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PhD Thesis Autoreport

Character of the host cell death induced by vaccinia virus and inhibitory effects of the lipoic acid on vaccinia virus growth

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S disertací je možno se před obhajobou seznámit na oddělení pro vědeckou činnost a zahraniční styky děkanátu 1. lékařské fakulty Univerzity Karlovy v Praze.

Content

Súhrn

Vírus vakcínie je typickým zástupcom poxvírusov. V minulosti bol ako vakcína používaný na úspešnú eradikáciu pravých kiahní (ang. smallpox). V súčasnosti je používaný ako vektor pre profylaktické aj experimentálne účely.

Táto dizertačná práca nadväzuje na výsledky publikovaných prác z nášho laboratória a je zčasti zameraná na bližšie charakterizovanie vplyvu infekcie vírusom vakcínie na typ bunkovej smrti hostiteľskej bunky. Výsledky ukazujú na to, že počas infekcie vírusom vakcínie sa aktivujú dráhy apoptózy, apoptóza ale nie je dokončená a bunka umiera nekroticky. V našich pokusoch sme farmakologickou inhibíciou aktivity enzýmu PARP (Poly-(ADP-ribóza) polymeráza) nedokázali zmeniť formu bunkovej smrti indukovanú vírusom vakcínie z nekrózy na apoptózu. Efekty anti-apoptotických faktorov kódovaných vírusom vakcínie pravdepodobne hrajú významnejšiu úlohu.

Ďalšia časť práce sa venuje sledovaniu inhibičného efektu redox-modulujúcej látky, kyseliny lipoovej, na infekciu vírusom vakcínie. Výsledky ukázali jej inhibičný vplyv v bunkových líniach rôzneho embryonálneho pôvodu. Naše výsledky ukazujú na to, že inhibičný efekt kyseliny lipoovej je na úrovni expresie neskorých génov alebo morfogenézy vírusových častíc. Kyselina lipoová tak ponúka možnosť použitia ako podpornej látky pri liečbe infekcie poxvírusmi.

Summary

Vaccinia virus is a typical member of the poxvirus family. It had been successfully used during the worldwide smallpox eradication campaign. Currently, it is used as vector for prophylactic, as well as experimental purposes.

This PhD thesis stems from the findings previously published in our lab. It is partially focused on a further characterization of vaccinia virus effects on the type of host cell death. The results point to the activation of apoptosis during vaccinia virus infection, but it cannot be completed and the cell dies by necrosis. Our attempts to shift the necrotic type of cell death induced by vaccinia virus infection towards apoptosis using a pharmacological inhibition of activity of a key enzyme PARP (Poly-(ADP-ribose) polymerase) remained unsuccessful. The effects of vaccinia virus-encoded anti-apoptotic factors appear superior to the inhibition of this single enzyme.

The second part of the thesis is focused on inhibitory effects of a redox-modulating compound, lipoic acid, on vaccinia virus infection. Our results demonstrated its inhibitory effects in cell lines of different embryonic origin. It appears that the lipoic acid inhibits vaccinia virus growth at the stage of late gene expression or possibly later, during virus morphogenesis. Lipoic acid could be potentially used as a supportive treatment in therapy of a poxvirus infection.

1. Introduction

1.1 Vaccinia virus

Vaccinia virus (VACV) is a typical member of the poxvirus family. VACV genome consists of one molecule of dsDNA, approximately 195 kb long that encodes about 200 proteins. VACV replication takes place in the host cell cytoplasm.

VACV has been widely used because of its properties like high stability, easy and inexpensive preparation, ability to induce robust and long-lasting humoral and cellular immune responses together with a capacity to integrate big portions of DNA. It serves as a constituent of several recombinant vaccines and vectors, and it also offers opportunity for the study of host cell interactions (Condit andNiles, 2002; Pastoret andVanderplasschen, 2003). Thanks to these properties VACV was successfully used during the worldwide smallpox eradication campaign (Buller andPalumbo, 1991).

VACV is morphologically one of the largest viruses. It produces two major infectious forms, intracellular mature virion (IMV) and extracellular enveloped virion (EEV; (Schepis et al., 2006). The VACV virion core is released after virus entry into the cell. Almost immediately after the entry, transcription of about 100 different molecules of early mRNA's occurs, employing the transcriptional apparatus included in the virion. Early mRNA's are then released into the cytoplasm and viral proteins are synthesized (Schramm andLocker, 2005). Viral DNA replication is independent of the host cell nucleus and it takes place in the cytoplasm parts called virosomes or viral factories. After DNA replication, synthesis of mRNA's encoded by the intermediate genes

occurs (Baldick andMoss, 1993). Consequently, expression of VACV late genes takes place. Some of the late proteins are then glycosylated, phosphorylated and cleaved before or during their incorporation into virus particles (Buller andPalumbo, 1991). Assembly of viral particles is complex. Stepwise, structural proteins, enzymes and viral DNA are surrounded by immature viral membranes. Subsequently, virions are transported from the cytoplasm.

1.2 Effect of the vaccinia virus infection on the host cell metabolism

VACV, like a typical poxvirus, causes a cytopathic effect of the host cells, the nature of which is dependent on the type and origin of the host cell.

There are significant changes in the metabolism of host cells during VACV infection. Host cell DNA synthesis is inhibited soon after the entry of the virus and the host DNA is hydrolysed by an endonuclease present in the virion.

Host cell RNA synthesis and processing is also inhibited, but it occurs only after viral protein synthesis. The host mRNA half-life, as well as the viral RNA, is decreased during the course of VACV infection. VACV itself inhibits also the host protein synthesis by various mechanisms. The rate of inhibition depends on the type of infected cell and the multiplicity of infection (Buller andPalumbo, 1991). On the other hand, VACV infection is in turn affected by the the energy metabolism of the host cell (Vrbacky et al., 2003).

1.3 Virus interaction with the host cell defense system

The poxviruses are successful pathogens thanks to several mechanisms, which help them to resist and overcome host cell defense system.

VACV has developed a strategy against the host complement system. VCP, VACV complement control protein, is a 35-kDa protein secreted by VACV-infected cells that inhibits activation of the classical complement cascade (Girgis et al., 2008; Kotwal et al., 1990). Poxviruses overcome the host interferon (IFN) system by secretion of soluble homologues of IFN receptors. Those receptors are highly species-specific. VACV expresses a B8R binding to human and rat but not mouse IFNγ, and B18R binding to IFN α/β of several species (Alcami andSmith, 1995). In addition, VACV also interferes with the effector phase of the interferon-inducible defense mechanisms (e.g. inhibitors encoded by the E3L and K3L genes (Johnston andMcFadden, 2003).

Similarly, proteins encoded by VACV affect other parts of the IFN pathways. H1L gene of VACV expresses phosphatase, which inhibits IFN-induced activation of the transcription factor STAT-1. Products of VACV genes A46R and A52R contain domains homologous to the Toll-like/IL-1 receptor, enabling them to interrupt IL-1 signaling pathway and inhibit NF-κB activation induced by IL-1 (Johnston andMcFadden, 2003).

1.4 Apoptosis

A programmed cell death – apoptosis, plays an important role in the elimination of infected cells. The infected cells are controlled by two mechanisms directing them to apoptosis. The first mechanism consists in the presentation of viral

peptides on the surface of infected cells in complex with the major histocompatible antigens. The complex is then recognized by cytotoxic T lymphocytes that induce apoptosis of the infected cells. The second mechanism is linked to the control of viral infection by the cell itself. The cell detects a virus-induced activation of the cell cycle proteins and starts the process of apoptosis through the p53 protein (O'Brien, 1998).

Cascade cleavage and activation of caspases, cysteine proteases, is a central process of apoptosis (Kumar, 2007). Caspases are involved in the initiation and effector phases of apoptosis (caspases 8, 9, 10, and caspases 3, 6, 7, respectively). The efector caspase action results in cleavage of several cellular proteins, leading to the cell death. Caspase or death substrate cleavage is then responsible for the execution of apoptosis, including the morphological changes (Fischer et al., 2003).

Apoptosis can be triggered by extracellular or intracellular signals. The extracellular receptor pathway is activated by a death ligand binding to the corresponding cell death receptor. The binding is followed by a signal transduction via adapter proteins and subsequently activation of caspase 8. Active caspase 8 may activate effector caspase 3 directly or via protein Bid and mitochondria (Danial andKorsmeyer, 2004).

The intracellular apoptotic pathway is initiated by stress signals leading to mitochondrial permeability transition and the release of cytochrome c and/or apoptosis inducing factor (AIF). The release of cytochrome c leads through its interaction with the adapter molecule Apaf-1 to the activation of caspase 9 in apoptosome, resulting in the activation of effector caspases (Ghobrial et al., 2005).

A third, newly described, apoptotic pathway is initiated in the endoplasmic reticulum (ER). Changes in Ca^{2+} ion homeostasis and accumulation of the missfolded proteins leads to the ER stress, which may result in the activation of caspase 12, leading to the activation of caspase 9 (Rao et al., 2004).

Mitochondria represent a key checkpoint in apoptosis. Induction of apoptosis leads to the reduction of mitochondrial membrane potential ($\Delta\Psi_m$) and release of many pro-apoptotic factors (Green andKroemer, 2004). Release of these factors from mitochondria is strictly regulated by proteins of the Bcl-2 family.

Poxviruses have developed several mechanisms to interfere with the processes of apoptosis directly or indirectly. They encode a first inhibitor caspases to be described, CrmA (Taylor andBarry, 2006). This protein, also known as SPI-2 (serin protease inhibitor 2), inhibits caspase 1 and 8. Its homologue SPI-1 prevents apoptosis by binding to catepsin G (Guo et al., 2005; Shisler et al., 1999). Several other proteins that interfere with apoptosis are encoded by VACV. Among them, F1L is unique for its inhibition of the loss of $\Delta\Psi_m$ and the release of cytochrome c from mitochondria provoked by various inducers of apoptosis. A possible mechanism of its anti-apoptotic action consists in the interaction with BH3-proteins of the Bcl-2 family (Stewart et al., 2005; Wasilenko et al., 2003).

1.5 Vaccinia virus-encoded redox system

Certain VACV-encoded proteins contain stable disulfide bonds in their domains. The disulfide bonds formation occurs through the virus-encoded redox pathway in the host cell cytoplasm. The importance of this pathway for a successful virus replication has been demonstrated by disabling each of

its three key protein constituents, resulting in the inability to form viral particles in each case (Senkevich et al., 2002a; Su et al., 2006).

1.6 Smallpox virus – potential for misuse

Initial attempts to protect against smallpox date back to the ancient Egypt. In the 18th century, Edward Jenner introduced vaccination (Lat. vacca = cow) consisting in the application of the material from the cowpox lesions as a preventive measure against smallpox. VACV became the invariable part of the smallpox vaccines only at the end of the 19th century. Vaccination with various strains of this virus enabled smallpox eradication by 1980 (WHO, 1980). Since then, vaccination of the general population was stopped.

However in recent years, a threat of a potential misuse of smallpox as a biological weapon appeared. Due to the lack of immunity in the general population, high transmissibility of variola and mortality on the disease, the impact of smallpox release in today's population would be much more disastrous than in the past century. On the other hand, vaccination with VACV is associated with a risk of side effects higher than any other vaccine and they are 10-times more likely to occur in the first-time adult vaccinees, which is an obstacle to reintroduction of vaccination.

In addition to misuse of poxviruses as biological weapons, there is also a threat emerging from the natural environment. Monkeypox virus causes a disease in human that is similar to smallpox.

1.7 Post-vaccination complications caused by vaccinia virus

Post-vaccination complications caused by VACV relate mainly to the excessive VACV multiplication at the vaccination site or in other parts of the body. The most frequent complication consists in an occasional inoculation in other parts of the skin due to virus transfer from the vaccination site. Generalized vaccinia, a condition when the virus spreads by blood and the pustules appear on distant parts of the body, is less common but usually without further complications. Both forms could result in serious consequences in immunocompromised individuals, though. Serious complications of vaccination occur mostly in people with deffects in natural or specific immunities and they include eczema vaccinatum, progressive vaccinia and post-vaccination encephalitis. Other complications comprise fetal vaccinia, skin erythema, myopericarditis and bacterial superinfections (Bray, 2003; Fulginiti, 2003).

1.8 Therapy of postvaccination complications caused by vaccinia virus

No specific therapy for postvaccination complications associated with VACV vaccination was available until the 50-thies when VACV immunoglobulin and tiosemicarbozone derivatives were introduced. During many years of research, VACV used to be included among viruses that were tested for sensitivity to different kinds of chemicals. Most of the substances that have proven efficacy against VACV are nucleoside analogues. The only substance approved by the U.S. FDA for the treatment of VACV postvaccination complications, cidofovir (Vistide ®, HPMPC) was discovered

by prof. Holý and it belongs also to a group of nucleoside analogues (De Clercq et al., 1986). The disadvantage of cidofovir is an intravenous administration and nephrotoxicity (Safrin et al., 1997). However, the unique antiviral activity of cidofovir led to a development of its oral form CMX001 (Sliva andSchnierle, 2007) and other derivatives (Krecmerova et al., 2007).

Recently described substances active against VACV include also those that inhibit its spread: Gleevec (STI-571, Glivec) and ST-246 (Sliva andSchnierle, 2007).

1.9 Redox-modulating agents

Part of this work is focused on inhibitory effects of thiol-reactive substances on VACV growth and replication, in particular, on lipoic acid. Lipoic acid (LA) is a disulfide derivative of the octanoic acid, which forms intramolecular disulfide bond in its oxidised form. Inside the cell, a probable site of LA synthesis and action are mitochondria (Bilska andWlodek, 2005).

Increased interest of current medicine in this substance results from its unique reduction capacity. Reduced LA participates in the neutralizing reactions of oxygen radicals, as well as in the reducing reactions of oxidized forms of other antioxidants. In addition, LA has another special feature – it is soluble not only in the water but also in fat. All these features contribute to the fact that LA is often called "the antioxidant of antioxidants" (Bilska andWlodek, 2005).

Currently, LA raises considerable interest for its therapeutic use and positive impact on the treatment of several diseases such as diabetes, atherosclerosis, degenerative nerve disease or AIDS (Bilska andWlodek, 2005).

It has been shown that the LA inhibits HIV replication by preventing activation of NFκB in cell cultures (Pande andRamos, 2003).

2. Aims and hypothesis

In this work, we focus on further elucidation of previously published findings showing activation of caspases during VACV infection and on the effects of redox-modulating agents on the VACV infection.

Specific aims of the work:

1. Analysis of the protease substrates cleaved during VACV infection.
2. Study of the inhibition of caspase and PARP activities and the effects on VACV infection.
3. Preparation of cell lines with an inducible expression of Bcl-2 and analysis of effects of the expressed Bcl-2.
4. Characterization of the effect of redox-modulating agents, especially LA, on VACV infection.

3. Material and methods

Chemicals. All cultivature media and growth supplements used were from Invitrogen, Gibco or PAA Laboratories. Other chemicals were from Sigma, Fluka, Promega, R&D Systems, Applied Biosystems and Molecular Probes.

Cell lines. The following cell lines were used in experiments: human epithelial cell line HeLa-G, green monkey kidney cell line BSC-40, mouse fibroblast cell line L929,

mouse monocyte/macrophage cell line J774.G8 and human cell line Jurkat.

Preparation and screening of stably transfected cell lines. Preparation of stably transfected cell lines was performed according the Tet-On transfection system manufacturer protocol. The Superfect (Qiagen) was used as a transfection agent. Stably transfected cell lines were selected with G.418 and hygromycin, respectively. Transfected cells were screened using determination of luciferase activity after transient transfection of a reporter plasmid or using western blot analysis.

Viruses. Wild-type VACV (WT-VACV), strain Western Reserve (WR; ATCC VR-119) and recombinant VACV expressing chloramphenicol acetyltransferase (CAT), Bcl-2, PKR, inducible NO-synthetase (iNOS) and luciferase (Luc) were used in the experiments.

Flow cytometry. The flow cytometer FACScan (Beckton Dickinson) was used for flow cytometric analyis. Data analysis was performed using the software WinMDI v2.8.

Fluorescence microscopy. Cultured cells were examined directly in culture plates using an inverse fluorescence microscope Olympus IX-70.

Western blot. SDS-PAGE a Western blotting were performed in accordance with published protocols (Ausubel et al., 2002; Harlow andLane, 1988; Laemmli, 1970).

DNA isolation and real-time PCR. For real-time PCR analysis, the cells were prepared according to a published protocol (Schmidtmayerova et al., 1998). Viral DNA was quantified using the Applied Biosystems 7300 Real-time PCR System. Primers and probes were prepared by Applied Biosystems as Custom TaqMan® Gene Expression Assay. Primers set and probe were selected for the terminal part of

VACV genome. Results were analyzed by the Sequence Detection Software (Applied Biosystems).

Statistics. Results were presented as averages +/- S.E.M. (standard error of mean). Statistical differences within the single group were calculated using ANOVA. Statistical differences between the group and controls or between the two different groups were analyzed by Student t-test.

4. Results

4.1. Lytic cell death in vaccinia virus-infected cells

VACV causes lysis, an equivalent of necrosis, in most of the infected cells. Bcl-2 expressed by VACV reveals two opposite effects repeatedly described in the past: it shows a typical anti-apoptotic effect in Hela G cells, while it induces apoptosis in BSC-40 cells. (Kalbacova et al., 2008; Kalbacova et al., 2003; Melkova et al., 1997; Vrbacky et al., 2003). These results were confirmed also in this work (Kalbacova et. al. 2008).

4.2. Caspase activity during a lytic infection with vaccinia virus

Caspase activation and/or activity detected by flow cytometry were observed in VACV-infected HeLa G and BSC-40 cells (Kalbacova et al., 2008; Kalbacova et al., 2003).

As these earlier results were obtained only from infectious experiments, we decided to supplement them and compare with those obtained after induction of apoptosis with non-infectious inducers under the same experimental conditions. Based on our results, we can conclude that the induction of apoptosis by ionomycin and staurosporine caused

significantly increased caspase activation/activity. The level of activation and activity of caspases in this non-infectious model was comparable to the levels found in both VACV-infected cell lines tested (Kalbacova et al., 2008).

4.3. Cleavage of caspase substrates during vaccinia virus infection

To supplement the flow cytometric measurements, we decided to characterize caspase activity using western blot analysis of caspase substrates cleavage.

4.3.1. Cytokeratin 18

First, we examined a cleavage of cytokeratin 18. An obvious decrease in levels of the whole-size cytokeratin 18 was observed in VACV infected Hela G cells and BSC-40 cells (Fig. 1). We can conclude that infection with both VACV recombinants used led to the reduction in cytokeratin 18 levels in both tested cell lines.

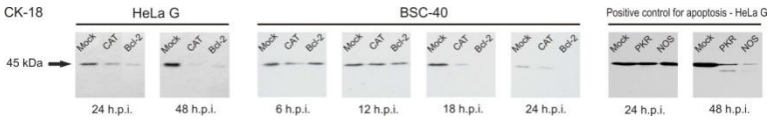

Fig. 1. Western blot analysis of the cytokeratin 18 cleavage in VACV-infected cells.

4.3.2. Actin

Actin, one of the cell cytoskeleton components, is also a caspase substrate. Its cleaved fragments contribute to the changes of apoptotic cell morphology (Chang andYang, 2000; Stroh andSchulze-Osthoff, 1998).

Infection with both VACV recombinants tested results in a decrease of actin levels in Hela G cells at 24 h.p.i. VACV infection has not led to changes in actin levels in BSC-40 cells (Fig. 2).

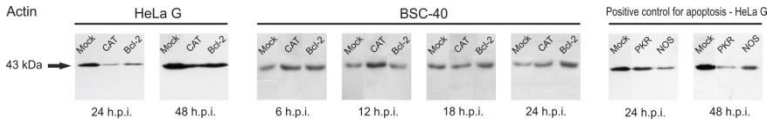

Fig. 2. Western blot analysis of the actin cleavage in VACV-infected cells.

4.3.3. PARP

We further analyzed a cleavage of the poly(ADP-ribose) polymerase (PARP) protein, a typical caspase substrate. PARP is inactivated by cleavage to an 85 kDa fragment during apoptosis (Kaufmann et al., 1993).

No significant PARP cleavage was found in VACV-infected Hela G cells in comparison with uninfected controls (Fig. 3). Similarly, PARP was not cleaved in uninfected nor VACV-CAT-infected BSC-40 cells. In contrast, the 85 kDa PARP cleavage fragment was detected in VACV-Bcl-2-infected BSC-40 cells. This finding is consistent with the apoptotic morphology of these cells detected since 12 h.p.i.

The obtained results show that the cleavage of PARP typical for apoptotic cells was observed only in those VACV-infected cells that exhibit morphological signs of apoptosis.

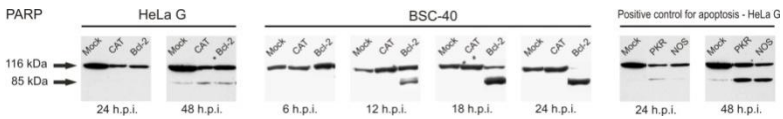

Fig. 3. Western blot analysis of the PARP cleavage in the VACV-infected cells.

4.4. Effect of the caspase inbition on vaccinia virus infection

Based on our previous results, it seems possible to suggest that caspases might have a special role during the VACV infection. Therefore we decided to characterize the effect of caspase inhibition on VACV infection.

Fig. 4 shows that the administration of a pan-caspase inhibitor z-VAD-FMK did not reveal any statistically significant effect on VACV growth in Hela G nor BSC-40 cells.

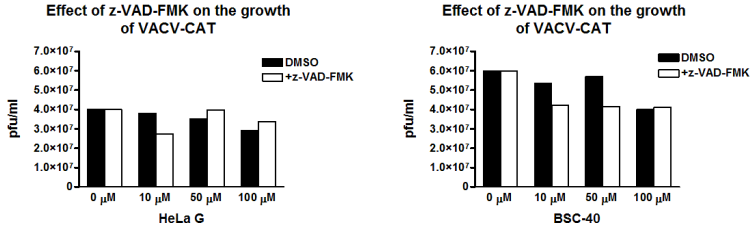

Fig. 4. Effect of a pan-caspase inhibitor z-VAD-FMK on VACV growth.

Additional analysis of the cleavage of cytokeratin 18 in the presence of z-VAD-FMK shows that cytokeratin 18 is most likely cleaved by proteases other than caspases.

4.5. Effect of PARP inhibition on vaccinia virus infection

Based on the previous results, we can speculate that there is a shift in the type of the on-going cell death during VACV infection. The apoptotic program at the beginning of infection, demonstrated by caspase activation, seems to change and continue as necrosis. It is possible that this change occurs because of a reduced availability of energy supplies. PARP

cleavage is a critical event that decides whether the cellular signals link to apoptosis or necrosis (Nicotera andLeist, 1997). PARP activation leads to a reduction of available ATP, which results in necrosis. In contrast, when PARP is cleaved, sources of ATP are preserved and the cell has enough energy for the process of apoptosis.

As PARP remains uncleaved during the infection with VACV, it is likely to be active. Its pharmacological inhibition could therefore result in preservation of ATP and increased chances of the cell to execute apoptosis.

To examine the effects of PARP inhibition on VACV-induced type of cell death, we used a potent PARP inhibitor PJ 34. We did not observe any changes in PARP cleavage in infected cells treated with the inhibitor. On the contrary, we observed a significant cleavage of cytokeratin 18 in Hela G cells infected with VACV and treated with PJ 34. As VACV infection induces a decrease of $\Delta\Psi_m$ and/or a decrease of total cellular levels of ATP, changes in mitochondrial function were also analyzed in the presence of PJ 34. However, addition of the PARP inhibitor during VACV infection had no significant effect on the changes of $\Delta\Psi_m$ induced by VACV.

In summary, we were not able to demonstrate any significant effects of PARP inhibition on VACV infection and the type of cell death in our experiments. VACV-encoded anti-apoptotic activities probably affect the cell death machinery more strongly and their role is superior to the effects of PJ 34.

4.6. Stably transfected cell lines

A unique pro-apoptotic activity of the protooncogene Bcl-2 expressed by VACV (Kalbacova et al., 2008; Kalbacova et al., 2002) can consist in the properties of the protein itself or in its behavior and/or interactions in a specific cellular

environment, but also in the type of the expression system. We have therefore tried to examine the behavior of Bcl-2 using its expression using the expression system other than VACV. We have chosen the Tet-On system, which should allow a high level of induction of expression compared to the basal conditions and a tight concentration-dependent regulation.

4.6.1. Effect of the inducible expression of Bcl-2 on BSC-40 cells and their infection with vaccinia virus

We have generated a stably transfected BSC-40 cell line expressing Bcl-2 under the inducible promoter Tet-On. However, the induction of expression of Bcl-2 did not lead to any significant morphological changes in the stably transfected BSC-40 cell line and the cells revealed a typical morphology similar to the parental BSC-40 cells.

We also analyzed the effect of Bcl-2 expression on the VACV infection. The plasma membrane integrity analysis by flow cytometry shows changes typical for the lytic VACV infection. However, we did not observe any significant changes due to the inducible Bcl-2 expression by the Tet-On system. Similarly, we did not experience any significant changes in $\Delta\Psi_m$ after inducible Bcl-2 expression.

Based on our results, we can conclude that in our experimental conditions and at the levels of expression achieved using the Tet-On expression system, expression of Bcl-2 had no significant effect on the stably transfected BSC-40 cell line morphology nor the VACV infection.

4.7. Effect of redox-modulating agents on vaccinia virus infection

The VACV structure is dependent on the disulfide bonds which are formed during the virion morphogenesis by several VACV redox proteins (Locker andGriffiths, 1999; Senkevich et al., 2002a; Senkevich et al., 2002b). It is possible that agents affecting the redox environment of infected cells could also affect the VACV infection.

4.7.1. Effect of different redox-modulating agents on vaccinia virus infection

We analyzed the effect of five different redox-modulating agents (β-mercaptoethanol, DTT, ascorbic acid, LA and Ethacrynic acid) on the VACV infection in our experiments (Spisakova et al., 2009). First, we analyzed the effect on the growth of VACV characterized by luciferase activity in cells infected with VACV expressing luciferase (Fig. 5). We measured a statistically significant increase of luciferase activity for the three agents tested - β-merkaptoethanol, DTT and ascorbic acid. Other two substances, LA and ethacrynic acid, resulted in a statistically significant reduction in luciferase activity.

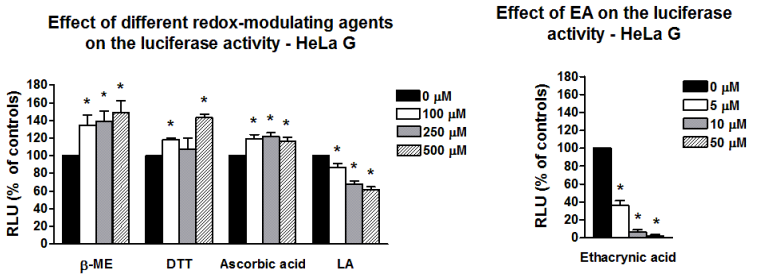

Fig. 5. Effect of different redox-modulating agents on VACV infection.

Based on these results we decided to further characterize the inhibitory effect of LA on VACV infection. In a series of experiments with LA, we experimentally confirmed its inhibitory effect on the growth of VACV in cell lines of different origin.

4.7.2. Effect of the lipoic acid on the early and late vaccinia virus genes expression

First, we characterized in which phase of VACV growth cycle inhibitory effects of LA occur. To distinguish the effect on the synthesis of VACV either early or late genes, VACV recombinants expressing luciferase under two different VACV promoters (early/late - VACV E/L, and late - VACV L) were used. A DNA polymerase inhibitor, cytosine arabinoside (Ara C), was also added to the experimental samples. As Ara C inhibits VACV DNA synthesis, expression of VACV late proteins does not occur. Therefore in the presence of Ara C, we have determined activity of luciferase expressed only under control of the VACV early promoter.

The results showed that LA had no inhibitory effect on the VACV early gene expression, while the late gene expression was blocked (Fig. 6).

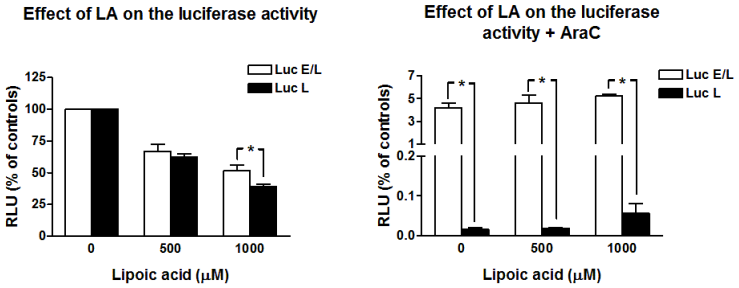

Fig. 6. Effect of the LA on VACV early and late gene expression.

4.7.3. Effect of the lipoic acid on the overall vaccinia virus protein expression

In another series of experiments, we analyzed the effect of LA on the overall expression of VACV proteins in HeLa G cells using western blot analysis. Addition of 250 and 500 μM LA revealed no visible effect on the distribution or levels of VACV proteins in both VACV recombinants tested. We observed only a slight decrease of viral protein levels in the presence of 1 mM LA.

Based on these results, we can conclude that LA affects VACV growth cycle at the level of viral DNA synthesis, viral late gene expression or virion morphogenesis.

4.7.4. Effect of the lipoic acid on vaccinia virus DNA replication

The effect of LA on VACV DNA replication was analyzed by the real-time PCR. The results in Fig. 7 show that LA does not inhibit viral DNA synthesis. On this basis, it can be concluded that LA exerts its effect at the later stages of the VACV growth cycle, i.e. at the level of VACV late gene expression or morphogenesis.

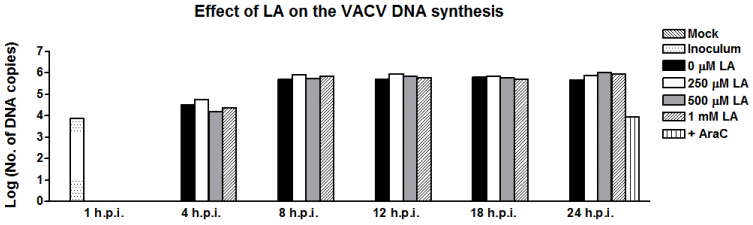

Fig. 7. Effect of the LA on VACV DNA synthesis.

4.7.5. Effect of the lipoic acid on vaccinia virus infection depending on the time of addition

Finally, we decided to confirm the stage of VACV growth cycle by adding the LA at different times after infection. Fig. 8 shows a strong inhibitory effect of LA on the VACV infection when added at or before 4 h.p.i., which is a time when viral DNA synthesis starts. Lower, but also statistically significant inhibition was observed when the LA was added at 8 h.p.i. As the viral DNA replication should be over at this time post infection, the results further support the previous conclusion that LA inhibits VACV growth at the level of the late gene expression.

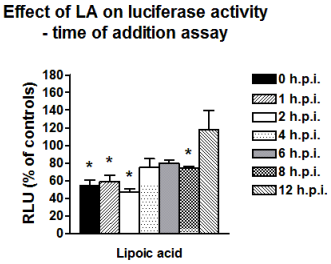

Fig. 8. Effect of the LA on VACV infection depending on the time of its addition.

In conclusion, we have demonstrated the inhibitory effect of LA on VACV growth in cell lines of different origin. According to our results, the inhibition takes place at later stages of VACV growth cycle - at the level of VACV late gene expression or possibly at the stage of virion morphogenesis.

5. Discussion

5.1. Type of cell death induced by vaccinia virus infection

VACV causes lysis in the most of infected cells. This type of cell death is considered as equivalent to necrosis. Morphology of infected epithelial cells used in our experiments confirms the necrotic type of cell death. The exception is the apoptosis induced after VACV-Bcl-2 infection of BSC-40 cells. However, this is a previously described phenomenon induced by VACV-driven overexpression of Bcl-2 in these cells. Necrosis and apoptosis differ in several morphological and biochemical processes. The most typical for apoptosis, but not for necrosis, is the activation of caspases. My predecessor and colleague in the lab, Marie Kalbáčová, observed the activation of caspases during the lytic infection

with VACV (Kalbacova et al., 2008). Based on these unexpected findings, we decided to further analyze the type of cell death caused by VACV infection.

The cleavage of specific caspase substrates in VACV infected cells was studied by Western blotting. Our results confirmed the cleavage of cytokeratin 18 during the lytic infection with VACV in both cell lines tested, while opposite results were observed in case of PARP. We did not observe any PARP cleavage in either of VACV infected cell lines. Therefore, we can assume that PARP is active in these cells and participates in the consumption of cellular ATP, leading to the necrosis of infected cells. However, the reasons why cytokeratin 18 is cleaved during the infection with VACV, while PARP remains uncleaved, remain to be explained.

Thus, it seems that apoptosis is activated during the infection with VACV, but it can not be completed and the cell dies due to necrosis. A possible explanation may consist in the initiation of the apoptotic program by infection with VACV, but soon after, the apoptosis is inhibited by various caspase inhibitors and other proteins affecting apoptosis that are encoded and expressed by VACV (Stewart et al., 2005; Taylor et al., 2006; Wasilenko et al., 2003). VACV infection results in apoptosis in certain immune cell types (macrophages, dendritic cells and B lymphocytes) (Baixeras et al., 1998; Engelmayer et al., 1999; Humlova et al., 2002). In these cases, apoptosis might probably serve as a strategy how to overcome immune defense mechanisms of the host. These examples also suggest that in some cases, sufficient energy supplies to execute apoptosis might be present .

5.1.1. Effect of caspase inhibition on vaccinia virus infection

Based to our results, it is possible to expect that caspase activation might play some role during the infection with VACV. Therefore we decided to analyze the possible effect of caspase inhibition on VACV infection. However, the results obtained revealed only a marginal affect of a pan-caspase inhibitor on VACV growth. Unexpectedly, we observed a more pronounced reduction in levels of the whole-size cytokeratin 18 after the inhibition of caspases. Therefore, it seems that cleavage of cytokeratin 18 could be executed also by proteases other than caspases.

5.1.2. Effect of the PARP inhibition on the cell death induced by Vaccinia virus

The intracellular pool of ATP is one of the key determinants of the cell death type. It has been shown that if the level of ATP was reduced, a typical apoptotic stimulus resulted in necrosis (Leist et al., 1997). PARP plays an important role in consumption of ATP, and it is likely to be activated due to VACV-induced DNA damage.

Therefore, our aim was to prevent the enzymatic activity of PARP by a specific PARP inhibitor PJ 34, and thus save energy supplies for the processes of apoptosis. However using western blot analysis, we did not observe any changes either in levels of PARP or in its cleavage in VACV-infected cells treated with PARP inhibitor. There were no morphological signs of apoptosis either. In the experiments performed, it is likely that PARP activity was inhibited, but a pharmacological inhibition of this single protein was not sufficient to change the type of cell death. In summary, a contribution of various

VACV-encoded inhibitors of apoptosis is probably superior to the effect of PJ 34.

5.2. Stably transfected cell lines

Upon expression of a particular recombinant protein by VACV, virus interactions with the protein itself and/or with the host cell also play an important role. The observed effects of Bcl-2 protein expressed by VACV differ in dependence on the host cell type and metabolism. Therefore, we decided to use another expression system for its expression to distinguish the effects caused by the interactions of Bcl-2 and VACV from the effect of a different intracellular microenvironment.

After a successful preparation of the cell line BSC-40 expressing Bcl-2 under control of a promoter inducible by doxycycline, we characterized the effects of Bcl-2 on the processes of apoptosis. The experiments performed did not prove any effect of the expressed Bcl-2 on cell morphology, changes of metabolism or VACV infection in stably transfected cell lines. However, further characterization of the effects caused by Bcl-2 overexpression was hampered by low and decreasing levels of Bcl-2 expression. The increased expression of Bcl-2 is probably detrimental for BSC-40 cells, which is in line with the observed apoptosis of BSC-40 cells infected with VACV-Bcl-2.

The reason why we did not observe any effects of Bcl-2 expressed by stably transfected cell lines could consist in a lower level of its expression compared to the VACV expression system. Therefore, it might be more appropriate to use a stronger expression system, e.g. adenovirus, to study the effects of Bcl-2 independently of VACV.

5.3. Effect of the redox-modulating agents on the growth of vaccinia virus

We focused on the characterization of redox-modulating agents, especialy LA, on the growth of VACV. First, we experimentally analyzed the effect of five different redox-modulating agents, β-merkaptoethanol, DTT, ascorbic acid, LA and EA, on VACV infection. We have observed an inhibitory effect only after the treatment with LA and EA. The effects were concentration-dependent and the LA was effective in high micromolar range. The mechanism of the LA-mediated inhibitory effect could consist in the interference with disulfide bond formation and/or exchange or with redox cycling of a thioredoxin, glutaredoxin, or similar compounds. LA could directly target VACV redox-dependent proteins or change the intracellular redox potential. VACV morphogenesis is dependent on a cytoplasmic redox pathway encoded by VACV that consists in a sequential disulfide bond formation. The change in function of any of the three key virus proteins results in an incorrect virus maturation (Su et al., 2006).

LA-mediated inhibition of VACV growth could also consist in the inhibition of activation of the transcription factor NF-κB by decreasing levels of oxygen free-radicals. Nevertheless, VACV itself inhibits NF-κB activation and translocation into the nucleus (Graham et al., 2008).

In our experiments, we have confirmed the inhibitory effect of LA in cell lines of different embryonic origins. In the same time, the effective concentrations of LA did not reveal any cytotoxic effects in cell lines tested, as assessed by the MTT test. The IC50 of LA ranges in 200-500 μM as determined for VACV titer. Published IC50 values of cidofovir for VACV are 9.8 μM for human embryonic fibroblasts and 54

- 62 μM for Vero cells. Although the results obtained by different tests under different conditions can be compared only with some limitations, we realize that LA inhibitory concentrations are probably therapeutically uninteresting. However, our aim was to describe this new inhibitory effect of LA on VACV growth and to further characterize the step in VACV growth cycle that is inhibited. The results showing that LA inhibits VACV late gene expression or possibly a later step in virus morphogenesis point to the new possibilities in the inhibition of poxvirus infection.

Despite the high effective concentrations of LA against VACV, it reveals many favorable properties that make it an attractive target worthy of further investigation and development. LA is well absorbed from the intestine, it passes well the blood-brain barrier, it is non-toxic even at high concentrations for most cells, and it is used as a food supplement. Even if LA is not likely to be used as a single therapy of the poxvirus infection, it is possible to imagine that it could serve as a supportive treatment in combination with other antiviral agents.

6. Conclusions

This PhD thesis has focused on detailed characterization of the previously demonstrated events in VACV infection and on the presentation and analysis of new inhibitory properties of certain redox-modulating agents. The results can be briefly summarized as follows:

1. In the experiments characterizing cleavage of caspase substrates, we confirmed the cleavage of cytokeratin 18. On the other hand, the cleavage of another caspase substrate,

PARP, was not observed. The results point to the activation of apoptosis during the VACV infection. However, apoptosis can not be completed and the cell dies by necrosis.

2. Experiments characterizing the effect of caspase inhibition during VACV infection were performed with a pan-caspase inhibitor z-VAD-FMK. However, this inhibitor revealed only a marginal effect on VACV growth and a more pronounced effect on the cleavage of cytokeratin 18, suggesting that proteases other than caspases can be involved in its cleavage.

Attempts to inhibit activity of PARP and thus shift the necrotic type of cell death during VACV infection towards apoptosis remained unsuccessful. The effects of other VACV-encoded anti-apoptotic factors appear superior to the inhibition of PARP activity.

3. We prepared a stably transfected cell line BSC-40 expressing Bcl-2 under control of the inducible Tet-On expression system. In the experiments performed, we did not observe any significant changes in the type of cell death due to Bcl-2 expression. However, further characterization of the effects caused by Bcl-2 overexpression was hampered because of a low and decreasing level of Bcl-2 expression. The increased expression of Bcl-2 is probably detrimental for BSC-40 cells.

4. In experiments focused on the effects of five redox-modulating agents on VACV growth, we observed a stimulatory effect exerted by β-mercaptoethanol, DTT and ascorbic acid, and a concentration-dependent inhibitory effect exerted by lipoic and ethacrynic acids. In this work, we further

analyzed the inhibitory effects of LA. It appears that LA inhibits VACV growth at the stage of late gene expression or later during virus morphogenesis.

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