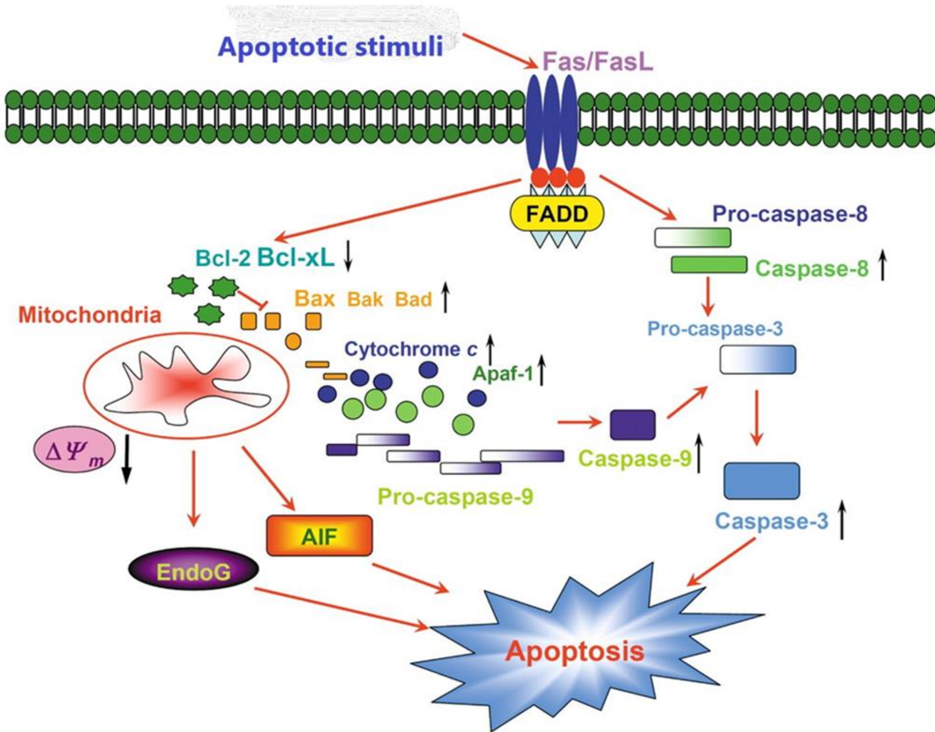


Figure 5. Schematic representation of apoptotic activation. The pro-apoptotic signals could be distincted in intrinsic and extrinsic; both extrinsic and intrinsic pathways converge in the activation of caspases.

3.1.1 Regulatory mechanisms in apoptosis signalling

Commonly, the activation of apoptosis is regarded to occur when a cell encounters a specific death-inducing signal such as the ligation of a death receptor by its analogous ligand or if cells are treated with a cytotoxic drug. This suggests that the apoptosis signalling pathways in viable cells occur in response to a death stimulus. At the same time, cell death is continously repressed by survival signals such as provided by other cells of the organism, e.g. growth factors, hormones, nutrients, adhesion molecules. Those survival signals enhance the expression and/or activity of antiapoptotic regulatory molecules thereby keeping in check the activation of proapoptotic factors (Raff et al.;1993; Ameisen; 2002).

The main protagonists of this modulation are proteins belonging respectively to the Bcl-2 and p53 families respectively.

-Bcl 2 family: 22 members of Bcl-2 family (B-cell lymphoma gene 2) have been identified. They were classified on the basis of preserved helix sequences, known as homology domains Bcl-2 (BH, Bcl-2 Homology domains) (Scorrano & Korsmeyer; 2003) (Figure 6). In addition, there are a number of other prosurvival proteins, e.g. Bcl-XL, Bcl-w, A1, and Mcl-1, which all possess the domains BH1, BH2, BH3, and BH4. The proapoptotic group of Bcl-2 members can be devided into two subgroups: the Bax-subfamily consists of Bax, Bak, and Bok that all possess the domains BH1, BH2, and BH3, whereas the BH3-only proteins (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, Puma, Blk, BNIP3, and Spike) have only the short BH3 motif, an interaction domain that is both necessary and sufficient for their killing action (Cory et al.; 2002; Mund et al.; 2003). The pro-apoptotic elements block the action of anti-apoptotic proteins (Bcl-2,

Bcl-XL) or activate the pro-apoptotic proteins Bax and Bak (Ward et al.; 2004). The proteins of this family have a location dependent from the role played. The anti-apoptotic elements Bcl-2 and Bcl-XL are located above the membranes of the organelles (endoplasmic reticulum, nucleus and outer membrane of the mitochondria). Bax, instead, is mainly located in the cytoplasm, anchored to the cytoskeleton structures (Desagher & Martinou; 2000). Apoptotic stimuli, discovering the C-terminal hydrophobic residue, determine the dimerization of Bax that can, therefore, interfere with the lipids of the Bax external mitochondrial membrane (Fig. 7). It follows the fall of the mitochondrial potential (□□m) and the release of not only molecules like Smac/ Diablo, which inactivated caspase inhibitors, but also of calcium ions, free radicals and glutathionees, responsible of the amplification of the signal. On the other hand, Bax and Bak can act on the endoplasmic reticulum by controlling calcium homeostasis (Scorrano L & Korsmeyer SJ; 2003). Finally, it must be remembered that mitochondria are involved by apoptosis even when the signal starts from the receptors of membrane, since caspase initiator 8 determines the cleavage of Bid, a pro-apoptotic modulator, which is located on this organelle (Figure 7). This is an example of "crosstalk" among the receptor and mitochondrial pathways (Elmore et al.; 2007).

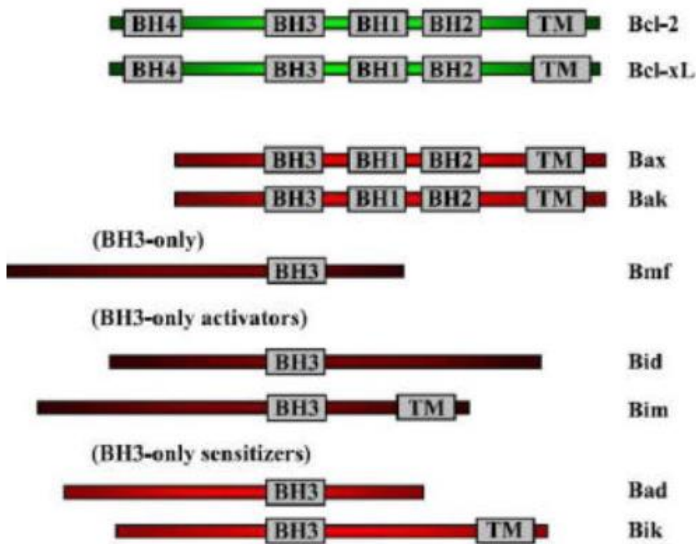


Figure 6. Structure of the members of the Bcl-2 family members. TM: transmembrane domain (Martin & Vuori, 2004).

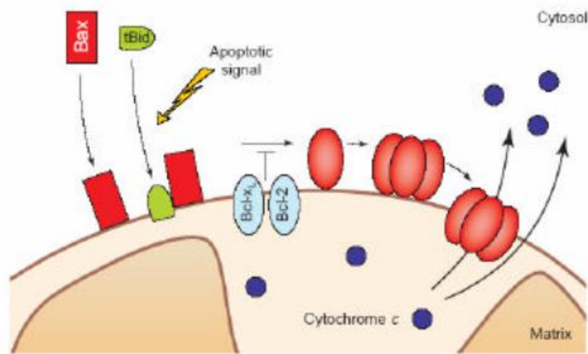


Figure 7. Mechanism of action of pro-apoptotic proteins belonging to the Bcl-2 family (Desagher & Martinou, 2000).

-P53: the coding gene for p53 protein is an oncosuppressor, the whose main role is to prevent inappropriate proliferation of cells in response to DNA damage. Activated p53 can cause arrest, depending on the circumstances, of the cell cycle in G1 or apoptosis.
The induction of programmed cell death is dependent on or less from transcriptional activity (Moll & Zaika; 2001) (Fig. 9). In the first case, p53 activates transcription of death receptors (DR5, CD95, Fas), proapoptotic proteins of the Bcl-2 family (Bax, PUMA, NOXA, Bid), as well as apoptotic adapters such as Apaf-1. Furthermore, p53 represses the transcription of anti-apoptotic proteins such as Bcl-2 and Bcl-XL (Sionov & Haupt; 1999). If the induction is independent from the transcription, p53 could act as a sensitizer of apoptosis, like Bad and Bik, linking directly to Bcl-2 and Bcl-XL and then activating only indirectly Bak and Bax (Mihara et al.; 2003).

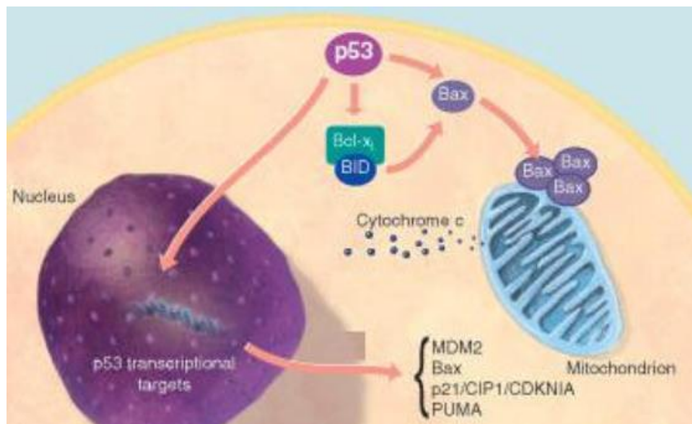


Figure 8. Apoptosis induction by p53.

3.1.2 Viral infection and apoptosis

Apoptosis plays an important role in cellular response to different insults, including viral infections. The virus-induced apoptosis, associated with a significant decrease in the number of viral progeny production, was first described in cells infected with a mutant adenovirus (Pidler et al.; 1984). Subsequently, several studies support the theory according to which programmed cellular death represents an innate defense mechanism against infections; through premature cell lysis the host blocks viral replications (Clem et al.; 1991; Koyama et al.; 1998). There are many interactions between virus and apoptosis; the virus-induced apoptosis is considered a host mechanism of defense since it allows phagocytosis of infected cells reduced to apoptotic bodies, restricting inflammatory response and stimulating the specific immune responses (Vaux et al.; 1994). However, the relationship between viruses and host cells is a classic conflict of interest, where the cell tries to minimize the damage of the infection while the virus tries to get the most benefit from the cell replication apparatus (Krakauer & Payne; 1997). Viruses, in fact, can influence the apoptotic process triggering the signal transduction until programmed cellular death (for example TNF receptor) or, sometimes, they have genes or proteins that act the action of apoptotic regulatory molecules. Sometimes apoptosis represents a favourable mechanism for virus since programmed cell death promotes, after the assembly, the spread of virions within apoptotic bodies or associated to them (Mi et al.; 2001). The ability to inhibit apoptosis is, however, necessary to prevent premature death of host cells, to facilitate spread of viral progeny in lytic infections and to allow persistent infection (Shen & Shenk; 1995). Several studies have investigated the cell

sensitivity to the first phases of infections (adsorbition and virus entry) and have observed the proapoptotic nature of some virion structural proteins.

Evaluating Herpesvirus, it was showed that modulation of apoptosis through virus-encoded proteins is the key to the pathogenesis of infection (Longo et al.; 2009; Pagnini et al.; 2005; Wang et al.; 1997). The induction of programmed cell death by HSV represents an early event related to the expression of an IE gene or of a structural protein introduced into the infected cell (Leopardi & Roizman; 1996). If protein synthesis is inhibited, apoptosis does not occur. However, apoptosis is "suppressed" by the action of some anti-apoptotic viral genes (Koyama et al.; 1998) that interfere specifically with a protein kinase, PKR (dsRNA-dependent serine / threonine protein kinase) which normally does not only act as a mediator of the interferon antiviral functions, but it also induces apoptosis through the activation of the death domain associated with FAS (FADD) (Ubol et al.; 1994). It was demonstrated the bond between virus and death receptors belonging to the family Fas/TNF (Ashkenazi & Dixit; 1998; Schwarz; 1996). BHV-1 induced apoptosis through a structural constituent of virion which acts as a ligand for death receptors, since apoptosis was observed in cells exposed to inactivated virus. Furthermore, programmed cell death induced by BHV-1 can be considered a late event, since it occurs during the last stages of infection (Devireddy & Jones; 1999).

Often apoptosis is the basis of the pathogenetic mechanisms of different infections and also herpetic infections (Shen & Shenk; 1995; Teodoro & Branton; 1997). It was demonstrated the capacity of CpHV-1 of inducing apoptosis in mononuclear cells of sheep blood (Pagnini et al.; 2005); it was also demonstrated the proapoptotic potential of CpHV-1 in permissive

cells of the MDBK line (Longo et al.; 2009) and in neuroblastoma cell line (Neuro 2A) (Montagnaro et al.; 2013).

3.2. Autophagy (PCD II)

Autophagy is a lysosomal degradative mechanism occurring in different modes (chaperone-mediated autophagy, microautophagy, and macroautophagy) (Cuervo; 2004) (Figure 9). The discovery of autophagy by De Duve and Wattiaux (De Duve et al.; 1966) was contemporary with that of lysosomes. The physiological importance of autophagy in maintaining cell homeostasis in organs such as liver and in cultured cells rapidly emerged (Mortimore et al.; 1977; Mortimore et al.; 1994). At the same time, the term autophagic cell death or type II programmed cell death (PCD II) was introduced to describe a cell death different from apoptosis or type I PCD (PCD I) (Schweichel et al.; 1973; Clarke et al.; 1990).

Autophagy is a general and evolutionarily conserved vacuolar catabolic pathway terminating in the lysosomal compartment (Seglen et al.; 1992; Klionsky et al.; 2000). It contributes to the quality control of cytoplasmic components by recycling macromolecules (autophagy is responsible for the turnover of long-lived proteins) and removing organelles when damaged or in excess (peroxisomes, mitochondria). It enables cells to survive stress from the external environment, such as nutrient deprivation, as well as internal stresses like accumulation of damaged organelles and pathogen invasion. Autophagy is induced by starvation in all eukaryotic systems examined, including several species of fungi, plants, slime mold, nematodes, fruit flies, mice, rats and humans (Levine et al.; 2004). It aids in maintenance of homeostasis in cellular differentiation, tissue

remodeling, growth control (Tanida et al.; 2004; Meijer et al.; 2004). Several types of autophagy exist, which differ mainly in the site of cargo sequestration and in the type of cargo. These include micro- and macroautophagy, chaperone-mediated autophagy, micro- and macropexophagy, piecemeal microautophagy of the nucleus, and the cytoplasm-to-vacuole targeting (Cvt) pathway (Klionsky; 2004) (Figure 9). This process involves the sequestration of bulk cytoplasm within a cytosolic double-membrane vesicle termed the autophagosome, which ultimately fuses with the lysosome (or the vacuole in yeast). The delimiting membrane of the autophagosome is derived from an ‘isolation’ membrane or phagophore of unknown origin (Fengsrud et al.; 1995). Fusion results in the release of the inner vesicle, now termed an autophagic body, into the lysosome lumen. Within the lysosome the engulfed material is denatured because of the acidic environment.and the products are recycled. Microautophagy is the way used to remove cellular organelles no more useful for the turnover of basal epithelial cells.

Macroautophagy is a homeostatic way, divided into several steps, of extensive catabolism against older proteins and cellular organelles that are identified by the lysosomes and then, destroyed (Levine et al.; 2005; Levine et al.; 2008). Autophagy si mediated by chaperone proteins (CMA), a group of polypeptides (the main being hsc 70) that binds the soluble cytoplasmic proteins and makes them recognizable to the lysosomes and thus eliminates them. This is the only destructive way in mammals against cytoplasmic proteins. Autophagy has been implicated in several human diseases and conditions, including cancer, neurodegenerative disorders, certain myopathies, aging and defense against pathogens. Identification of

its molecular machinery and signaling pathways has shed some light on the importance of autophagy in physiological processes and diseases (Klionsky et al.; 2000; Cuervo et al.; 2004; Levine et al.; 2004).

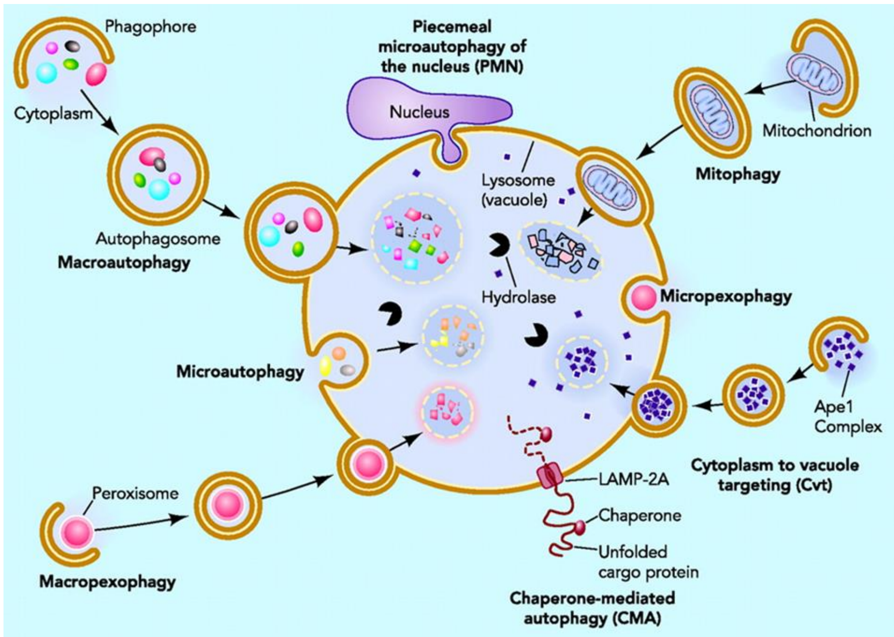


Figure 9. Schematic representation of autophagy.

3.2.1 Morphology and mechanistic aspects of Autophagy

At least 30 genes (ATGs) were identified in yeast that function in the regulation and execution of autophagy (Xie et al., 2007). Some of these genes are known homologous in mammalian cells. Classical autophagy can be subdivided into four main phases that are performed by specific ATG genes (Levine et al.; 2008; Xie et al.; 2007). Among the autophagy-related (ATG) genes, discovered in yeast and almost integrally conserved in all eukaryotic phyla, which control the formation of the autophagosome (Klionsky et al.; 2003), beclin 1 (the mammalian ortholog of the yeast ATG6) is a tumor suppressor gene that contributes with the class III phosphatidylinositol-3- kinase (PI3K) to the formation of the autophagosome (Liang et al.; 1999; Kihara et al.; 2001). Other tumor suppressor gene products, such as p53, PTEN, TSC1/TSC2, death-associated protein kinase (DAP kinase), are involved in the control of autophagy (Botti et al.; 2006). Interestingly, autophagy is also stimulated in cancer cells by ceramide (Scarlatti et al.; 2004; Daido et al.; 2004), a tumor suppressor lipid (Hannun et al.; 1993).

Although autophagy is a dynamic process, the pathway was delineated into several static steps for the convenience of description: induction and nucleation, phagophore expansion, autophagosome targeting, docking and fusion, and cargo degradation and recycling.

Many signaling pathways and proteins seem to be involved in this first phase, but the esact mechanism of action and/or interaction is unknown. Autophagy may be induced as a response to a change in the extracellular environment of a cell, and the target of rapamycin complex 1 (TORC1) is one of the signaling pathways that plays a primary role in sensing the shift

in nutrient availability. Nutrient starvation, in particular nitrogen and/or amino acid limitation, initiates an intracellular signalling cascade by discontinuing TORC1 stimulation, resulting in the activation of the Atg1 kinase complex. The Atg1 kinase complex works directly downstream of the TORC1 pathway and it consists of Atg1, Atg13, and a scaffold subcomplex that includes Atg17- Atg31-Atg29 (Kamada et al.; 2000). Assembly of this complex is crucial for autophagy because it plays a role in recruiting other Atg proteins to the phagophore assembly site (PAS) and activating downstream targets through phosphorylation (Suzuki et al.; 2007; Papinski et al.; 2014). Protein kinase A (PKA) is another negative regulator of the Atg1 kinase complex, in this case primarily in response to carbon source, whereas the energy sensor Snf1/AMP-activated protein kinase (AMPK) acts as a positive regulator. Although the associations among these proteins have been demonstrated as biomolecular interactions, it is unknown whether all of these proteins are ever present in a single complex. In autophagy, nucleation refers to the process of mobilizing a small group of molecules to the PAS; the phagophore is the active sequestering compartment of autophagy. The class III PtdIns3K complex I, which is employed specifically for autophagy, is one of the key complexes that are recruited to the PAS upon induction of autophagy. This complex is constituted of five distinct proteins: the lipid kinase Vps34, the regulatory kinase Vps15, Vps30/Atg6, Atg14 and Atg38, which are all necessary for autophagy (Schu et al.; 1993; Kihara et al.; 2001). In brief, the class III PtdIns3K is responsible for the production of phosphatidylinositol-3-phosphate (PtdIns3P) directly from phosphatidylinositol (Burman et al.; 2010). This PtdIns3P is important for

the correct localization of some of the Atg proteins including Atg18 and Atg2, which enables the recruitment of Atg8, Atg9 and Atg12 to the PAS (Obara et al.; 2008). Other proteins and molecules that play a role in regulating this first autophagic phase are p53, c-jun-N-terminal kinase1 (JNK1), eucariotic initiation factor 2α (elF2α), GTPase and intracellular calcium (Levine et al 2008; Talloczy et al.; 2009;). All these molecules and/or signaling pathways appear to promote autophagy by activating class III PtdIns3K Cps3, which promotes the formation of phosphatidylnositous 3-phosphate (PIP3) on lipids. Autophagosomes, which correspond to the mature form of the phagophore, is essentially a terminal compartment that does little more than fuse with the vacuole; formation of the phagophore and sequestration by the phagophore are the truly dynamic steps of autophagy (Baba et al.; 1994). There are two essential ubiquitin-like (Ubl) conjugation systems that are necessary for phagophore expansion and these involve the Ubl proteins Atg12 and Atg8 (Ohsumi; 2001); these two proteins have structural similarity to ubiquitin, but are not actual homologs. Atg12 is conjugated to Atg5 via the action of the E1 and E2 enzymes Atg7 and Atg10, and this conjugate binds Atg16 to form the dimeric Atg12–Atg5–Atg16 complex; Atg8 undergoes a different type of conjugation, being covalently attached to the lipid phosphatidyl-ethanolamine (PE). The generation of Atg8–PE involves the protease Atg4, Atg7 as an E1 enzyme and Atg3 as an E2 enzyme, with the Atg12–Atg5–Atg16 complex participating as an E3 enzyme, although the latter is not absolutely required for conjugation to occur (Cao et al.; 2008). A detailed mechanism in which these conjugation systems operate along with other complexes to enlarge the phagophore is currently an on-going

research topic. Atg9 functions in some manner as the membrane transporter for the growing phagophore, but direct evidence or a mechanistic explanation are not available. Nonetheless, Atg9 has multiple functions; first, Atg9 is the only transmembrane protein that is essential for phagophore expansion (Noda et al.; 2000); second, Atg9 is found to be highly mobile in the cytosol upon rapamycin treatment (Yamamoto et al.; 2012); third, this protein is capable of binding with itself and appears to transit to the PAS as part of a complex (Reggiori et al.; 2005). While none of these studies directly proves the role of Atg9 in membrane shuttling, researchers have begun identifying the machinery that is involved in Atg9 trafficking. Upon completion of the autophagosome, it fuses with the vacuole. This fusion allows the release of the inner autophagosome vesicle into the vacuole lumen where it is now termed an autophagic body. Note that mammalian cell lysosomes are generally smaller than autophagosomes so autophagic bodies are not a general feature of autophagy in most of the more complex eukaryotes. The mechanism that controls the fusion is unknown at present; however, there are regulatory mechanisms to prevent premature autophagosome fusion with the vacuole, which would prevent delivery of the cargo into the vacuole lumen. Other cellular processes that also deliver their cargo to the vacuole employ similar components that facilitate fusion including SNARE (SNAP= Soluble NSF Attachment Protein Receptor) proteins and those involved in the homotypic fusion and vacuole protein sorting (HOPS) pathway (Jiang et al.: 2014). After the cargo is delivered inside the vacuole, the autophagic body membrane is degraded by a putative lipase, Atg15, (Epple et al.; 2001; Teter et al.; 2001) followed by cargo degradation by resident hydrolases. Once

degraded, the resulting macromolecules (aminoacids, proteins and lipids) are released back into the cytosol through various permeases including Atg22 (Yang et al.; 2006).

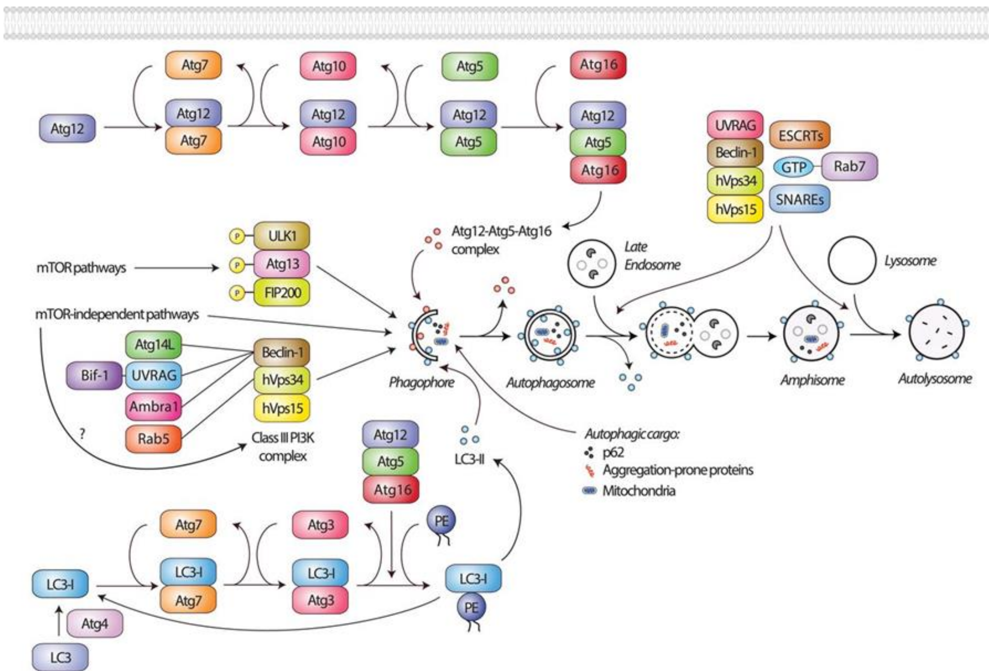


Figure 10. Regulatory mechanisms of autophagy. (http://www.biochemsoctrans.org).

3.2.2 Viral infection and autophagy

Different studies demonstrated an interaction between viral infection and autophagy (Kirkegaard et al.; 2004; Deretic et al.; 2009). Some viruses can modulate autophagy; Furaka et coll. have analysed the role of autopaghy in oncolytic herpes simplex virus type 1-induced cell death in squamous cell carcinoma cells (Furaka et al.; 2017). Montagnaro et coll. have demonstrated the impact of BHV-4 infection on autophagy in BHV-4 infected Madin Darby bovine kidney (MDBK) cells, suggesting that the virus developed mechanisms for modulation of autophagy, probably to enhance viral replication and to evade the immune system (Montagnaro et al.; 2013). Autophagy protects host cells from infection activating the virus degradation through auto-lysosomies or triggering innate immune response (Talloczy et al.; 2006; Delgado et al.; 2008). It was demonstrated that autophagy can have a dangerous effect on the pathogenesis of virus Sindbis (Li et al.; 2005). Autophagy is also implicated in the adaptive immune responses later viral infection providing viral antigens to MCH I and II (Paludan et al.; 2005; English et al.; 2009; Gannage et al.; 2009). Some viruses demonstrated different strategies to antagonize autophagy triggered by host cells (Cavignac et al.; 2010).

3.3 Necrosis

Necrosis is a passive, catabolic, pathological cell death process which generally occurs in response to external toxic factors such as inflammation, radiations, viral infections, immune responses, ischaemic or toxic injury (Dive et al.; 1992). Necrosis is characterized by the swelling of mitochondria, early break of the plasma membrane, dispersed chromatin

and early destruction of the intact structure of the cell. During necrosis, the cellular contents are released uncontrolled into the cell environment which results in damage of surrounding cells and a strong inflammatory response in the corresponding tissue (Leist et al.; 2001). As such, necrosis is often detected in infections and inflammatory diseases. This association has led to the popular view that necrosis represents pathological cell death. Recent advances have showed the existence of dedicated molecular pathways controlling necrotic cell death. Notably, the classical markers that define apoptosis can sometimes be detected in necrosis. For example, Annexin V staining is a commonly used method to detect exposure of phosphatidyl serine (PS) on the outer leaflet of the plasma membrane in early apoptotic cells. In some necrotic cells, PS exposure can be detected without significant plasma membrane leakage (Sawai et al.; 2001). PS exposure is supposed to mark apoptotic cells for clearance by phagocytes. However, researchers have also described scavenger receptors that recognize necrotic cells (Yamasaki et al.; 2008; Sancho et al.; 2009). Recent studies have identified several forms of regulated necrosis, including phosphoribosyl pyrophosphate (PPRP)-1-mediated necrotic death (Yu et al.; 2002), p53-mitochondrial function–mediated necrosis (Vaseva et al.; 2012), pyroptosis (Cookson et al.; 2001), ferroptosis (Gao et al.; 2015) and necroptosis (Moriwaki et al.; 2015). Thus, caspase-dependent apoptosis is not the only form of programmed cell death. Necroptosis has recently gained increasing attention as a novel but well-studied form of programmed cell death. Degterev et coll. (Degterev et al.; 2005), who originally coined the term "necroptosis," showed that necroptosis is characterized by a necrotic cell death morphology and by activation of autophagy. This form of

programmed cell death can occur when the cells suffer from severe stress or are treated with chemotherapy or inflammatory factors (Chan et al.; 2003; Long et al.; 2012).

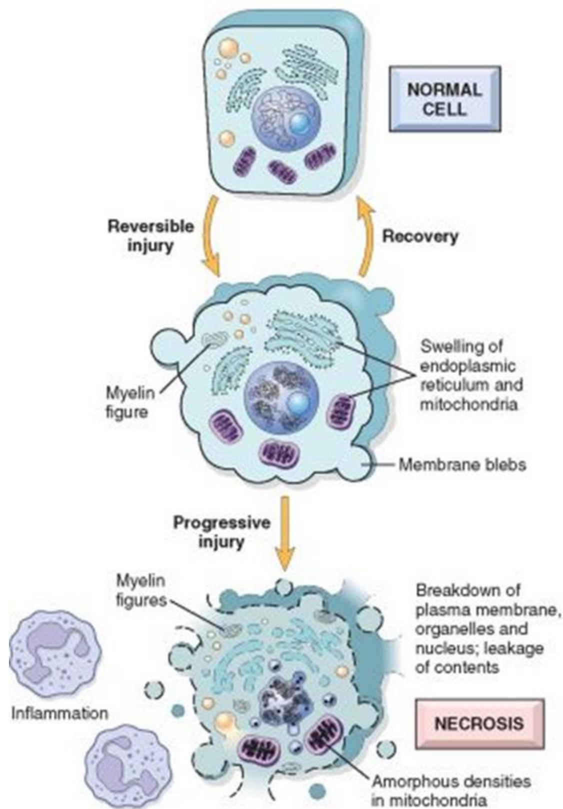


Figure 11. Morphological representation of necrosis. (https://www.studyblue.com).

The first discovery of necroptosis can be tracked to the 1988 observation that tumor necrosis factor (TNF) triggers necrotic death in multiple cell types (Laster et al.; 1988). For a long time, it was believed that this type of TNF-triggered cell death was apoptosis. In 1998, Vercammen et al. reported that TNF induced the rapid death of murine L929 fibrosarcoma cells in a necrotic manner when cellular caspases were repressed by inhibitors. Around the same time, Kawahara et coll. observed that Fas-associated protein with death domain (FADD)-mediated cell death was independent of caspase-8 activation. These studies suggested that the apoptosis-related molecules regulated a novel form of cell death that was different from apoptosis and more similar to necrosis. This novel finding motivates investigators to explore the potential mechanism behind it. A landmark study of necroptosis was published in 2000 when receptor-interacting protein kinase 1 (RIP1) was identified as the critical regulator of necrotic death in caspase-inhibited cells (Holler et al.; 2000). In 2005, Degterev et al. (Degterev et al.; 2005) called this Fas/TNF receptor (TNFR) receptor family-triggered, nonapoptotic form of cell death "necroptosis" and invented a small molecule, necrostain-1, that blocked necroptosis. In 2009, three independent studies proved that RIP3 was required for necroptosis and was the downstream target of RIP1 (Cho et al.; 2009; Zhang et al.; 2009). In 2012, mixed lineage kinase domain–like protein (MLKL) was identified as a necroptosis executor, which functioned after interacting with and being phosphorylated by RIP3 at its threonine 357 and serine 358 residues (Sun et al.; 2012). The discovery of MLKL enabled a rough mapping of the necroptosis regulatory pathway. As with apoptosis, the goal of necroptosis is to eliminate unnecessary or

abnormal cells from the body. This is important for embryonic development and disease defenses. Cancer is a disease that is closely related to cell death, and resistance to cell death is a hallmark of cancer. As one of multiple forms of cell death, necroptosis has been recognized for its critical roles in defending against cancer. Numerous studies have suggested that necroptosis suppresses the initiation and progression of cancer and facilitates its therapy. The anti-tumor effects of necroptosis have been recently reviewed (Chen et al.; 2016). However, emerging data have revealed that necroptosis can promote cancer progression, which suggests that necroptosis is a double-edged sword in cancer progression.

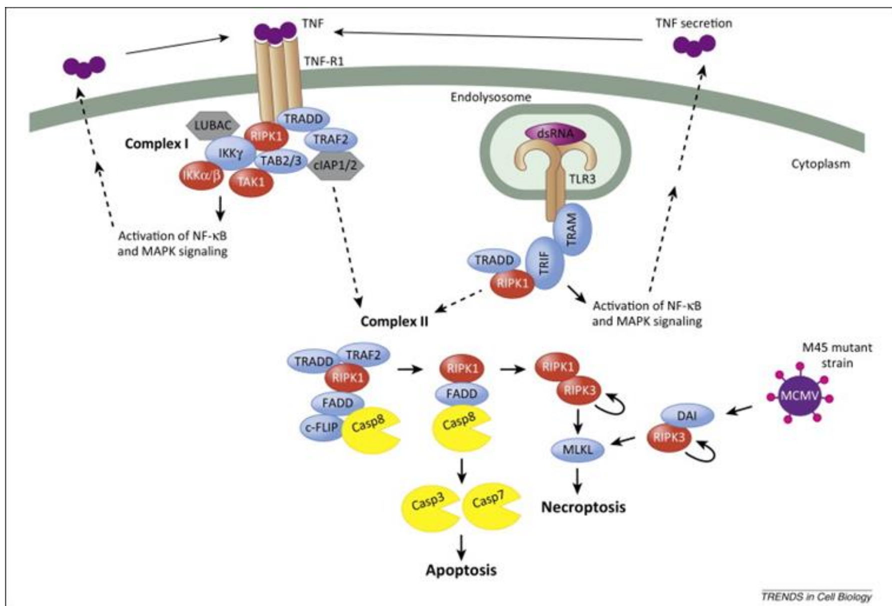


Figure 12. Activation of necroptosis process. (www.cell.com).

3.3.1 Viral infections and necrosis

Necroptosis can be initiated following viral infection by externaland internal stimuli, including activators of cell death receptors, viral nucleic acids, and direct sensing of viral proteins. TLRs that sense viral nucleic acids (TLR3, TLR7, TLR9) induce necroptosis upon ligand binding and caspase-8 inhibition, but these receptors elicit this response via different mechanisms. For example, similarly to apoptosis, only TLRs that rely on TRIF (i.e., TLR3) promote necroptosis through an intrinsic mechanism (Kaiser et al.; 2010; He et al.; 2011). TLRs induce necroptosis indirectly through the expression of TNF-α (Kaiser et al.; 2010). While TNFR signalling is normally associated with apoptosis, this receptor can also induce necroptosis under conditions of caspase-8 inhibition. When caspase-8 is inhibited, TNFR signalling induces the formation of a protein complex known as the necrosome, which includes the RIPK family members RIPK1 (Holler et al.; 2000) and RIPK3 (Cho et al.; 2009). The strongest argument for an antiviral role of TNFR-mediated necroptosis derives from studies of vaccinia virus (VV). VV encodes the B13R protein which blocks caspase-8 activity and sensitizes infected cells to TNF-α-mediated necroptosis in vitro (Zhang et al.; 2009). Interestingly, no viral infection has been shown to induce TLR3-dependent necroptosis in vitro, and elucidation of the necroptotic signalling pathway initiated by TLR3 has been performed solely through use of synthetic RNAs that mimic viral nucleic acids (Kaiser et al.; 2010; He et al.; 2011). Influenza A virus (IAV) infection induces cell death in MEF sand human lung epithelial cells via a DAI/RIPK3-dependent signalling axis (Kuriakose et al.; 2016). Viral nucleic acids can initiate necroptosis in response to DNA and RNA virus

infection. RIPK3 has also been shown to promote cell death-independent neuroinflammation following subcutaneous West Nile virus (WNV) infection (Daniels et al.; 2017).

The ability of viral proteins, rather than viral nucleic acids, to initiate necroptosis was first demonstrated in response to HSV infection. HSV-1 and HSV-2 induce necroptosis in murine cells, and the viral RHIM domain-containing proteins ICP6 (HSV-1) and ICP10 (HSV-2) mediate this response (Wang et al.; 2014; Huang et al.; 2015). However, in contrast to observations made in murine cells, HSV does not induce necroptosis in human cells. Simultaneously, these viral proteins bind to and inactivate caspase-8, indicating HSV can directly antagonize apoptosis and necroptosis through a single viral protein. Furthermore, these observation ssuggest that necroptosis may represent a species-specific barrier to HSVinfections (Guo et al.; 2015).

Capitolo 4

Aim of the study

4. Aim of the study

Oncolytic virus immunotherapy is a therapeutic approach to cancer treatment that utilizes native or genetically modified viruses that selectively replicate within tumour cells while displaying minimal adverse effects in normal healthy cells. In recent years, virus therapy has become a promising prospect for the treatment of cancer, particularly in the cases where traditional approaches are not feasible or are unlikely to succeed. The ability of viruses to kill cancer cells has been recognized for nearly a century, but only over the past decade have clinical trials documented a therapeutic effect in the treatment of malignancies. The mechanisms through which oncolytic viruses mediate tumour rejection are incompletely understood. To date, a wide variety of viruses have been evaluated for their oncolytic potential, including DNA viruses such as herpesviruses, adenoviruses, and vaccinia virus and RNA viruses such as reoviruses and poliovirus. HSV-1 vectors have generated much interest in the field, as HSV-1 was the first virus used to show that a genetic mutation could render a virus oncolytic. To date, Phase I and II clinical trials have been conducted with various HSV-1 mutants, showing the safety of administering various oncolytic mutants of HSV-1 in humans. However, HSV-1 is a human pathogen, so it must be genetically manipulated to attenuate the virus sufficiently for it to preferentially replicate in tumor cells; attenuation makes viral replication less efficient, compromising the success of the therapy. Furthermore, it has been estimated that 40–90% of the population has preexisting immunity to HSV-1, which precludes efficient systemic delivery of HSV-1 oncolytic vectors. Consequently, the use of wild-type viruses that are not human pathogens is being developed

as an alternative approach for oncolytic virotherapy. Exploiting the correlation between the members of Alphaherpesvirinae family, we aim to investigate the potential of Caprine Herpesvirus 1 (CpHV-1) as an oncolytic vector. Like herpes simplex virus 1 (HSV-1), the prototype member of Alphaherpesvirinae family, CpHV-1 establishes life-long, non-productive latent infections in ganglionic sensory neurons. Our research group previously demonstrated that CpHV-1 has the capacity to induce apoptosis in goat peripheral blood mononuclear cells; moreover, we have investigated on the pro-apoptotic potential of CpHV-1 in a permissive cell line (Madin Darby bovine kidney cells, MDBK). Recently, we have characterized in more detail the intracellular pathway by which CpHV-1 induce apoptosis by analyzing the gene expression response during the apoptotic phase of CapHV-1 infection in a murine neuroblastoma cell line (Neuro 2A). Thus, the aim of this study is to investigate the ability of CapHV-1 to replicate, cause cytopathic effects and affect cellular viability in a panel of human cancer cells lines (HeLa, U2OS, MDAMB-468, A549, PC3, K562) from a variety of histological origins. In a first series of experiment we have analyzed the effect of CpHV-1 infection on cell viability at different time post infection (p.i.) and several multiplicity of infection (MOI). Then, we investigated virus induced cytotoxicity, viability, and apoptosis. The final results of this study will allow to demonstrate the effect of CpHV-1 infection in neoplastic cell lines in terms of caspase activation and apoptosis modulation, to suggest CpHV-1 as a novel candidate oncolytic virus.

Capitolo 5

Material and methods

5. Material and methods

5.1 Cell lines

All cell types were maintained at 37°C with 5% CO2 in medium supplemented with 2 mM L-glutamine, 100 U penicillin and 100 U streptomycin and were purchased from the American Type Culture Collection (ATCC Manassas, VA). Human breast adenocarcinoma (MDA-MB-468), Human cervical adenocarcinoma (HeLa), Human osteosarcoma (U2OS), Human prostatic adenocarcinoma (PC3) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, Human lung carcinoma (A549) were cultured in F-12K Medium supplemented with 10% FBS, and Chronic Myelogenous Leukemia (K562) cell lines were maintained in Iscove medium (IMDM) (Gibco, Carlsbad, CA, USA). Madin Darby bovine kidney (MDBK) cell lines were maintained in DMEM supplemented with 10% FBS and used as control.

5.2 Viruses

The reference Swiss strain E/CH (Mettler F, et al.; 1979) of CpHV-1 was used. It was multiplied on MDBK, and cell extracts, obtained by three cycles of freezing and thawing, was be pooled, collected, and stored in aliquots at -80°C. Infectivity titres was expressed as median tissue culture infectious doses (TCID 50)/ml (Reed and Muench, 1938).

5.3 Cytopathic effect assay

Before investigating the oncolytic capacity of CpHV-1, we first confirmed the restriction of selected human cancer cells against CpHV-1 replication and cytopathic effect (CPE) induction. A panel of six cell types (HeLa, U2OS, MDAMB-468, A549, PC3 and K562) were infected with increasing amounts of CpHV-1, and CPE was monitored. MDBK cell line was used as control.

Briefly, for adherent cells, 90 – 95% confluent cell monolayers were infected with a different multiplicity of infection (MOI) between 0,5 and 10 in serum free medium. After 1 hour of viral adsorption at 37°, cell monolayers were maintained in medium with 5% of FBS. At 24, 48 and 72 hours post infection cells were fixed in methanol and stained with Giemsa to observe and score CPE.

Otherwise for K562 suspension cell, to assess cell viability trypan blue assay was performed. Briefly, cells were collected and an aliquot of the cell suspension was mixed with an equal volume of 0.2% Trypan-blue (Sigma, St. Louis, MO) in 1X phosphate-buffered saline (PBS). After 10 min, cells were counted using TC20 automated cell counter (Biorad).

5.4 Viability

Virus viability was assayed by means of the 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) assay as previously described (Pagnini et al.; 2004). The principle of this method is that MTT, a soluble tetrazolium salt, is converted to insoluble formazan by active mitochondrial dehydrogenases of living cells. Such conversion from yellowish soluble tetrazolium to purple formazan can be assayed

spectrofluorimetrically. MTT was added to cultured cells at different hours p.i. Cultures were incubated for an additional 2 hours, the medium was removed and replaced with dimethyl sulfoxide (DMSO) to solubilise the MTT formazan crystals. The spectrophotometer adsorbance at 570 nm was determined.

Data are presented as a percentage of the control, and results are the mean ± SD of three experiments performed in duplicate.

5.5 Apotox-glo™triplex assay

The ApoTox-Glo™Triplex assay was used to assess viability, cytotoxicity, and caspase activation events within a single assay well. In the first part of the assay, it measures two protease activities simultaneously; one is a marker of cell viability and the other is a marker of cytotoxicity. Peptide substrate (glycylphenylalanyl-aminofluoro-coumarin; GF- FC) enters intact cells where it is cleaved by the live-cell protease activity to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. Peptide substrate (bis-alany-lalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. Bis-AAF-R110 is not cell-permeable, so no signal from this substrate is generated by intact, viable cells. The live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously. In the second part of the assay, the Caspase Glo® 3/7 Reagent (Promega Corporation), add in an “add-mix-measure” format,

results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal produced by luciferase. Human cancer cells of approximately 500/well were seeded in a flat 96-well micro-plate (Becton Dickinson Labware, USA) as triplicates. Four different types of controls, namely: positive, infected, negative, and background controls were used throughout the study. Positive control had cells with culture medium treated with Staurosporine of 10 mM final concentration for 24 h to induce apoptosis. Infected cell cultures contained cells treated with CapHV-1 at MOI 2.5 for 24. Negative control consisted of mock infected neoplastic human cells and no-cell control (background) containing only culture medium without cells. After 24 h post-infection 20 µl of viability/cytotoxicity reagent containing both GF-AFC and bis-AAF-R110 substrates was added to each well and briefly mixed by orbital shaking at 300–500 rpm for 30 s and then incubated at 37°C for 30–180 min. Fluorescence was measured at 400Ex/505Em (Viability) and 485Ex/520Em (Cytotoxicity) by using Glomax Multi Detection System multiwell plate reader (Promega Corporation, USA). After that 100 µl of Caspase-Glo 3/7 reagent was added to each well, and briefly mixed by orbital shaking at 300–500 rpm for 30 s and then incubated at room temperature for 30–180 min. Luminescence was measured using a Glomax Multi Detection System multiwell plate reader (Promega Corporation, USA) by Luminescence protocol which is proportional to the amount of caspase activity present.

5.6 Western Blot analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot analysis were performed on CpHV-1 infected neoplastic cells and on mock infected neoplastic cells. HeLa, U2OS, MDAMB-468, A549, PC3 and k562 cells in 75 cm2 flask, at confluency, were infected with CpHV-1 at MOI 2.5. At 12, 24, and 48 h post-infection, adherent cells were washed twice with PBS and scraped, while K562 suspension cells were collected and washed with PBS. Cells were then mixed with cells previously collected by centrifugation from supernatant of the same flask and resuspended in PBS. While K562 suspension cells were collected and washed with PBS. The pellets, obtained by centrifugation, were stored at 20°C and then processed by Western blot analysis as previouslydescribed (Montagnaro S, et al.; 2013). To measure caspases activation at least 1x106 cells were used. Cells were homogenized directly into lysis buffer 50 mM Tris pH 7.5; 150 mM NaCl; 1 mM EDTA; 0.25% deoxycholic acid, 1% Triton X-100) with 20 mM sodium pyrophosphate, 0.1 mg/mL aprotinin, 2 mM phenylmethylsulphony fluoride (PMSF), 10 mM sodium orthovanadate (Na2VO3), and 50 mM sodium fluoride (NaF). Protein concentrations were determined by a protein assay kit (Bio-Rad Laboratories). Equal amounts of lysate samples were boiled, loaded on bis/acrylamide gels, separated by electrophoresis and proteins were blotted from the gel onto PVDF membranes. The membranes were blocked with 5% nonfat dry milk in Tris buffered saline (TBS: 12.5 mM Tris–HCl pH 7.4; 125 mM NaCl) at room temperature, washed with TBS-0.1% Tween and incubated with primary antibody. The primary antibodies used were rabbit anti-cleaved caspase 3 (Cell Signaling Technologies catalog no.

9662; 1:1,000 diluition), anti-LC3I/II (Cell Signaling Technologies catalog no. 4108; 1:1000 diluition) and anti-SQSTM1/p62 (Cell Signaling Technologies catalog no. 5114; 1:1000 diluition). After appropriate washing steps, peroxidase-conjugated anti-rabbit IgG (GE Healtcare, UK, catalog no. NA934) (GE Healtcare, UK, catalog no. NA931) was applied for 1h at 1:1000 dilution. The blots were stripped and reprobed against mouse anti-actin antibody (Calbiochem, San Diego, CA; catalog no. CP10) at 1:2000 dilution to confirm equal loading of proteins in each lane. Protein expression levels were quantitatively estimated by densitometry using a Gel Doc scanner (BioRad) equipped with a densitometric work station. The protein concentrations were normalized to the actin level and expressed as relative band density (arbitrary units).

Capitolo 6

Results

6. Results

6.1 CpHV-1 lytic capacity in various cancer human cell types

Unlike many herpesviruses, CpHV-1 has a restricted host range and is unable to productively infect humans, despite its similarities with HSV-1. Before investigating the oncolytic capacity of CpHV-1, we first assessed the capacity of virus to induce CPE in a panel of human cancer cells from multiple histological origins. As shown in Figure 13, at 24h post infection CPE was observed in different cell lines (PC3, MDA-MB-468, U2OS and HELA) in wich more than 50% CPE was observed at MOIs comprised between 2.5 and 10; MDBK cell line have shown 50% CPE at MOIs 2,5, 5 and 10. Finally, in A549 cell line we have observed less than 50% CPE only at MOI 10. As shown in Figure 14, at 48h post infection we have observed more than 50% CPE in PC3, MDA-MB-468, U2OS, and MDBK at MOIs 1, 2,5, 5 and 10; in HeLa cell line we have observed more than 50% CPE between MOIs 0,5 and 10, while in A549 cell line there was 50% CPE at MOIs 5 and 10. Otherwise in K562 suspension cells we did not observe a decrease in number of living cells by Trypan blue assay (data not showed). These data suggest that CpHV-1 has the capacity to induce CPE in all adherent human cancer cell line used.

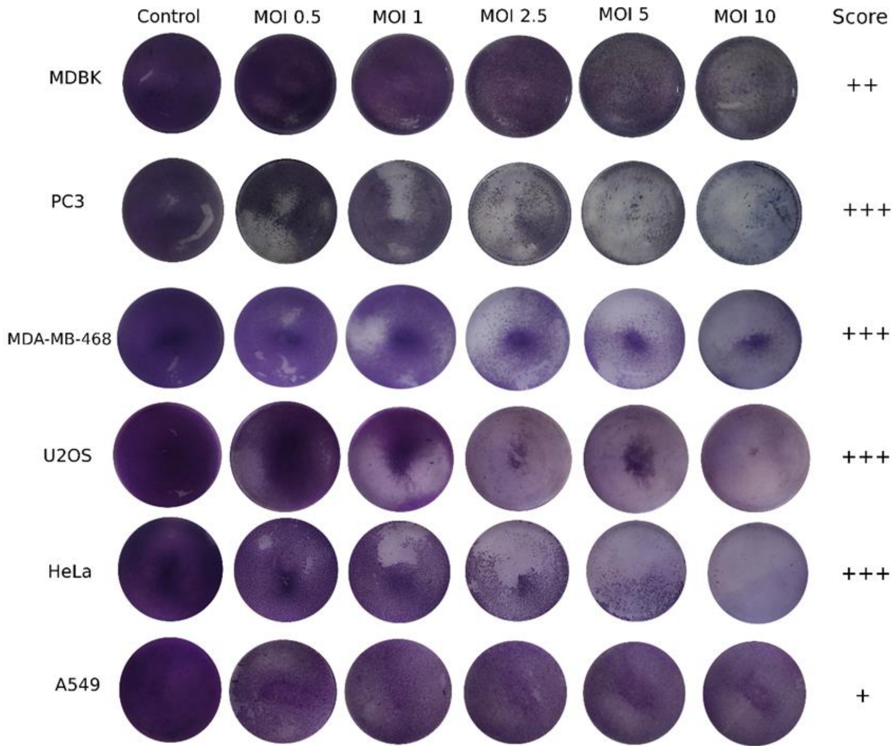


Figure 13. Permissiveness of immortalized human cancer cell lines to Caprine Herpesvirus 1 (CpHV-1). Cells were infected with the indicated MOI of CpHV-1 and 24h post infection, cell monolayers were stained with Giemsa to observe and score Cytopathic effect (CPE): +++ indicates very permissive cells, in wich more than 50% CPE was seen; ++ indicates a moderately permissive cell type, in wich 50% CPE was seen; + indicates a cell type that is not very permissive, in wich less tna 50% CPE was seen; - indicates a non-permissive cell type, in wich no CPE was observed.

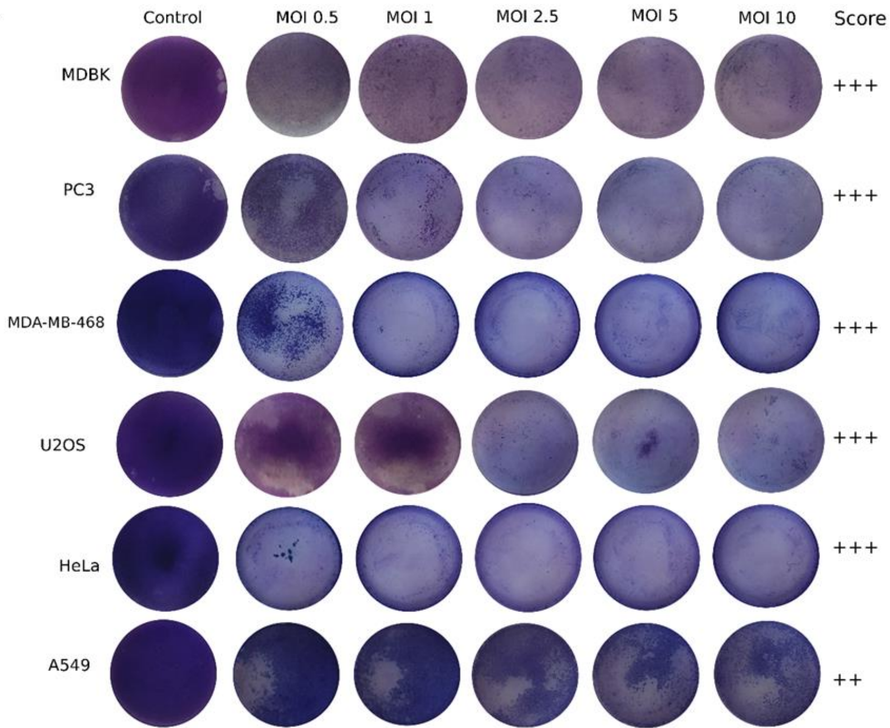


Figure 14. Permissiveness of immortalized human cancer cell lines to Caprine Herpesvirus 1 (CpHV-1). Cells were infected with the indicated MOI of CpHV-1 and 48h post infection, cell monolayers were stained with Giemsa to observe and score Cytopathic effect (CPE): +++ indicates very permissive cells, in wich more than 50% CPE was seen; ++ indicates a moderately permissive cell type, in wich 50% CPE was seen; + indicates a cell type that is not very permissive, in wich less tna 50% CPE was seen; - indicates a non-permissive cell type, in wich no CPE was observed.

6.2 Viability

Infection of seven human cancer cells with the CpHV-1 resulted in cell death in a time-dependent and dose-dependent manner, as detected by MTT assay (Figure 15). All cell lines, except A549 and K562 cells, showed a marked cytopathic effect (CPE), demonstrating an oncolytic potential of CpHV-1 in tested human cancer cells. In different cell lines, infected at various m.o.i., the decrease of viability appeared 24h and/or 48h post-infection. In particular, in MDA-MB-468 the viability started to decrease at 24h post infection until showing a reduction at 72h post infection; U2OS showed only a remarkable decrease of viability at 72h post infection; in PC3 we have observed an irregular trend, characterized by a reduction of viability at 48h post infection followed by an increase at 72h post infection. The same trend was observed in HeLa cell line which showed a decrease of viability observed at 24h and 48h post infection, while presenting an increase of viability at 72h post infection. In MDBK cell line we have observed a time and dose dependent decrease of viability from 24h to 72h post infection; in A549 cell line we did not have observed a significant decrease of viability. Finally, K562 cell line have showed increased viability at 24h, 48h and 72h post infection.

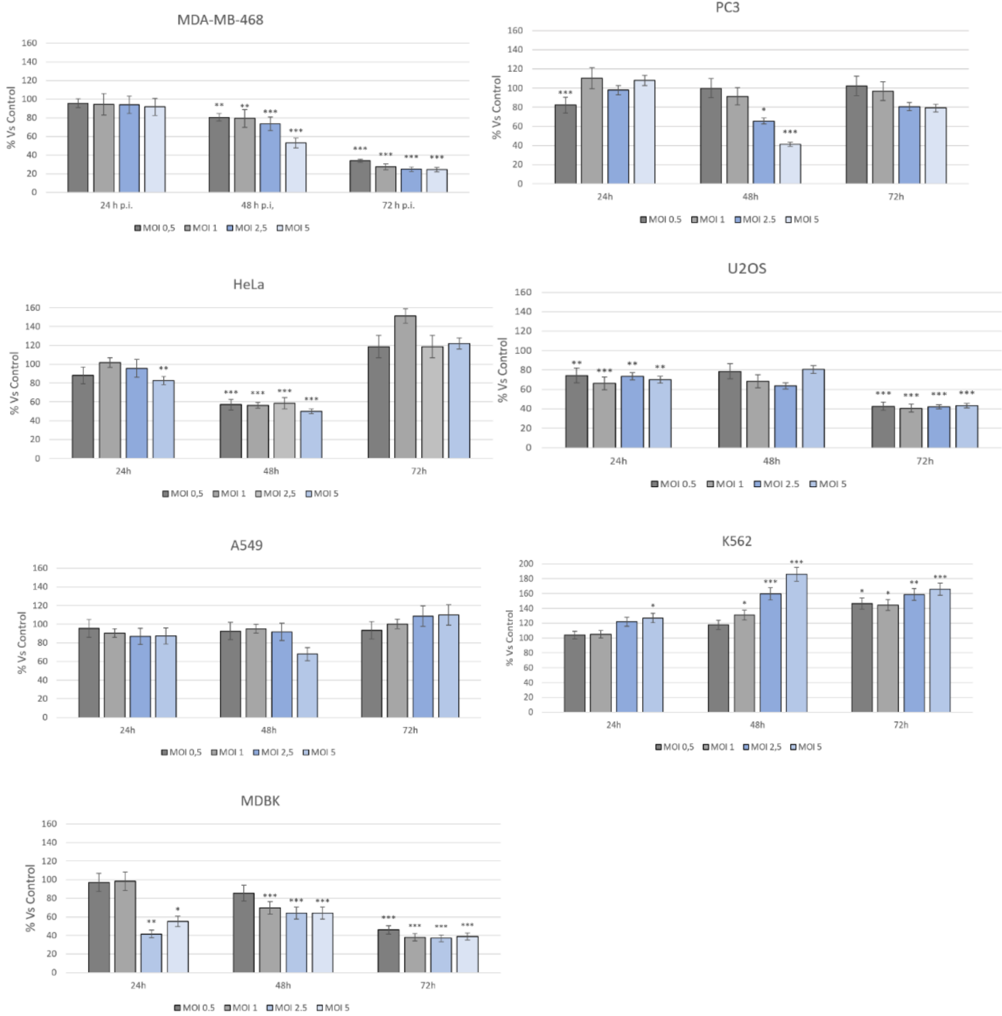


*Figure 15. Dose–response curve of human cancer cell lines infected with different MOI of CpHV-1 and observed at different time on cell viability. Viable cells were stained with MTT at different hours post infection and the absorbance assayed as described in the Materials and Methods Section. Data are presented as a percentage of the control, and results are expressed as the mean SE of three independent experiments performed in duplicate. Significant differences between control and TCDD-exposed groups are indicated by probability P. *P < 0.05, **P < 0.01, and ***P < 0.001.*

6.3 Time course of virus production and viral expression

We next determined whether cell death was related to a significant level of viral production. For this purpose, infection of CpHV-1-infected human cancer cell lines was evaluated as TCID50. Figure 16 shows a time course of virus yield, in supernatants and cell associated virus, following exposure to CpHV-1 for 24, 48 and 72h at a MOI 2.5. MDBK cell lines was used as control. The acme of viral production was observed at 24h post infection in MDA-MB-468 (108 TCID50/ ml) and HeLa (107 TCID50/ ml) cells and in PC3 (108 TCID50/ ml) cells at 48h post infection. The higher viral titres were observed, as expected, in MDBK (1015 TCID50/ ml) at 24h post infection. There was no efficient viral replication was in A549 cell line. Surprisingly, we have observed a very high viral titre in K562 (1010 TCID50/ ml) at 48h post infection. Conversely, U2OS and A549 showed low viral titres (105 TCID50/ ml, 103 TCID50/ ml respectively).

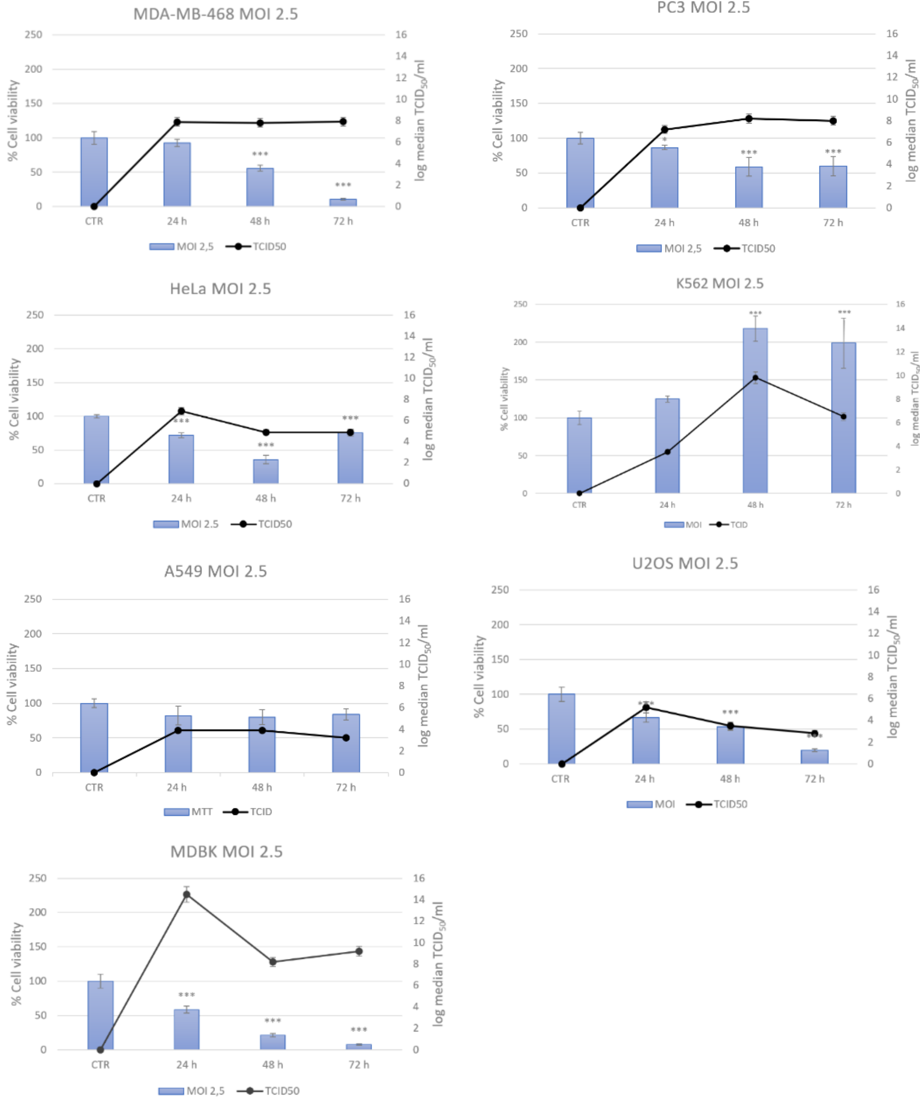


Figure 16. Time course of virus production and cell death in Human cancer cell lines infected with CpHV-1. Virus production was titrated at 24, 48 and 72h after infection with CpHV-1 at a MOI. of 2.5. Results are expressed as Mean SD of three.

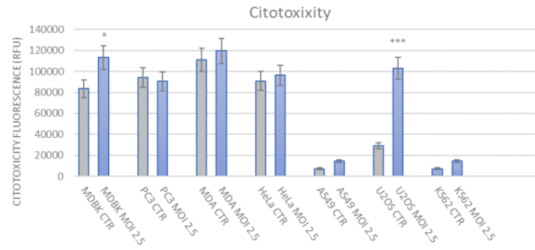
6.4 Apotox-glo™ triplex assay

We have analysed the effect of CpHV-1 infection on cytotoxicity, viability, and apoptosis within a single assay well, by using the ApoTox-Glo™Triplex assay. The graphs in Figure 17 depicts the results of an ApoTox-Glo™Triplex assay showing the cytotoxicity, viability, and apoptosis of HeLa, U2OS, MDAMB-468, A549, PC3, K562 and MDBK cells infected with CpHV-1 at MOI 2.5 at 24h post infection. As showed in Panel A, after 24h post-infection, the cytotoxicity increased significantly only in U2OS cell line, compared to mock infected cells.

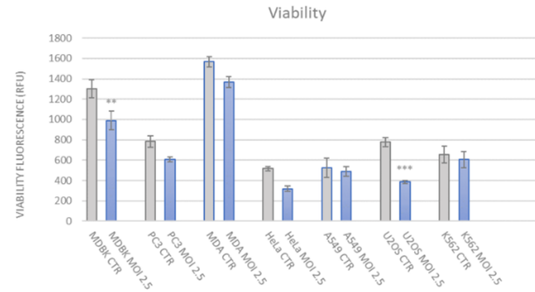
Panel B depicts the results of an Apo-Tox-Glo™ Triplex assay showing the viability of cells lines infected with CpHV-1 at MOI 2.5. After 24h post-infection, the viability CpHV-1 infected cells decreased respect to untreated control cells and was considered significant only in U2OS cell line compared to mock infected cells.

Panel C shows the results of an Apo-Tox-Glo™ Triplex assay showing the activation of caspase 3 in human cancer cell line infected with CpHV-1 at MOI 2.5 at 24h post-infection. CpHV-1 infected cells showed significant level of caspase 3 at 24h post-infection in PC3 and U2OS cells respect to mock infected control cells.

Panel A



Panel B



Panel C

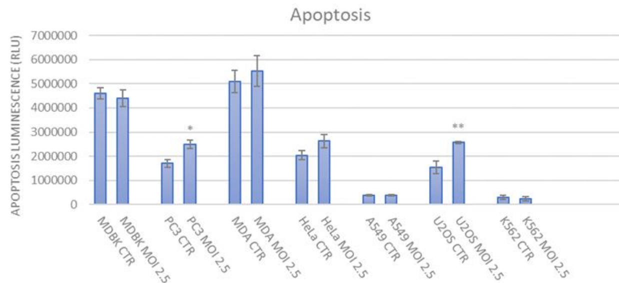
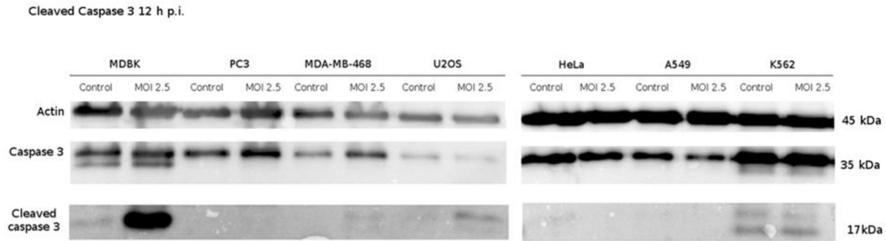


Figure 17. CpHV-1 induce apoptosis in human cancer cell lines. Replicate cultures of human cancer cell lines were mock infected or infected with CpHV-1 at MOI 2.5. At 24h after infection, the cytotoxicity (Panel A), viability (Panel B) and apoptosis (Panel C) were assayed using ApoTox-Glo™Triplex assay. Results are expressed as Relative Fluorescence Units (RFU) and Relative Luminescence Units (RLU) and reported as the mean SD of three separate experiments. A P-value <0.05 was selected as significant.

6.5 Western blot analysis

To investigate the modulation of apoptotic pathway in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines infected with CpHV-1, we also measured the cleaved form of caspase 3 expression, as apoptotic marker, by Western blot analysis. Activation of the DNase responsible for oligonucleosomal degradation of nuclear chromatin is dependent on cleavage of an inhibitor complexed with the DNase molecule (Sakahira et al.; 1998). The cleavage is mediated by caspase-3. A cleaved caspase-3 fragment corresponding to the 17-kDa protein band was detected at 12h post-infection in MDA-MB-468, U2OS cell lines (Figure 18). Instead, cleaved caspase 3 fragment was detected at 24 and 48h post infection in PC3 and K562 respectively (Figure 19 and Figure 20). Finally, we have investigated the activation of autophagic flow by analyzing the conversion of LC3I to LC3II and the simultaneous reduction of protein p62 (Figure 21). The decrease of LC3I, the increase of LC3II, and the reduction of p62 were observed only in K562 cells, the other cell lines did not show typical pathway of autophagy activation.



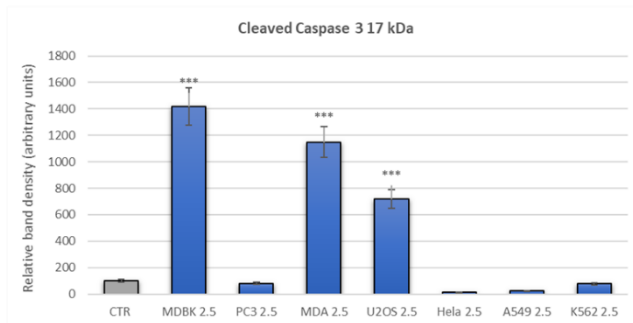


Figure 18. Caspases activation is induced in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines cells infected with CpHV-1. Replicate cultures of human cancer cell lines were mock infected or infected with CpHV-1 at MOI 2.5. At 12h after infection, cells were scraped, harvested, lysate, and subjected to Western blot analysis with antibodies against Actin and Caspase 3. Actin protein levels were detected to ensure equal protein loading. Densitometric analysis of blots relative to Caspase 3. Results are expressed as the mean SD of three separate experiments, a P-value <0.05 was selected as significant.

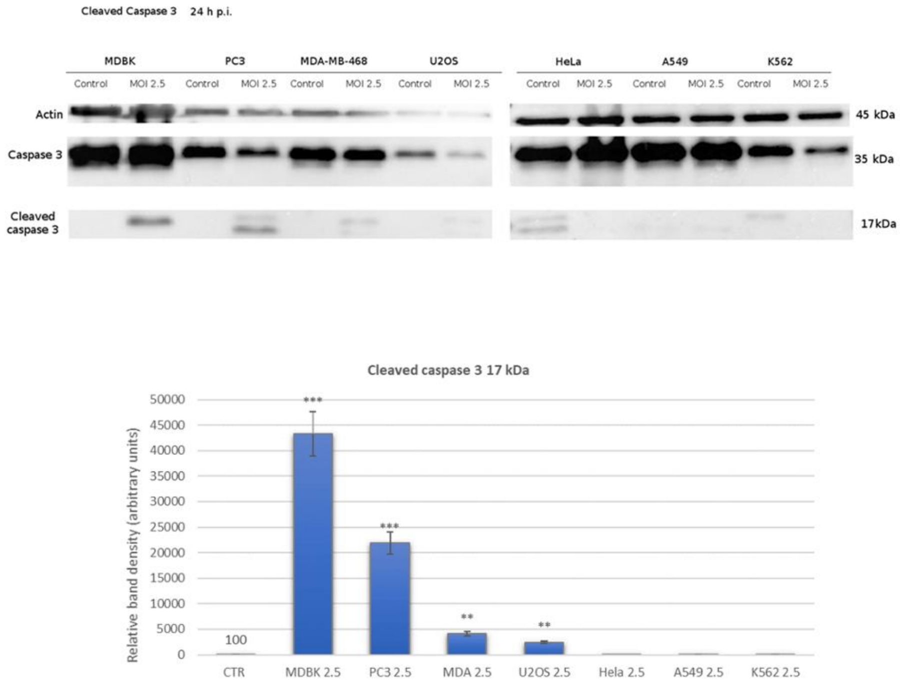


Figure 19. Caspases activation is induced in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines cells infected with CpHV-1. Replicate cultures of human cancer cell lines were mock infected or infected with CpHV-1 at MOI 2.5. At 24h after infection, cells were scraped, harvested, lysate, and subjected to Western blot analysis with antibodies against Actin and Caspase 3. Actin protein levels were detected to ensure equal protein loading. Densitometric analysis of blots relative to Caspase 3. Results are expressed as the mean SD of three separate experiments, a P-value <0.05 was selected as significant.

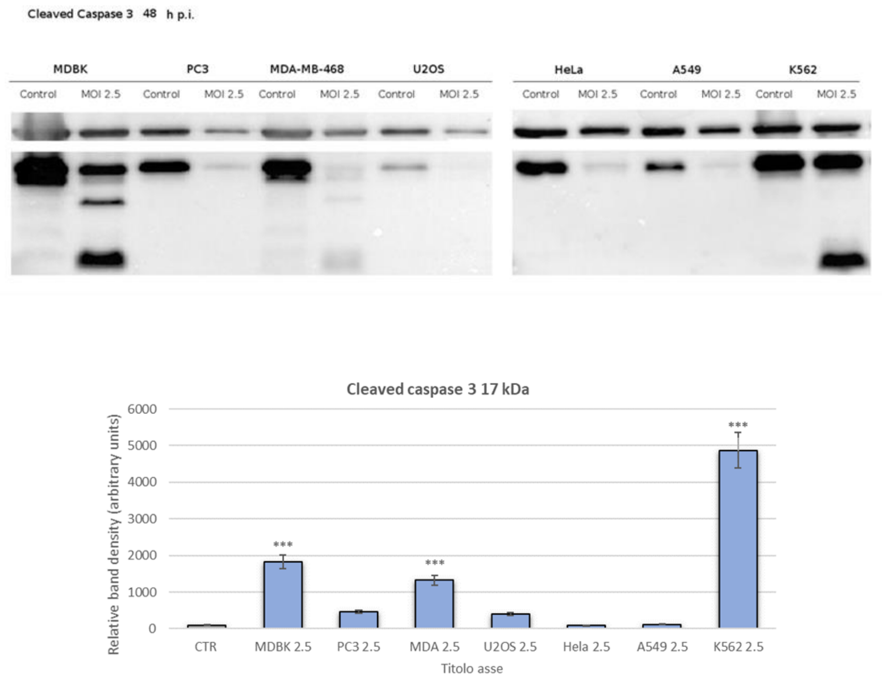
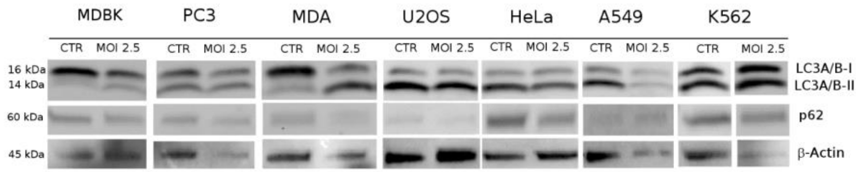


Figure 20. Caspases activation is induced in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines cells infected with CpHV-1. Replicate cultures of human cancer cell lines were mock infected or infected with CpHV-1 at MOI 2.5. At 48h after infection, cells were scraped, harvested, lysate, and subjected to Western blot analysis with antibodies against Actin and Caspase 3. Actin protein levels were detected to ensure equal protein loading. Densitometric analysis of blots relative to Caspase 3. Results are expressed as the mean SD of three separate experiments, a P-value <0.05 was selected as significant.



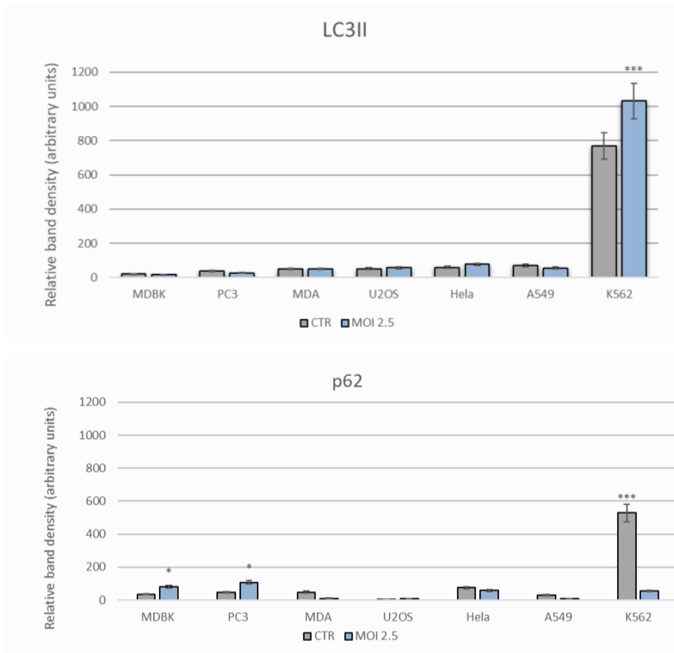


Figure 21. Autophagy activation in PC3, HeLa, MDA-MB-468, U2OS, K562 and in MDBK cell lines cells infected with CpHV-1. Replicate cultures of human cancer cell lines were mock infected or infected with CpHV-1 at MOI 2.5. At 24h after infection, cells were scraped, harvested, lysate, and subjected to Western blot analysis with antibodies against actin, LC3II and p62. Actin protein levels were detected to ensure equal protein loading. Densitometric analysis of blots relative to LC3II and p62. Results are expressed as the mean SD of three separate experiments, a P-value <0.05 was selected as significant.

Capitolo 7

Discussions

7. Discussions

Oncolytic viruses show the natural propensity to infect and kill malignant cells, leaving surrounding cells unharmed. Several oncolytic viruses have been shown to replicate preferentially in cancer cells (Kelly et al.; 2007; Bartlett et al.; 2013). Cancer cells evolve to resist apoptosis and growth suppression, evade immune mediated destruction and proliferate indefinitely (Nguyen et al.; 2008; Su et al.; 2015; Vähä-Koskela et al.; 2007); moreover, they have defects in host antiviral response pathways, mainly the interferon pathway, which makes these cells unable to efficiently fight and suppress viral replication. OVs offer many potential advantages over conventional cancer therapies, including the safety, the ability of oncolytic viruses to also function as tumour vaccines and to work in synergy with conventional cancer therapy (Cervantes-Garcia et al.; 2008; Li et al.; 2008; Rodrigues et al.; 2010; Vaha-Koskela et al.; 2007). Nonhuman wildtype oncolytic viruses show many advantages over human vectors, like the incapacity to induce infection in humans and the absence of pre-existing immunity (De Munck et al.; 2017; Filley et al.; 2017).

Thus, in the present study we have investigated the CpHV-1 oncolytic potential on a panel of human cancer cells. In a first part of the study, we demonstrated that CpHV-1 is able to induce CPE in PC3, MDA-MB-468, U2OS, HeLa. Otherwise, A549 and K562 cell lines showed low permissiveness to CpHV-1 infection. The same trend was observed in cytotoxicity assay experiments.

Virus growth assays in cancer cell lines demonstrated that CpHV-1 grows to a higher titre in PC3, MDA-MB-468, HeLa, U2Os and K562 but not in A549 cancer cell. These data confirmed that a reduction in viability

corresponded to an increase in viral replication, suggesting the permissiveness of infected cell lines.

These results agree to the data reported in previous studies, which demonstrated the permissiveness of PC3, U2OS and MDA-MB.468 cell lines to oncolytic BHV-1 (Rodrigues et al.; 2010), a genetically related alphaherpevirus. Several other Herpesviruses have been evaluated as oncolytic viruses, enclosing Herpes simplex virus type 1 (Dharmadhikari N, et al.; 2015), Bovine herpesvirus 1 (Rodrigues et al.; 2010), Bovine herpesvirus 4 (a Gammaherpesvirus of the genus Rhadinovirus) (Gillet et al.; 2005). Given the genetic similarity between BHV-1 and CpHV-1, we believe that CpHV-1 could offer advantageous properties that make it a potential new member in the collection of anticancer agents. CpHV-1 is a non-pathogenic virus for humans but seems to be able to replicate, with production of viral progeny, and kill different human cancer cell lines. The virus has a short, defined growth cycle that results in the death of infected cells and the virus can be grown to high titres. Another attractive feature of CpHV-1 as oncolytic virus is the absence of pre-existing immunity against the virus. This would consent the entry of CpHV-1 into human cancer cells and consequent replication and spread of the virus that would not be delayed by pre-existing antibodies or memory cytotoxic T cells. Moreover, CpHV-1 is a large virus and therefore has a wide packaging capacity. This feature would allow relatively easy insertion of therapeutic genes that could act to increase the oncolytic or the selective properties of an CpHV-1 oncolytic vector.

In our experiments, in order to elucidate the mechanism of cell death involved in the CpHV-1 oncolytic activity, we have investigated the ability

to induce apoptosis. Our experiments have demonstrated the activation of apoptotic process in MDA-MB-468 at 12h, 24h and 48h post infection, in U2OS at 12h and 24h post infection and in PC3 at 48h post infection. These results are in accordance with the decrease of viability and the high viral titres in these cell lines, confirming the ability of the virus to induce apoptosis. This is consistent with our previous studies which demonstrated that, CpHV-1 during lytic cycle exert its replicative potential inducing apoptosis in ruminant epithelial cells, in peripheral blood mononuclear cells and in a murine neuroblastoma cell line (Longo et al.; 2009; Montagnaro et al.; 2013) and agree with the results obtained by Cardoso et coll., who has demonstrated that oncolytic potential of BHV-1 is mediated by apoptosis induction in and glial-derived tumor cell cultures (Cardoso et al.; 2016).

As expected, we did not observe the activation of apoptotic process in A549 and this aspect is in line with the increase of cell viability and the weak viral yield observed in these CpHV-1 infected cells. In HeLa cells we didn't observe caspase 3 activation despite of the relatively high viral titre detected. Surprisingly, in K562 cell line we observed the caspase 3 activation at 48h post infection related to a high viral titre despite a low cytotoxicity and it is noteworthy that in K562 a very high viral titre was detected in presence of a significant increase of cell viability. Moreover, we showed that in CpHV-1 infected K562 cells an accumulation of the autophagosome-associated form of LC3, a marker of autophagy activation. The modification of LC3 correlated with increased formation of autophagosomes, with cytoplasmic depletion and with degradation of the autophagy marker p62/SQSTM1 polyubiquitin binding protein. Our results

are in agreement with other studies about herpesviruses. Infact, Takahashi et coll. (2009) have demonstrated that VZV infections induces autophagy in permissive cancer cells line at late stage of infection with depletion of p62. It was also demonstrated that HSV-1 infection induces autophagy in macrophages (English et al.; 2009).

Modulation of autophagy affords great advantages to the virus, facilitating virus replication, the spreading of progeny virus to neighbouring cells and providing the protection for the progeny virus against cellular enzymes (Deretic and Levine; 2009; Cavignac and Esclatine; 2010). Autophagy protects host cells against viral attack by degrading the viruses in autolysosomes, or by activating the innate immunity of the cells by loading viral components onto endosomal sensors such as TLRs (Talloczy et al.; 2006; Delgado et al.; 2008). It has been suggested that autophagy may have a deleterious effect on viral pathogenesis in the case of the neurotropic Sindbis virus (Liang et al.; 1998), and the role of autophagy has been clearly demonstrated to limit the replication of the tobacco mosaic virus (Liu et al.; 2005). Then, despite the ability of autophagy to function as an anti-viral mechanism, some viruses appear to up-regulate the process and/or subvert autophagic genes in order to enhance their replication (Kudchodkar et al.; 2009).

Deregulation of autophagy is implicated in several human diseases including cancers. Several studies have shown that tumour cells are defective in autophagy and/or apoptosis pathway (Chen et al.; 2009). Autophagy may protect against cancer by isolating damaged organelles, allowing cell differentiation, increasing protein catabolism, and even promoting cancer cell death. Autophagy-defective tumour cells also

display elevated genome damage with stress, suggesting that damage mitigation by autophagy is a cell-autonomous mechanism of tumour suppression (Karantza-Wadsworth et al.; 2007; Mathew et al.; 2007a, b). Several studies found that induction of autophagy may be a viable new therapeutic arm in fighting cancer. Although our findings have shown that the CpHV-1 was not able to kill K562 cells, it was able to induce the apoptosis and autophagy activation, restoring the defective pathways. It was demonstrated that that by introducing the Beclin-1 gene into the oncolytic adenoviral backbone, the antileukemia activity of the virus on multidrug-resistant cell lines can be significantly improved (Li et al.; 2014; Zhang et al.; 2006). These results could suggest that targeting the autophagic cell death pathway by CpHV-1 may represent a novel strategy for gene virotherapy, above all for combination with conventional cancer therapies and for chemotherapy resistance.

Several aspects of the CpHV-1-mediated cell death in neoplastic cells reported herein need to be addressed with further experimentation. In particular, the activation of intrinsic or extrinsic pathway of apoptosis, the role of INF in virus infection and the mechanism responsible for induction of autophagy in CpHV-1 infection as well as the impact of the CpHV-1 as oncolytic virus remains to be identified. These issues are currently under investigation in our laboratory.

Overall, in conclusion, our findings showed that CpHV-1 replicates and decreases the viability in several human cancer cells; furthermore, it induces apoptosis and the activation of autophagy pathway and it could represent a new candidate for the oncolytic virus immunotherapy.

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