EFFECT OF CORDYCEPIN ON HANTAAN VIRUS 76-118 INFECTION OF PRIMARY HUMAN EMBRYONIC PULMONARY FIBROBLASTS – CHARACTERIZATION OF APOPTOTIC EFFECTS

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Summary. – The cDNA microarray technique was used to study gene epression in human embryonic pulmonary fibroblasts (HEPF) infected with Hantaan virus (HTNV) under the influence of cordycepin (Cor), an inhibitor of post-transcriptional pre-mRNA polyadenylation. Four apoptotic genes, the insulin-like growth factor binding protein 1, NFkB inhibitor alpha, caspase-3 and NFkB1 were up-regulated in both infected and uninfected Cor-treated cells and two cell cycle-associated genes, CDC-like kinase and beta-induced transforming growth factor were up-regulated in Cor-untreated cells but down-regulated in Cor-treated cells. Cell morphology examination, quantitative RT-PCR, and immunofluorescence (IF) test suggested that following the Cor treatment the HTNV infection took place, but late viral gene expression was slightly reduced. Three parameters, namely caspase-3 activity, annexin V binding, and cell cycle were used to detect apoptosis. The results suggested that the induction of apoptosis in HEPF by HTNV started at 6 hrs post infection (p.i.). Following the Cor treatment, however, the caspase-3 activity began to increase at 24 hrs p.i. Thus it is suggested that inhibition of *de novo* late viral protein synthesis by Cor changes the apoptosis pathway and cell cycle by delaying caspase-3 gene expression and by up/down-regulating of expression of other apoptotic and cell cycle-associated genes. This implicates that HTNV can induce apoptosis in HEPF even without *de novo* viral protein synthesis and with a reduced and slowed viral maturation.

Key words: annexin V; apoptosis; caspase-3; cordycepin; flow cytometry; Hantaan virus; microarray; phosphatidylserine; RT-PCR

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Abbreviations: Cor = cordycepin; CPE = cytopathic effect; DMEM = Dulbecco's Modified Eagle's Medium; FACS = fluorescence-activated cell sorter; FBS = fetal bovine serum; HEPF = human embryonic pulmonary fibroblasts; HFRS = hemorrhagic fever with renal syndrome; HTNV = Hantaan virus; IF = immunofluorescence; MOI = multiplicity of infection; p.i. = post infection; PS = phosphatidylserine; PBMC = peripheral blood mononuclear cells; SEOV = Seoul virus

Introduction

HTNV (the *Hantaan virus* species, *Hantavirus* genus, the *Bunyaviridae* family) is the etiological agent of hemorrhagic fever with renal syndrome (HFRS). Hantaviruses (members of the *Hantavirus* genus) can replicate in primary cultures of animal cells with little or no cytopathic effect (CPE). Little is known of HTNV pathogenesis and molecular mechanisms underlying resistance to HTNV infection.

Elucidation of the mechanisms of virus-induced cell injury plays an important role in understanding the pathogenesis of virus infection. Apoptosis is defined as an

active physiological process of cellular self-destruction, characterized by specific morphological and biochemical changes (Steller, 1995; Spriggs, 1996). On the other hand, apoptosis is also a process regulating immune homeostasis and key mechanism of elimination of virus-infected cells (Steller, 1995). It has been demonstrated for a growing number of RNA and DNA viruses, both in cell culture and *in vivo,* that apoptosis is a common consequence of virus replication (Huang *et al.*, 2005). Apoptosis occurs either due to direct interactions of viral proteins with corresponding cellular ones or, indirectly, due to the immune response. There are several reports on HTNV-induced apoptosis in different cells. Thus Kang *et al*. (1999) have reported that cultured Vero E6 cells infected with Hantaan or Prospect Hill viruses exhibit characteristic features of apoptosis. Akhmatova *et al.* (2003) have suggested that the immune system reacts to hantavirus infection through apoptosis activation. According to Li *et al.* (2002) HTNV may interfere with the apoptotic pathway at the post-translation level and use the novel Fas-binding protein, Daxx, as a mediator. The mechanisms of control and realization of virus-induced apoptosis are, however, not completely understood because of the complexity of the underlying biochemical cascades (Budihardjo *et al.*, 1999), and because not all of the participating host factors have been identified (Teodoro and Branton, 1997).

Proliferating mammalian cells repeatedly undergo DNA synthesis and cell division during the cell cycle. The major phases in the cell cycle are G1 (gap), S (DNA synthesis), G2 (gap) and M (mitosis). At key transitions during the eukaryotic cell cycle progression, signaling pathways monitor successful completion of preceding events prior to proceeding to the next phase. Hartwell and Weinert (1989) have referred to these regulatory pathways as cell cycle checkpoints. Cells can be temporarily arrested at these checkpoints to repair cellular damage, dissipate exogenous cellular stress signals or allow for availability of essential growth factors, hormones and/or nutrients. Checkpoint signaling may also result in activation of pathways leading to apoptosis if cellular damage cannot be properly repaired (Pietenpol and Stewart, 2002).

Caspase-3 and annexin V are markers of immediate early and early phases for apoptosis detection. Moreover, there is a close relationship between the cell cycle and apoptosis. Many studies have focused on the relationship between virus infection, cell cycle arrest (George *et al.,* 2003; Song *et al.*, 2003; Venkatesan *et al.*, 2003) and apoptosis (Akhmatova *et al.*, 2003; Li *et al*., 2002); however, little is known about the variation of cell cycle after HTNV infection. To investigate the effect of HTNV on primary HEPF, cDNA microarray technique was used in our study to monitor changes in the mRNA levels of apoptotic and cell cycle-associated genes. Caspase-3 activity, annexin V binding and DNA histogram of HEPF infected with HTNV were examined to analyze the apoptosis status. Furthermore, Cor was used to elucidate the possible apoptotic effects of the *de novo* viral components.

Materials and Methods

Virus and cells. HTNV strain 76-118 was propagated in cultured Vero E6 cells (Yoshimatsu *et al.*, 1993). As the lungs are one of the major target organs for HTNV infection, primary HEPF were used in the experiments. These cells were kindly provided by Prof. H. Changshou, Institute of Virology, Chinese Academy of Preventive Medicine, Beijing, P.R. China. The cells that had underwent 15–25 passages were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) in 5% CO_2 at 37°C.

Virus infection. HEPF were infected with HTNV at a multiplicity of infection (MOI) of 0.5–1.0 $TCID_{50}$/cell throughout the study. DMEM supplemented with 5% of FBS with or without Cor (200 μmol/l) was added after 2 hrs of adsorption. The cultures were subjected to cell morphology examination, IF test, and cell cycle analysis at 6, 24 and 96 hrs p.i. The cells were collected for RNA preparation, caspase-3 activity and phosphatidylserine (PS) assays. Time points of 6 and 24 hrs p.i. were chosen to represent the early and late stages of the HTNV replication cycle; and 96 hrs p.i. had to correspond to that more than one virus replication cycle.

Cell morphology examination. Characteristic CPE included changes in cell morphology, cell lysis, ballooning, vacuolization, clumping and syncytium formation.

IF test was performed according to Yoshimatsu *et al*. (1993). Briefly, HEPF were cultured on 8-well chamber slides. Two-hundred μl of mouse anti-SEOV monoclonal antibody (NPs of HTNV and SEOV are highly homologous) (Clone A35, Chinese Center for Disease Control and Prevention, Beijing, P.R. China) diluted 1:400 in PBS supplemented with 10% of FCS was added to each well for 45 mins at 37°C. A secondary goat anti-mouse FITC-conjugated antibody (Chemicon, USA) diluted 1:200 in PBS supplemented with 10% of FCS was added to each well for 45 mins at 37°C. After washing 3 times in PBS, the cells were observed under a fluorescent microscope (Olympus BH-2, Japan).

Quantitative RT-PCR. RNA was extracted according to the method of Chevillard (1993) and its purity and concentration were assayed by agarose gel electrophoresis and spectrophotometry. To quantify viral RNA, a competitive RT-PCR construction kit (Ambion, USA) for internal standard RNAs was employed. The kit contained [α-^{32}P]GTP for labeling RNA. The RT step was done according to Celi *et al*. (1993). The RT mixture (20 μl) contained 20 pmoles of a primer (5'-TGAGAAATGTGTATGACATGA-3'), 1 μg of RNA and other RT ingredients. The resulting cDNAs were used as templates for PCR. The PCR mixture (50 μl) contained 20 pmoles of the upstream primer 5'-TGAGAAATGTGTATGACATGA-3' (nt 96–116) and downstream primer 5'-ACTAGACACTGTTTCA AATGA-3' (nt 323–343) and other PCR ingredients.

M segment. The samples were amplified using a 'hot start' at 94°C for 5 mins, followed by 35 cycles of a three-stage thermal

cycle consisting of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. Both HTNV-positive and HTNV-negative controls were included. The HTNV-specific product of 248 bp mapped at the M segment of HTNV genome. The PCR products were electrophoresed in a 1.5% agarose gel and scanned by a pdi scanning densitometer.

Microarray analysis. Total RNA was extracted from cells using the Easy Extract Kit from United Gene Ltd. (P.R. China) according to the manufacturer's protocol. The RT, second DNA strand synthesis, and probe generation were also accomplished using standard United Gene protocols. The Biostar H-II Gene Chip (United Gene Ltd.) contained 2304 genes including full-length and partial cDNAs. About 2200 cDNAs corresponded to the genes for apoptosis (60 spots), iron channel and transportation protein (ICTP) (130 spots), cell cycle (150 spots), skeleton (170 spots), metabolism (100 spots), receptor and signal transduction (870 spots) and others. Control spots included genes for house keeping (48 spots), internal (20 spots) and negative controls (16 spots), and spotting solution alone without DNA (16 spots). The chips were hybridized, washed, and scanned at the United Gene Ltd. Shanghai facility. Expression values were normalized by a single multiplicative normalization factor and applied to all Cy5/Cy3 ratios so that the median normalized Cy5/Cy3 ratio became 1.0. All values were calculated for two individual samples under the same conditions. Some values were excluded due to a relative error rate of more than 0.5-fold change.

Caspase-3 assay. Cells were collected and homogenized in a lysis buffer according to the protocol for the BD ApoAlert Caspase-3 assay (BD Biosciences Clontech; USA) as described before (Huang *et al.*, 2005). Triplicate cell plates were set up for the following samples: infected/uninfected (negative control); infected plus Cor; and, infected without substrate. Briefly, A_{405} values of samples were read in a microplate reader (BIO-RAD, USA). A calibration curve was generated using 0, 25, 50, 100, 200 µmol/l NA and A_{405} values for the X and Y axes, respectively. The caspase-3 activity waas calculated from the slope of this curve.

Annexin V binding. The cells (10^5 –10^6) in triplicate were washed with PBS, detached from culture plates with 0.25% trypsin, gently washed with a serum-containing medium, and incubated with annexin V and propidium iodide in the same medium according to the ApoAlert Annexin V protocol (BD Biosciences Clontech; USA) (Huang *et al.*, 2005). A binding buffer was used to adjust the reaction volume to 500 µl The cells were then analyzed by flow cytometry (Epics-XL MCL; Beckman Coulter, USA) using a single laser-emitting excitation light at 488 nm and the WinMID Version 2.8 software.

Cell cycle analysis. The cells (10^5–10^6) in triplicate were washed with PBS, detached from the culture plates with 0.25% trypsin, pelleted and fixed in 80% ethanol at 4°C overnight. They were then treated with 1 ml of a solution containing 50 µg/ml prodium iodide, 0.1% sodium citrate, 0.2% NP-40, and 0.25 mg/ml RNase for 30 mins at 37°C in the dark. The cells were then subjected to flow cytometry (Epics-XL MCL; Beckman Coulter, USA), with 10,000 events collected from each sample. The prodium iodide-positive cells in the G0/G1, G2/M, S and sub-G1 fractions were counted and modeled using a cell cycle-analysis program (Beckman Coulter, USA).

Statistical analyses. The Kruskal-Wallis test was used to test the significance of differences. The DNA content of the cells, as assayed by FACS, was analyzed by simple linear regression. A difference with a P value ≤0.05 was considered significant. The Wilcoxon rank sum test was used for evaluating the significance of differences between pair-wise samples.

Results

Virus multiplication in HEPF

To prove that HEPF are capable to multiply HTNV, they were infected and examined for CPE and viral nucleocapsid antigen. The cell morphology was not changed either by virus infection or Cor treatment within 96 hrs p.i. (Fig. 1a). IF test demonstrated that the viral antigen was produced in infected cells regardless of the Cor treatment (Fig. 1b). Using RT-PCR, a HTNV-specific DNA band was detected in infected cells at 6, 24 and 96 hrs p.i. (Fig. 2); the intensity of this band was increased by 12% and 26% as compared to non-infected controls, respectively. The Cor treatment reduced significantly ($P \leq 0.05$) the intensity of the DNA band to 84%, 58%, and 38% of corresponding controls at 6, 24 and 96 hrs p.i., respectively.

Differential apoptosis and cell cycle-associated gene expression in HTNV-infected HEPF

Of the 2304 human genes analyzed by the microarray technique, a total of 60 apoptotic and 150 cell cycle-associated genes were identified. In HTNV-infected HEPF, differential expression was demonstrated for a total of 13 apoptotic genes (21.7%) (Table 1). Most of the genes (10/13) changed during the first 6 hrs p.i. Only one of them, P13K alpha gene was down-regulated (Cy5/Cy3 ≤0.5), while up-regulation (Cy5/Cy3 ≥0.2) was noted for the genes for the growth factor receptor-bound protein 2, PI3K beta, protein phosphatase 1A (formerly 2C), ras homolog gene member A, insulin-like growth factor binding proteins 4 and 1, NFkB inhibitor alpha, ras homolog gene member C, I kappa B kinase complex-associated protein (IKBKAP), caspase-3, and B-cell nuclear factor of kappa light polypeptide gene enhancer 1 (NFkB1, p105).

At 24 hrs p.i., only the caspase-4 gene was up-regulated. At 96 hrs p.i., the genes for insulin-like growth factor binding protein 1 and GRB2-associated binding protein 2 were up-regulated. For cell cycle-associated genes in virus-infected cells, differential expression was found for five genes (3.3%). For most of them (4) the changes were apparent at 6 hrs p.i.; one gene (Cyclin L ania-6a gene) was down-regulated, while the genes for CDC-like kinase 3 (CLK3), protein phosphatase 1A (formerly 2C), and beta-induced transforming growth factor were up regulated. At 24 hrs p.i., only

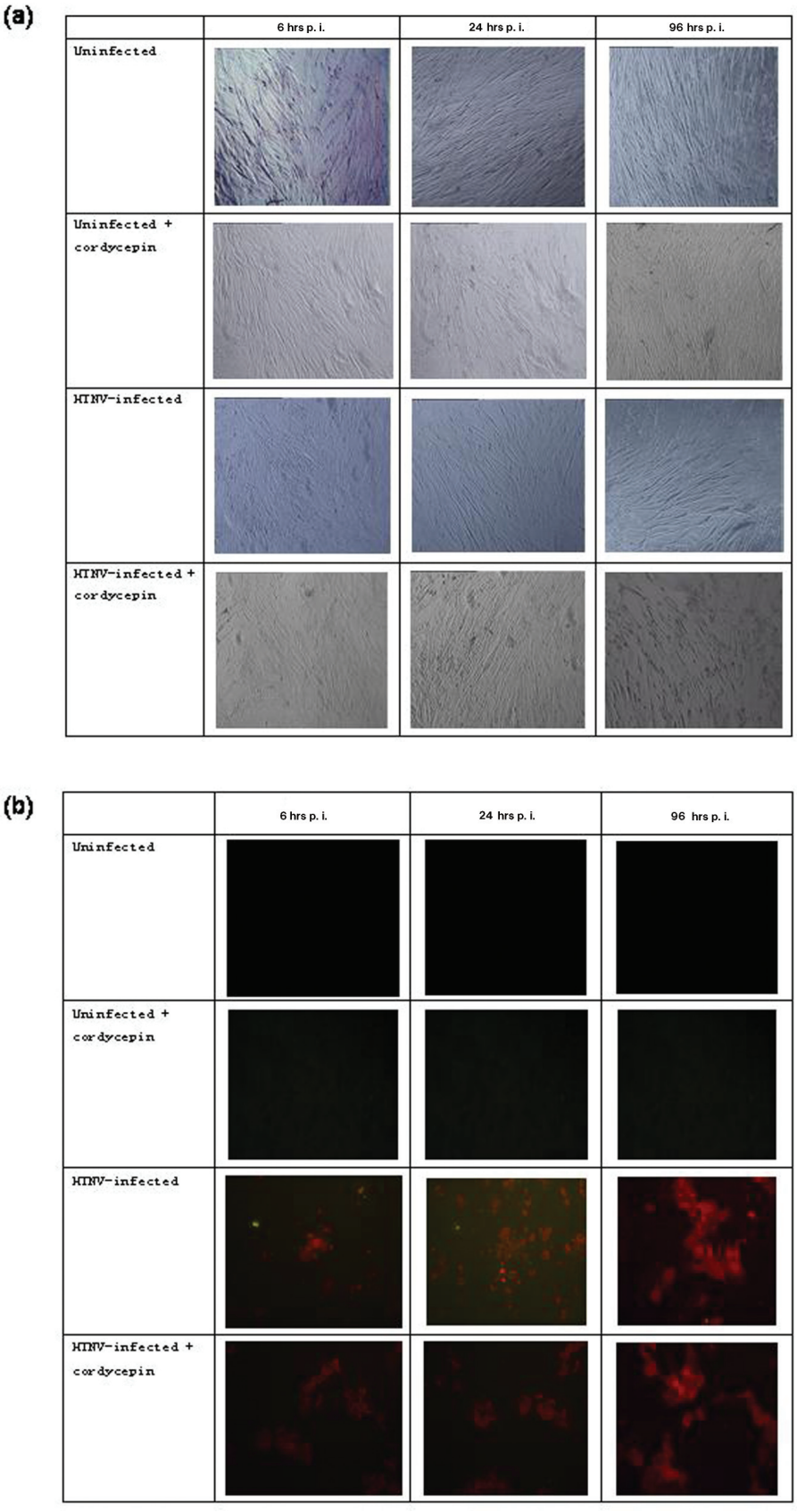


Fig. 1

Morphological appearance (a) and viral antigen expression (b) of HEPF, effects of HTNV infection and Cor treatment

Magnification 100x.

the growth-differentiation factor 5 gene was down-regulated; at 96 hrs p.i., however, there was no significant variation in any gene expression.

Effect of HTNV infection and Cor on apoptotic and cell cycle-associated gene expression in HEPF

In infected Cor-treated cells (Table 2), a significant differential expression was observed in 21 apoptotic genes (35.0%). Ten of them were up-regulated, seven were down regulated, two (the genes for SH3 domain GRB2-like 2 and insulin-like growth factor-binding protein 1) were first down-regulated and then up-regulated, and one (the gene for protein phosphatase 2 (formerly 2A)) were first up-regulated and then down-regulated.

At 6 hrs p.i., the genes for protein kinase C alpha, STK17A (apoptosis-induced) and insulin-like growth factor were down-regulated, while the genes for insulin-like growth factor binding protein 4, SH3 domain GRB2-like 2, death-associated protein, insulin-like growth factor binding protein 1, NFkB1 (p105), and NFkB inhibitor alpha were up-regulated.

At 24 hrs p.i., the genes for TRADD, death-associated protein, MAPK10 variant1 (JNK3 alpha1), and protein phosphatase 2 (formerly 2A) were down-regulated, while the genes for GDI, caspase-4, caspase-3, SH3 domain GRB2-like 2, protein phosphatase 1A (formerly 2C), caspase 6, insulin-like growth factor binding protein 1, NFkB1 (p105) and NFkB inhibitor alpha were-up regulated. At 96 hrs p.i., the genes for protein kinase C alpha, cathepsins D, caspase-2, SH3 domain GRB2-like 2 and insuli n-like growth factor

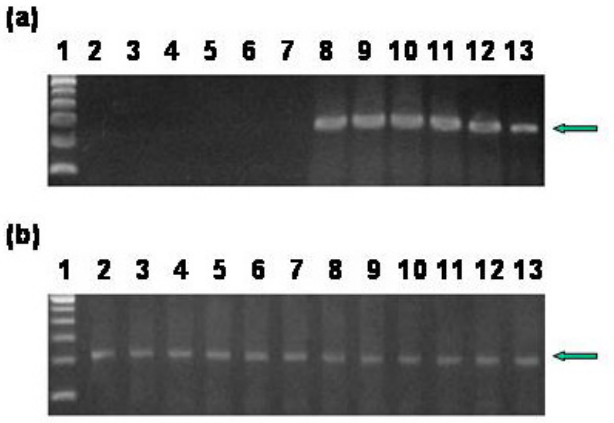


Fig. 2

Agarose gel electrophoresis of RT-PCR products

(a) Experimental samples. (b) β-globin as internal standard. DNA size marker, 100-bp ladder (lane 1); uninfected HEPF at 6, 24 and 96 hrs p.i., respectively (lanes 2–4); uninfected Cor-treated HEPF at 6, 24 and 96 hrs p.i., respectively (lanes 5–7); infected HEPF at 6, 24 and 96 hrs p.i., respectively (lanes 8–10); infected Cor-treated HEPF at 6, 24 and 96 hrs p.i., respectively (lanes 11–13). The specific band of 248 bp is indicated by arrowhead.

Table 1. Effect of HTNV infection on differential expression of apoptotic and cell cycle-associated genes in HEPF

Gene	GenBank ID	Cy5/Cy3 Hrs p.i. 6	24	96
Apoptotic genes				
PI3K, catalytic, alpha	NM 006218	0.49		
Caspase-4	NM 033306		2.04	
Growth factor receptor-bound protein 2	NM 002086	2.06		
PI3K, catalytic, beta	NM 006219	2.15		
Ras homolog gene family, member A	NM 001664	2.42		
Insulin-like growth factor binding protein 4	NM 001552	2.75		
Insulin-like growth factor binding protein 1	NM 000596	3.12		2.39
GRB2-associated binding protein 2	NM 003026			3.19
NFkB, inhibitor alpha	NM 020529	3.20		
Ras homolog gene family, member C	NM 005167	3.47		
I kappa B kinase complex-associated protein	AF153419	3.65		
Caspase-3	NM 004346	4.02		
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1(p105)	NM 003998	6.63		
Cell cycle-associated genes				
Growth differentiation factor 5	NM 000557		0.50	
Cyclin L ania-6a	NM 020307	0.50		
CDC-like kinase 3 (CLK3)	NM 003992	2.06		
Protein phosphatase 1A (formerly 2C)	NM 021003	2.19		
Transforming growth factor, beta induced	NM 000358	2.25		

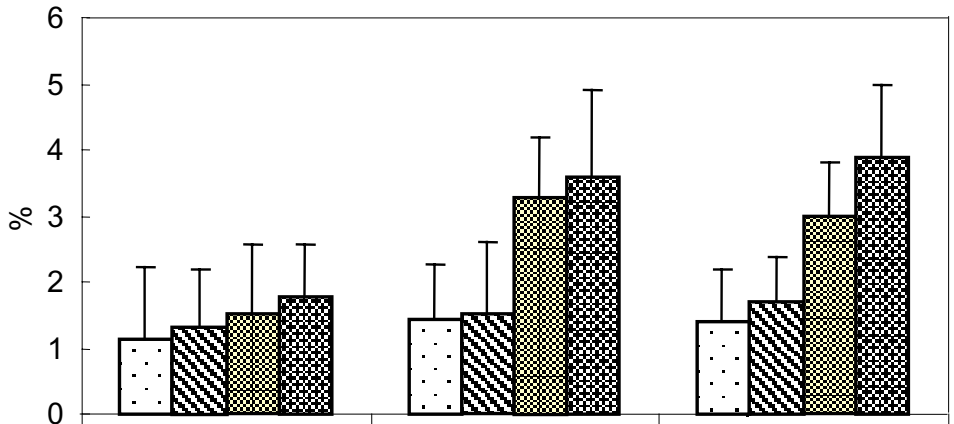
Table 2. Effects of HTNV infection and Cor on differential expression of apoptotic and cell cycle-associated genes in HEPF

Gene	GenBank ID	Cy5/Cy3 Hrs p.i. 6	24	96
Apoptosis-associated genes				
Protein kinase C, alpha	NM 002737	0.31		0.44
TRADD	NM 003789		0.35	
Cathepsins D	NM 001909			0.35
MAPK10 variant1(JNK3 alpha1)	NM 002753		0.42	
STK17A (apoptosis induced)	NM 004760	0.45		
Caspase-2	NM 032982			0.48
Protein phosphatase 2 (formerly 2A)	NM 004576		0.48	2.37
Insulin-like growth factor	X57025	0.48		
GDI	NM 001175		2.02	
Insulin-like growth factor binding protein 4	NM 001552	2.03		
Caspase-4	NM 033306		2.30	
Caspase-3	NM 004346		2.30	
SH3 domain GRB2-like 2	NM 003026	2.46	3.65	0.23
Protein phosphatase 1A (formerly 2C)	NM 021003		2.58	
SH3 domain GRB2-like 3	NM 003027			2.65
Death-associated protein	NM 004394	2.67	0.23	
P53 regulated PA26	NM 014454			2.68
Caspase-6	NM 001226		2.76	
Insulin-like growth factor binding protein 1	NM 000596	3.66	4.73	0.46
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NM 003998	3.99	2.10	
NFkB, inhibitor alpha	NM 020529	3.99	5.50	
Cell cycle-associated genes				
Cyclin B1	NM 031966		0.12	0.39
CDC7 cell division cycle 7-like 1	NM 003503		0.28	
Endonuclease G (Endo G)	NM 004435		0.22	
CDC20 cell division cycle 20 homolog	NM 001255		0.29	0.46
CDK7	NM 001799	0.30		
SOS1 (Cdc 25 similar)	NM 005633		0.35	0.37
Growth arrest-specific 1	NM 002048		0.36	
CDC2-related kinase	NM 003948	0.36		
Transforming growth factor, beta receptor 1	NM 004612			0.37
Transforming growth factor, beta induced	NM 000358			0.37
Growth arrest-specific 6	NM 000820		0.38	0.23
CDC-like kinase 3 (CLK3)	NM 003992			0.43
Transforming growth factor, beta 1	X02812			0.47
Cyclin D binding myb-like transcription factor 1	NM 021145	0.50		
CDK2	NM 001798		2.10	
E2F6	NM 001952		2.30	
Growth differentiation factor 8	NM 005259		2.60	
Cyclin D2	NM 001759			3.89

Table 3. Effects of HTNV infection and Cor on numbers of up- and down-regulated apoptotic and cell cycle-associated genes in HEPF

Character of regulation	Without Cor						With Cor					
	Hrs p.i.											
	6		24		96		6		24		96	
	A	CC	A	CC	A	CC	A	CC	A	CC	A	CCe
Up-regulated	10	3	1	0	2	0	6	0	9	3	3	1
Down-regulated	1	1	0	1	0	0	3	3	4	7	5	8
Total	11	4	1	1	2	0	9	3	13	10	8	9

A = apoptotic genes. CC = cell cycle-associated genes.



	6 hrs p.i.	24 hrs p.i.	96 hrs p.i.
Uninfected	1.13	1.42	1.41
Uninfected +Cor	1.31	1.51	1.69
HTNV-infected	1.52	3.28	2.98
HTNV-infected+Cor	1.78	3.59	3.87

Fig. 3

Annexin V binding in HEPF, effects of HTNV infection and Cor treatment

Mean percentage (total cell ± SD %) values.

binding protein 1 were down-regulated, while the genes for protein phosphatase 2 (formerly 2A), SH3 domain GRB2-like 3 and P53-regulated PA26 were up-regulated.

Of the cell cycle-associated genes, differential expression was observed for 18 (12.0%) genes. Most of them (15) showed changes between 24 and 96 hrs p.i. Among these 15 genes, 11 were down-regulated and 4 were up-regulated. At 6 hrs p.i., the genes for CDK7, CDC2-related kinase and cyclin D binding myb-like transcription factor 1 were down-regulated; while none was up-regulated. At 24 hrs p.i., the genes for CDC7 cell division cycle 7-like 1, endonuclesae G (Endo G) and growth arrest-specific 1 were down-regulated, while the genes for CDK2, E2F6 and growth differentiation factor 8 were up-regulated. At 96 hrs p.i., the genes for transforming growth factor, beta-induced transforming growth factor, CDC-like kinase 3 (CLK3) and transforming growth factor were down-regulated, and only the cyclin D2 gene was up-regulated. In addition, the genes for cyclin B1, CDC20 cell division cycle 20 homolog, SOS1 (Cdc 25 similar) and growth arrest-specific 6 were down regulated at both 24 and 96 hrs p.i.

Summing up the effect of Cor on the expression of apoptotic and cell cycle-associated genes in virus-infected cells, more genes showed a differential expression under the influence of the inhibitor.

Effect of HTNV infection and Cor on caspase-3 activity in HEPF

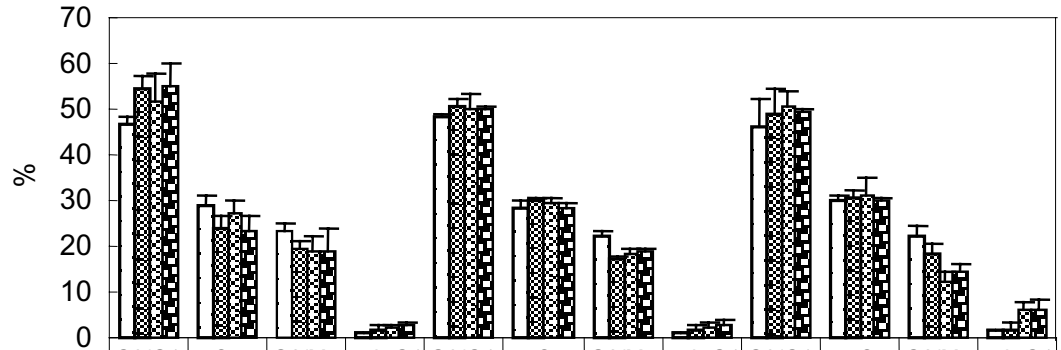
No significant changes were found in caspase-3 activity for both infected and uninfected cells treated with Cor (Table 3). However, there was a significant effect of Cor on the caspase-3 activity of infected cells at 6 hrs and 96 hrs p.i. The former finding was consistent with that from microarray analysis on a caspase 3 Cy5/Cy3 ratio of 4.02 at 6 hrs p.i. Similarly, the latter finding corresponded to a caspase 3 Cy5/Cy3 ratio of 2.30 at 96 hrs p.i.

Phosphatidylserine (PS) switch

PC exposed to the outer side of cell membrane can easily be detected by binding to annexin V, a 35.8 K protein with a strong affinity for PS. Fig. 3 shows that the annexin binding increased significantly in infected cells as compared to uninfected controls at 24 hrs p.i. Cor increased significantly the annexin V binding in infected cells as compared to uninfected controls at 24 and 96 hrs p.i.

Effect of HTNV infection and Cor on cell cycle in HEPF

Cell cycle analysis made by FACS is shown in Fig. 4. The virus infection caused a significant change (increase)



	6 hrs p.i.				24 hrs p.i.				96 hrs p.i.			
Uninfected	46.75	29.10	23.45	0.88	48.60	28.58	22.15	0.97	46.05	30.05	22.48	1.42
Uninfected+Cor	54.58	24.10	19.44	1.88	50.70	29.99	17.34	1.91	49.05	30.55	18.58	1.82
Infected	51.50	27.36	19.00	2.14	49.80	29.37	18.33	2.50	50.70	31.17	12.20	5.93
Infected+Cor	55.20	23.40	18.75	2.65	49.95	28.29	18.95	2.81	49.45	29.88	14.30	6.37

Fig. 4

Cell cycle phases in HEPF, effects of HTNV infection and Cor treatment

Mean percentage (ratio ± SD %) of cells in various cell cycle phases.

in sub G1 in Cor-untreated cells at 6 and 24 hrs p.i. only. On the other hand, the only effect of Cor was observed in sub G1 in infected cells at 6, 24 and 96 hrs p.i. In the remaining cell cycle phases, namely G0/G1, S and G2/M, there was not observed any effect of virus infection or Cor at any time point.

Discussion

Recently, apoptosis induction in host cells has been demonstrated for a growing number of RNA and DNA viruses. Additionally, involvement of caspase-3 activity has been observed in cells infected with a number of viruses, including HIV-1 (Banki *et al.,* 1998), adenoviruses (Chiou *et al.*, 1998) and Hepatitis C virus (Ruggieri *et al.*, 1997). In this study, the caspase-3 gene expression was highly activated by HTNV infection and apoptotic genes and their expression levels were altered by Cor treatment. Of the detected genes, a differential expression was noted in four apoptotic and two cell cycle-associated genes in HTNV-infected HEPF. Interestingly, the four apoptotic genes, namely insulin-like growth factor binding protein 1, NFkB inhibitor alpha, caspase-3 and NFkB1 (p105) (Gil *et al.*, 2001; Cory *et al.*, 2002; Bonni *et al.*, 1999; Ashkenzai *et al.*, 1998) were up-regulated in both infected and uninfected cells by the Cor treatment, while the two cell-cycle-associated genes, namely CLK3 and beta induced transforming growth factor, were up-regulated in infected cells in the absence of Cor, but down regulated in the presence of Cor. Although the genes for death-receptor and apoptotic regulators such as Fas/Fas-ligand, Daxx or bcl-2 have not been significantly modulated, all these six genes may play significant roles in the apoptotic processes in HEPF infected with HTNV.

In order to obtain insight into these complex virus-cell relationships, we attempted to define the role of Cor in them. Caspase-3 activity and annexin V binding are markers of immediate early and early phases of apoptosis, respectively. Taking caspase-3 as an indicator of the beginning of apoptosis in HTNV-infected HEPF, we asssume that the virus began to induce apoptosis in the cells at 6 hrs p.i., because, in comparison to uninfected controls, the caspase-3 gene expression was significantly highly activated at this time but not at 24 or 96 hrs p.i..

With the Cor treatment, however, the caspase-3 gene expression was also activated at 6 hrs p.i., but it continued so to 24 and 96 hrs p.i.

Further, PS translocation precedes other apoptotic events, thus allowing early detection of apoptosis. We observed that the annexin V binding was significantly increased by virus infection at 24 hrs p.i. Cor enters the cells rapidly and is phosphorylated to 3'-dATP with a concomitant drop in ATP levels. Treatment of HIV-1-infected H9 cells with 1 μmol/l Cor resulted in an almost 100% inhibition of virus production (Montefiori *et al.*, 1989). In this study, in the infected and Cor-treated cells, viral late gene (M segment) expression was only slightly reduced. It has been suggested that Cor, an inhibitor of mature mRNA production, causes a drop in the levels of mRNA for anti-apoptotis oncogene *c-myc* (Ioannidis *et al*., 1999). Besides, it has been reported that Cor either induces apoptosis in TdT-positive leukemia cells and causes a slight cytotoxicity (Koc *et al.*, 1996), but it does not increase apoptosis in human PBMC (Zhou *et al.*, 2002); an anti-apoptotic or delayed-apoptosis effect of Cor has never been suggested. Furthermore, we did not observe any significant variations in caspase-3 activity and annexin V binding due to Cor either in infected or uninfected cells, suggesting that Cor is unlikely to induce apoptosis in HEPF.

Regarding the cell cycle, FACS analysis revealed a significant increase in the percentage of cells in sub-G1 phase at 6 and 24 hrs p.i. Cor increased the sub-G1 value at 96 hrs p.i. Moreover, no obvious CPE of Cor on HEPF was observed. Concerning the Cor effect on the infected cells, the typical DNA fragmentation induced by the virus seems to be delayed until 96 hrs p.i. Summing the effects of Cor, it appears that the inhibitor delayed the apoptosis, causing a type of cell death similar to "a slower cell death" and increasing the percentage of the virus-infected cells in sub-G1 phase at 96 hrs p.i. This delaying effect on production of caspase-3 mRNA and protein may be due to Cordycepin inhibition of *de novo* viral protein synthesis; although we failed to demonstrate by IF test that the viral protein synthesis was blocked by Cor at 96 hrs p.i., Cor is known to inhibit polyadenylation of mRNA, thus blocking cellular mRNA maturation and protein synthesis (Person and Beaud, 1980). Thus we suggest that in this study the synthesis of late viral proteins was affected by changes in cellular mRNA maturation and protein synthesis and was related to apoptosis. The Cor inhibition of *de nov*o late viral protein synthesis may obviously change the apoptosis pathway and cell cycle phase by delaying the expression of caspase-3 gene and up/down-regulating other apoptotic- and cell cycle-associated genes. This would mean that HTNV is able to induce apoptosis in HEPF without *de novo* viral protein synthesis in a "slower manner".

Recently, it has been reported that hantaviruses cause CPE and induce apoptosis in HEK293 cells; however, the apoptosis appeared mostly in uninfected and rarely in infected cells (Markotic *et al.*, 2003). Moreover, CPE could be detected as early as 3–4 days p.i. with HTNV. In this study, we were unable to demonstrate CPE in HTNV-infected HEPF within 96-hrs p.i., although a low level of viral expression was detected by IF test. This inconsistence may due to different cells used; the absence of CPE in other types of cells has also been reported (Khaiboullina and St. Jeor, 2002). The study by Markotic *et al.* (2003) suggests that the apoptosis induction may occur independently of hantavirus replication and production of some soluble factors may contribute to the programmed cell death. This is consistent with our finding that HTNV induced apoptosis with or without *de novo* viral protein synthesis, suggesting that complete viral replication may not be necessary for apoptosis induction.

At present, there are virtually no data that could be used for formulation of mechanisms of immune regulation during human hantavirus infection. Little is known of the pathogenesis or molecular mechanisms underlying resistance to the virus infection. Recently, Kang *et al.* (1999) have suggested that hantaviruses induce apoptosis in Vero E6 cells through post-transcriptional regulation of the antiapoptotic protein Bcl-2. Akhmatova *et al.* (2003) have reported increased Fas/FasL and activation of caspases 2, 8 and 9 and effectors of caspases 3, 7 and 10 in PBMC, observed during acute and convalescent phases of the hantavirus infection. Nam *et al.* (2003) have confirmed that IFN plays a role in cellular defense against HTNV infection at an early stage and described the mechanism of resistance to HTNV infection. It has also been reported that Puumala (a hantavirus) viral nucleocapsid protein (PUUVN) interacts with Daxx, a Fas-mediated apoptotic enhancer, and that this PUUVN-Daxx interaction could be important for host defense (Li *et al.*, 2002). The reason for this immunological reaction may reside in the assumption that, in order to induce apoptosis an interaction of hantavirus nucleocapsid protein with Daxx is required (Tura *et al*., 2001). Li *et al*., 2002) have reported on a significant contribution of TNF-α to the apoptosis induced by Tula (a hantavirus) in Vero E6 cells. The analysis of the data from these studies suggests that, as for many other virus infections, the immune system reacts to hantavirus infection through induction of apoptosis. These immune reactions may be directed at preservation of immune homeostasis, development of optimal immune protection, and/or elimination of virus-infected cells. Markotic *et al*. (2003) have suggested, however, that members of the TNF-receptor superfamily do not contribute to the apoptosis observed in infected HEK293 cells. Our microarray data from this study are consistent with this finding, showing no obvious differences in the expression of TNF-α receptor superfamily mRNA levels in infected HEPF.

Apoptosis in infected cells facilitates host survival by diminishing pathogen production. We suggest that the HTNV-induced apoptosis in HEPF is caspase-3-dependent and may involve the active inhibition of anti-apoptotic

components, accomplished by either promotion of synthesis/induction or activation of caspase-3. On the basis of this study we suggest that Cor affects cellular genes that somehow influence HTNV protein expression and thus the apoptotic effect of the virus on the cells can be realized without *de novo* viral protein synthesis and viral maturation, but in a reduced and slowed manner. Further studies are needed to elucidate the apoptotic pathway induced or enhanced by hantavirus proteins.

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