

Modulation of GAPDH expression and cellular localization after vaccinia virus infection of human adherent monocytes[★][✉]

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Vaccinia virus is able to replicate in many cell types and is known to modulate apoptosis in infected cells. In this study, expression of apoptosis-related genes was screened in human adherent monocytes after vaccinia infection using a DNA array. A marked increase of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was found. Increased expression and nuclear translocation of GAPDH have recently been reported to participate in apoptosis of many cell types. To confirm the array results, levels of GAPDH mRNA were estimated by RT-PCR, showing an increase at 4 h p.i. followed by a slight decrease, which correlated with the viral anti-apoptotic *E3L* gene transcript levels. Subcellular localization of the enzyme in human monocytes was examined by Western blot and immunostaining of the infected cells. Both experiments revealed accumulation of GAPDH in the nucleus at 14 h p.i., which was completely suppressed at 24 h p.i. This might indicate GAPDH as a novel target for vaccinia anti-apoptotic modulation.

Vaccinia virus, a member of the *Poxviridae* family, is a DNA virus which probably evolved from the cowpox virus, formerly used as an anti-smallpox vaccine (Moss, 2001). At present, vaccinia is used as a tool in molecular biol-

ogy (Henderson & Moss, 1999), a vector for a number of therapies, including HIV and cancer therapies, and as a recombinant vaccine for other diseases (Moss 1996, Cooney *et al.*, 1993). Therefore examining cellular response

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Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; p.i., post infection; PMSF, phenylethylsulfonyl fluoride.

to vaccinia infection and the interactions of the virus with cellular signaling is of vital interest.

Apoptosis, or programmed cell death, is a common response to viral infection. Poxviruses, replicating in many cell types of the host organism, have developed a variety of factors modulating the cellular apoptotic response triggered by infection. These include many apoptosis inhibitors, among them caspase inhibitors (serpins), bcl-2 homologues and inhibitors of PKR kinase (reviewed by McFadden & Barry, 1998). Vaccinia itself carries several genes whose products exhibit anti-apoptotic activity: B13R is a specific serpin (Kettle *et al.*, 1997) while E3L and K3L act as inhibitors of PKR kinase (Kibler *et al.*, 1997).

Human adherent monocytes are permissive for vaccinia virus: it replicates within these cells, preventing apoptosis. It was previously shown that infection with the virus stimulates interleukin 12 and 10 expression in human monocytes (Ślęzak *et al.*, 2000). In this study we aimed to investigate the modulation of cellular apoptotic pathways by vaccinia Western Reserve strain. Screening the levels of expression of apoptosis-related genes in infected human adherent monocytes with a commercial DNA array revealed, among others, a significant change in the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) mRNA after infection.

GAPDH is a key glycolytic enzyme, examined for its pivotal role in energy production. Recently, however, a number of diverse non-glycolytic activities of GAPDH in mammalian cells have been reported. It is involved in membrane fusion (Morero *et al.*, 1985), microtubule bundling (Sirover, 1999) and nuclear tRNA transport (Singh *et al.*, 1993). It also exhibits activity of uracil-DNA glycosylase (UDG) important in DNA repair (Baxi *et al.*, 1995) and even phosphorylating activity (reviewed in Sirover, 1999), although the physiological importance of these activities is not clear. Involvement of the enzyme in apoptosis was first suggested by Ishitani &

Chuang (1996), who showed that over-expression of GAPDH accompanies cerebellar neuron cell death and GAPDH antisense oligonucleotides prevent apoptosis. Since then, involvement of GAPDH in apoptosis in many cell types, mostly of neuronal origin, has been proved by independent research (Sawa *et al.*, 1997, Ishitani *et al.*, 1998). The present study is the first to investigate the changes in GAPDH expression in cells infected by an apoptosis-modulating virus.

MATERIALS AND METHODS

Cell culture. Human peripheral blood leukocytes (PBL) were isolated from blood obtained from healthy donors by centrifugation in Ficoll Paque gradient (Amersham Pharmacia), according to the method of Shringer & Weiss (1969). Cell culture was established at a concentration of 2×10^7 total PBL cells/5.5 cm dish for protein isolation and 8×10^6 total PBL cells/3.5 cm dish for RNA isolation. Cells were then cultured for 10–14 days in RPMI medium (Gibco BRL) with 10% human AB serum and inspected for signs of undesired stimulation (i.e. differentiation into dendritic cells) with medium change every 48 h, until monocytes reached full adherence (Guzdek *et al.*, 2000). The adherent monocytes make 10% of the total PBLs plated on the dish.

Virus propagation and infection. Vaccinia virus Western Reserve strain was propagated in VERO-B4 cells and titrated according to the Reed & Muenh method (Burleson *et al.*, 1991). Adherent monocytes were infected with the virus at a concentration of 1–5 pfu/cell and incubated for 1 h at 37°C before changing the medium.

RNA isolation. Total cell RNA was isolated using the TRIzol reagent (Gibco BRL), according to the modified method of Chomczynski & Sacchi (1987). Concentration and purity of the isolated RNA was measured spectrophotometrically at 260 nm and 280 nm and checked by denaturing gel electrophoresis (Ogden & Adams, 1987).

DNA array analysis. The radioisotope DNA array assay for apoptosis-related genes (Sigma-Genosys Panorama Human Apoptosis DNA Array Kit) was performed according to the manufacturer's protocol. Radioactive probes were prepared by reverse transcription of total cell RNA, using Human Apoptosis cDNA Labeling Primers included in the kit and 20 μ Ci of [32 P]dCTP (ICN).

Membrane was exposed on a Personal Molecular Imager FX screen (Bio-Rad) for 24 h and the collected signal was analysed and quantified using Quantity One (Bio-Rad). Because the results obtained by this method are semiquantitative, reflecting mainly the tendency of change (increase or decrease of gene expression) rather than amplitude, they were later confirmed using standard RT-PCR analysis of the genes of interest.

RT-PCR. Reverse transcription was performed using M-MLV reverse transcriptase (Gibco BRL). A mix of 2 μ l oligo(dT)₁₂₋₁₈ (Sigma), 5 μ g total RNA and 2 μ l 10 mM dNTPs in a total volume of 24 μ l was incubated for 5 min at 65°C and cooled on ice. After adding 8 μ l \times 5 First Strand Buffer (Gibco BRL), 4 μ l \times 100 mM DTT (dithiothreitol) (Gibco BRL) and 2 μ l ribonuclease inhibitor (Sigma), the mix was incubated for further 2 min at 37°C. Finally, 2 μ l M-MLV RT was added and the reaction mix was incubated for 50 min at 37°C, followed by termination of reaction for 15 min at 70°C.

PCR reactions were performed using RedTaq (Sigma) or DyNAzymeII (Finnzymes), in reaction mixes containing 2.5 U polymerase, 10 nmol dNTPs, 10–20 nmol of each primer (IBB, Warszawa), 2 μ l of cDNA and PCR buffer as supplied by the manufacturer, in a total volume of 50 μ l. The PCR conditions were the same for all samples: initial denaturation for 3 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 55°C and 1 min at 72°C, followed by final extension for 15 min at 72°C.

PCR primers used for the analysis were designed based on sequences deposited in the GenBank database, their sequences are listed

in Table 1. The expected lengths of the PCR products were 307 bp for β -actin, 360 bp for E3L and 283 bp for F17R and 402 bp for GAPDH. Average intensity of the signal was quantified using Quantity One (Bio-Rad).

Table 1. Sequences of primers used in RT-PCR analysis

Primer	5' – 3' sequence
β -actin forward	GCCGGGAAATCGTGCGTG
β -actin reverse	GGGTACATGGTGGTGCCG
E3L forward	TATATTGACGAGCGTTCTGAC
E3L reverse	ACTCATTAAATAATGGTGACAGG
F17R forward	ATTCTCATTTGCATCTGCTC
F17R reverse	AGCTACATTATCGCGATTAGC
GAPDH forward	GAAGGTGAAGGTCGGAGTCA
GAPDH reverse	TTCACCCCATGACGAACAT

Protein isolation. Total cellular proteins were isolated as follows: cells were washed with 1 ml cold phosphate buffered-saline (PBS) and harvested into 2 ml eppendorf tubes in 1–2 ml of PBS. Harvested cells were centrifuged at 200 \times g, 4°C for 2–5 min. The cell pellet was suspended in 150 μ l of extraction buffer (50 mM Tris pH 8.0, 10 mM Chaps, 2 mM EDTA, 1 mM Na₃VO₄, 5 mM DTT, 1 mM PMSF, 10% glycerol) (Dignam, 1990), and left on ice for 20 min. After pressing through a 0.6 mm syringe needle, the cell lysate was centrifuged at 12 000 \times g, 4°C for 5 min and stored at –20°C. Nuclear and cytosolic protein fractions were isolated according to the method described by Suzuki *et al.* (1994). Protein concentration was measured using BCA Assay (Sigma) based on bicinchoninic acid method (Smith *et al.*, 1985). Absorbance was measured at 562 nm in a SpectraMax 250 microplate reader.

Western Blot. SDS/PAGE was performed in 10% SDS/polyacrylamide gel as described by Davis *et al.* (1986). Protein transfer to a Hybond polyvinyl membrane (Amersham Pharmacia) was performed in a semi-dry blotting system Fastblot B31 (Biometra) (Kyhse-Andersen, 1984) in Tris/glycine/methanol transfer buffer, at 35 V for 30 min. The membrane was blocked

with 5% powdered milk in TST buffer (10 mM Tris/HCl, pH 7.5, 0.9% NaCl, 0.05 % Tween 20) for 1.5 h, followed by a 20 min. wash in the TST buffer. The membrane was then incubated for 1 h with primary mouse anti-human GAPDH monoclonal antibodies (Chemicon) diluted 1:1000 in TST buffer with 2% BSA (bovine serum albumin). After 4 washes in TST buffer the membrane was incubated with secondary anti-mouse IgG coupled with horseradish peroxidase (Sigma) diluted 1:80 000 in TST with 2% BSA for 1 h, followed by another 4 washes in TST. Protein was detected using ECL Plus chemiluminescence kit (Amersham Pharmacia) and the membranes were exposed on X-ray film for 5 s to 2 min. The GAPDH band was expected at 37 kDa (the molecular mass of the monomeric form).

Immunofluorescence cell staining. Monocytes were cultured as described above on sterile glass coverslips mounted in culture dishes. After viral infection the cells were fixed with 3% paraformaldehyde in PBS for 15 min at 37°C and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After blocking with 3% BSA in PBS for 15 min the cells were stained with mouse anti-human GAPDH monoclonal antibodies (Chemicon) diluted 1:1000 in PBS for 1 h and sheep anti-mouse FITC-conjugated antibodies (Sigma) diluted 1:60 in PBS for 1 h. The coverslips were mounted on microscopic glass slides using Vectashield (Vector Laboratories). Microphotographs were taken using an inverted system microscope IX 50 with epifluorescence optics with excitation maximum at 494 nm and emission maximum at 523 nm. The microscope was equipped with a CCD camera.

RESULTS

DNA array analysis

Total RNA was isolated from infected cells at selected time points after infection. Results of DNA assay for control cells and cells 4 h or 24

h post infection (p.i.) with 5 pfu of the virus were compared. Among the 198 apoptosis and cell cycle-related genes analysed by the DNA array, several groups of genes exhibited significant changes in expression. As genes associated with early steps of the cell death pathway are most likely to exhibit changes in expression before the suppression of apoptosis by vaccinia, they were of special interest to this study. One of them is the *GAPDH* gene, which increased substantially at 4 h p.i., especially with respect to its high cellular content was chosen for further analysis. Table 2 shows changes in the expression of selected genes associated with early steps of apoptosis.

Table 2. Apoptosis-related gene expression analysis using human apoptosis DNA array.

Only selected genes, both of pro- and anti-apoptotic function are shown. V₄, change at 4 h p.i. compared with uninfected cells; V₂₄, change at 24 h p.i. Cells were infected with 5 pfu of the virus. The results come from one representative set of hybridizations (control *versus* V₄ and control *versus* V₂₄).

	V ₄		V ₂₄	
	change	factor	change	factor
ARC	↑	5.15	↑	1.57
Bcl-2	↑	1.712	↑	6.120
eNOS	↑	1.82	↑	1.54
FLASH	↑	2.08	↑	1.94
FLIP	↑	2.38	↑	2.40
GAPDH	↑	8.01	↑	1.81
Sentrin	↑	2.06	↑	2.23
p53	↑	1.09	↓	2.67

GAPDH mRNA content increases at 4 h after infection

To confirm the semi-quantitative results of the DNA array analysis, changes in *GAPDH* mRNA content were investigated by RT-PCR of total RNA isolated from uninfected cells and monocytes infected with either 1 or 5 pfu of the virus at 4 and 24 h p.i. (Fig. 1). In this experiment, accumulation of *GAPDH* transcript at 4 h p.i. followed by a slight decrease

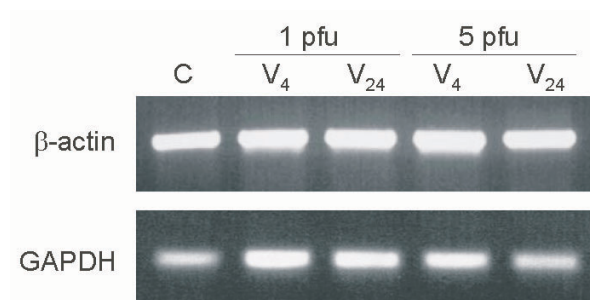


Figure 1. Changes in *GAPDH* mRNA level in adherent monocytes after viral infection.

Agarose gel electrophoresis of *GAPDH* RT-PCR products from control and infected monocytes. β -Actin gene product was used as a control. C, uninfected cells; V₄, 4 h p.i.; V₂₄, 24 h p.i.

at 24 h p.i. could be observed for both infectious doses of the virus used. However, the overall mRNA levels were slightly higher (increasing approx. 2–3-fold) after infection with 1 pfu of the virus than with 5 pfu.

GAPDH transcription is correlated with the viral life cycle

In order to check the viral infection itself, two vaccinia genes were chosen for analysis: the early gene *E3L*, involved in the vaccinia virus anti-apoptotic defence on the interferon pathway (Kibler *et al.*, 1997), and a late viral gene, *F17R*, the product of which takes part in the mature virions assembly (Moss, 1994). To confirm that the monocytes used for the experiment were not previously stimulated (e.g. by an infection of the donor), RT-PCR of the cellular stress and heat shock-related gene *Hsp70* and transcription factor HSF 1 were performed (not shown). Cells were infected with 4 pfu of the virus (to ensure total infection of cells in culture) and total RNA was isolated at four different time points.

As can be seen in Fig. 2, the *F17R* late viral transcript was still present in the infected cells after 48 h, indicating that viral replication was not stopped. An increase of *GAPDH* transcript level can be observed at 4 h p.i., remaining elevated over 48 h. The observed accumulation of *GAPDH* mRNA at 4 h p.i. corre-

lated with appearance of the early viral transcript *E3L*. On the other hand, a slight decrease in *GAPDH* level was observed at 14 h p.i., when the late viral transcript *F17R* appeared in the cells at a high level and a further slight increase at 48 h p.i., when *F17R* level dropped, showing a negative correlation.

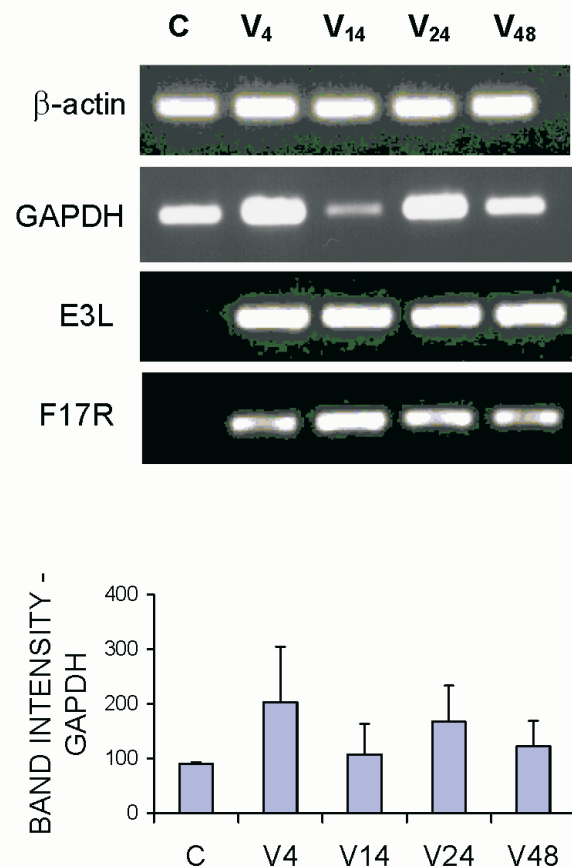


Figure 2. *GAPDH* mRNA and viral gene transcripts content in infected monocytes.

Top panel: agarose gel electrophoresis of *GAPDH*, *E3L* and *F17R* RT-PCR products from control and infected monocytes. β -Actin gene product was used as a control. C, uninfected cells; V₄, 4 h p.i.; V₁₄, 14 h p.i.; V₂₄, 24 h p.i.; V₄₈, 48 h p.i. Bottom panel: average band intensity of four (V₁₄ and V₄₈) or five (C, V₄ and V₂₄) independent *GAPDH* RT-PCR experiments. Labels as above.

GAPDH protein levels reflect the content of the transcript

The level of *GAPDH* protein in the control and infected cells and their whole cell, nuclear and cytosolic fractions was determined by Western blot. The analysis showed an initial

slight increase of the cellular GAPDH protein level at 4 h p.i., followed by more prominent and stable protein accumulation at 24 h p.i. (Fig. 3A). These results remain in accord with the previous findings, showing a long delay between *GAPDH* mRNA induction and the increase in protein levels in apoptotic cells (Ishitani & Chuang, 1996). Therefore, it is most likely that only the second phase of the protein level increase results from elevated transcription of the *GAPDH* gene, and the early increase (at 4 h p.i.) might be caused by an other phenomenon like higher protein stability.

***GAPDH* is reversibly translocated into the nucleus of infected cells**

Western blot analysis of the total protein extracts from cell fractions revealed a more complex picture of GAPDH protein localization after viral infection. Figure 3B shows that GAPDH level in the nuclear fraction begins to rise at 4 h p.i., with maximum at 14 h p.i., but falls down again at 24 h p.i. until only traces of GAPDH can be detected in the nuclear fraction at 72 h p.i. (note that the NE image is already amplified). The cytosolic protein level changes in a similar fashion as total cellular GAPDH, exhibiting two-phase accumulation with a maximum after 24 h p.i. which is understandable as it represents the major fraction of the cellular pool of the enzyme.

To confirm these findings and further investigate the spatial organization of GAPDH protein in the infected cells, immunostaining of control and infected adherent monocytes using anti-GAPDH antibody was performed. The results are shown in Fig. 4. In uninfected cells, GAPDH is present at a low level throughout the cells, but at 14 h p.i. it is selectively translocated into the nuclei in large amounts, appearing also to a smaller extent at the plasma membrane. Similarly to Western blot analysis, this process begins at the early stages of the infection, but the number of cells with nuclear GAPDH translocation increases in time. At 24 h p.i. GAPDH is no longer pres-

ent at a distinctively higher concentration in the nuclei than in the rest of the cells. Interestingly, it seems to remain associated with the cell membrane.

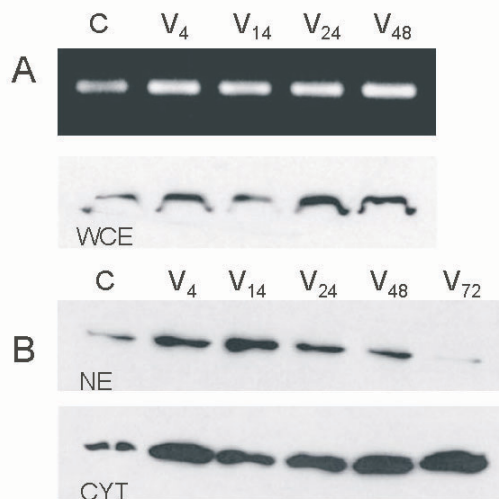


Figure 3. Western blot analysis of GAPDH protein concentration in adherent monocytes after viral infection.

A, *GAPDH* mRNA level (top) and total cellular protein level (bottom, WCE, whole cell extract). B, GAPDH protein levels (NE, nuclear fraction extract, CYT, cytoplasmic fraction extract). Proteins were extracted from uninfected cells and monocytes infected with 4 pfu of the virus. Ten micrograms of total protein was loaded per lane, NE image was amplified by longer exposure of the film. The results shown in the figure come from one of two independent experiments.

DISCUSSION

GAPDH overexpression and nuclear translocation have been reported to be involved in apoptosis of multiple neuronal and non-neuronal systems (Sawa *et al.*, 1997; Saunders *et al.*, 1999), suggesting that it is a general mediator of cell death. This study is the first to report an involvement of GAPDH in vaccinia virus infection *in vitro*, and its potential role in apoptosis of human monocytes.

GAPDH expression in infected cells is associated with the viral life cycle

In apoptotic cells, GAPDH expression is up to three times higher than in non-apoptotic

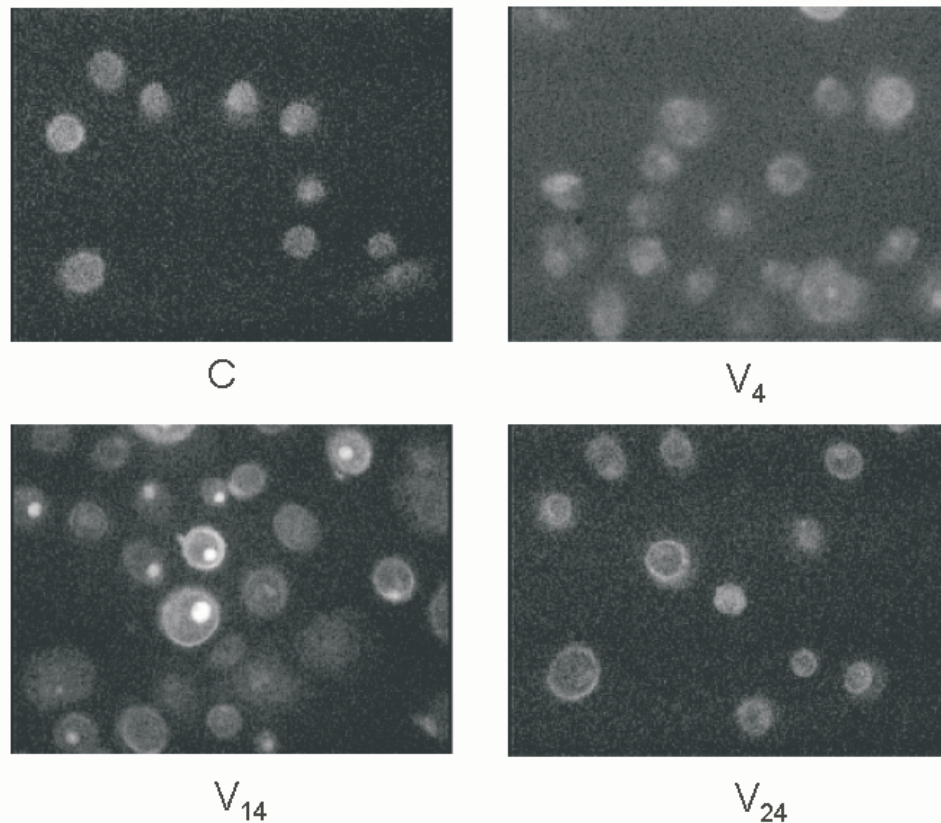


Figure 4. Reversible translocation of GAPDH to the nucleus after viral infection.

Immunostaining of GAPDH with FITC-conjugated antibodies in fixed and permeabilised adherent monocytes. C, uninfected cells; V₄, 4 h p.i.; V₁₄, 14 h p.i.; V₂₄, 24 h p.i. The results come from a single experiment.

cells (Dastoor & Dreyer, 2001). In this study, a similar level of stimulation was observed shortly after infection, followed by a consistent decrease throughout the infection. However, the RT-PCR method of transcript quantitation used in the study is not fully quantitative and these results should be treated as an estimation. Two viral transcripts were used in this study to control the virus life cycle: the early *E3L* gene and the late *F17R* gene. A correlation of *GAPDH* expression with both of these transcripts could be observed. *E3L* is involved in vaccinia anti-apoptotic defense and codes for two proteins binding double-stranded RNA (Xiang *et al.*, 2002). *E3L* appearance at the same time as high levels of *GAPDH* mRNA, typical for apoptotic cells, shows that its anti-apoptotic function is not immediate after the release from the virus particle. The levels of *GAPDH* transcript do fall down 10 h after the initial appearance of *E3L*, marking the

activation of virus protective mechanisms. *F17R*, however, is not associated with apoptosis and its correlation is much weaker, which might be an accidental result.

GAPDH is translocated to the nucleus at the early stage of infection

It is still speculated whether the nuclear *GAPDH* accumulation results from translocation of pre-existing protein in the cytosol or from newly synthesised protein. Our results suggest the latter, considering the decrease in total protein level at the time of accumulation. The opposite was suggested in previous research using GFP-fusion *GAPDH* protein (Shashidaran *et al.*, 1999), so this matter still remains to be settled.

Elevated *GAPDH* levels in the nuclear fraction during apoptosis become apparent after

about 30 h after the treatment with apoptotic stimuli in some cell types, but as early as 3 h after the treatment in primary thymocytes (Sawa *et al.*, 1997). It is feasible that a similar mechanism is employed in human monocytes and that the initial accumulation of GAPDH in the nucleus at 14 h after infection reflects the early apoptotic response of the virally-infected cells.

Association of GAPDH with the membrane was reported before and is thought to play a role in vesicular transport in the early secretory pathway (Tisdale, 2002). The role of such association in the viral infection remains unclear, however. It was not observed at a significant level in previous studies of cell death and need not be ascribed to the apoptotic function of the protein at all.

Nuclear translocation of GAPDH is completely reversed in the late stages of infection

Accumulation of GAPDH in the nucleus of apoptotic neuronal cells can be prevented by an anti-Parkinson drug, rasagiline (Maruyama *et al.*, 2001). Bcl-2 overexpression is able to suppress the nuclear translocation of endogenous and overexpressed GAPDH in many types of cells (Dastoor & Dreyer, 2001; Maruyama *et al.*, 2001). This suggests that the enzyme accumulates in the nuclei as a result of signal transduction which is antagonized by anti-apoptotic bcl-2 protein family. The DNA array screen undertaken in this study revealed a significant increase in bcl-2 levels at 24 h after infection (Table 2). These results were further confirmed by RT-PCR and Western blot studies conducted in our group (Piróg *et al.*, submitted). Bcl-2 accumulation is parallel to the reversing point of GAPDH translocation observed in this study, suggesting that the viral infection could lead to reversing the translocation by inducing the expression of endogenous bcl-2. Vaccinia does not express bcl-2 homologues found in some other poxviruses, but our results indicate that the

virus acts in the apoptotic pathway by modulating the cellular gene instead.

To obtain an integrated view of GAPDH function in human monocytes, further studies involving both apoptotic and infected cells and quantitative analysis of expression should be undertaken. The results of this study, nevertheless, constitute a next step in determining novel interactions between the virus and apoptotic pathways in the infected cells at the early stage of signaling.

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