Ph.D. Thesis

IN VITRO ANALYSIS OF NEWCASTLE DISEASE VIRUS-INDUCED CELL DEATH IN CANCER CELLS

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Ph. D. School: Multidisciplinary Medical Sciences

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INTRODUCTION

Almost a century ago Dock and DePace reported that their patients, suffering from gynecological cancers and vaccinated with Pasteur's rabies vaccine simultaneously, showed tumor regression suggesting that vaccination can alter the progression of human cancers. Since the idea of therapeutic use of viruses in humans arose in the early 1960's, the oncolytic potential of some of these viruses has been confirmed in several human trials, involving patients with cancers resistant to the traditional therapeutic modalities. Although the oncolytic viruses represent a promising possibility to find effective therapeutic strategies against resistant cancers, only limited investigations have been conducted so far to explain the mechanisms of the oncolytic cytotoxic effect. Two major mechanisms can be accounted for the possible molecular basis of the oncolytic effect. Oncolytic viruses can cause cell death by their replication in tumor cells, although it is not clear why they show selectivity to neoplastic versus non-transformed cells. Alternatively, immunological processes induced by virus infection are responsible for the cytotoxicity observed. To date almost 40 DNA and RNA viruses have been described with ability to selectively kill cancer cells. Among them viruses of human (such as smallpox, rabies, mumps) or animal diseases (e.g.: Newcastle disease virus) can be found.

THE NEWCASTLE DISEASE VIRUS

The Newcastle disease virus (NDV) belongs to the family of Paramyxoviruses in the Mononegavirales order. It is closely related to the infectious agent of human mumps. The structure of NDV is well characterized. Virions usually have spherical shape with a diameter between 150-500 nm. On their surface haemagglutinine-neuraminidase (HN) and fusion proteins (F) can be found. The neuraminidase activity present in all paramyxoviruses is thought to be responsible for the elimination of cell surface receptors of the host cell. There are at least three additional proteins in the virions. The matrix protein (M) that is present at the inner side of the lipid envelope of virions and probably plays structural role. The nucleoprotein (NP) is the main component of the nucleocapside and has a critical role in the viral replication and transcription. In the nucleocapside few copies of the „large" (L) and phosphoprotein (P) proteins are also present. They together make up the viral RNA dependent RNA polymerase. The nucleocapside is usually 13-18 nm in diameter, and has helical symmetry. Particles contain a completely sequenced 15 kb long, single-stranded, non-segmented negative-sense RNA genome coding for six viral proteins. Coding regions are separated by short repetitive sequences. At the end of the individual genes polyA signals can be found, the replication, the expression and the order of the genes is highly conserved and very similar to the Rhabdo- and Filoviruses. The replication of the viral genome takes place in the cytoplasm. Upon viral replication viral proteins are integrated in the cell membrane of the host cell, then newly produced nucleocapside proteins are connected to the modified cell membrane to form the virions including a small part of the host's cell membrane. While NDV has a strong cytotoxic potential against different tumor cells, it is one of the few oncolytic viruses that naturally do not infect humans. No serious human infection was ever described, except mild conjunctivitis or tracheitis in people working with live NDV vaccines.

Although the molecular mechanism of the oncolytic action of NDV remains unclear at the present time, therapeutic trials have been performed with promising results. In these early trials different NDV isolates were found to be effective in some human tumors as diverse as hematological, gastrointestinal cancers, and glioblastomas. The NDV variant MTH-68/H used in the present study was generated by several passages in chicken embryos of the original Hertfordshire strain of NDV designated Herts'33 and described in the early 1930's in England and was selected for its oncolytic capacity. The attenuated, highly purified MTH-68/H strain was found to have beneficial effects in patients with advanced cancer. Although many publications about the NDV-related clinical observations can be found in the literature, the mechanism of the NDV-induced beneficial oncolytical effects is still unclear.

AIM OF OUR STUDY

1. Is there any proliferation-inhibiting effect of MTH-68/H in cultured cells?
3. How could we screen the MTH-68/H sensitivity of cell cultures?
4. Which cell lines are more or less sensitive to MTH-68/H?
5. What is the molecular mechanism of action of MTH-68/H infection in cancer cell cultures?
6. Is viral replication necessary to MTH-68/H-induced effects?

MATERIALS AND METHODS

Materials. All biochemichals and culture media were purchased from Sigma-Aldrich Hungary (Budapest, Hungary), unless otherwise stated. MTH-68/H is a highly purified, attenuated NDV strain developed for human use by United Cancer Research Institute (UCRI Hungary, Budapest)

Cell Lines and Culture. The cell lines and their maintaining conditions used in this study are summerized in the following table.

Cell type	*Culturing feature*	*Medium*	*Final concentration*	*Manufacturer*	*Cat #*
Panc-1 (pancreas adenocarcinoma)	Adherent	RPMI 1640-Medium without phenolred FCS (Fetal Calf Serum) MEM Na-piruvát MEM non-essential amino acid solution	- 10 % 1 mM 1x	GIBCO GIBCO Sigma Sigma	11835-030 26010-074 S8636 M7145
HCT 116 (colon carcinoma) HT-25 (colon carcinoma) HT-168-M1/9 (melanoma) HT199 (melanoma) WM983B (melanoma)	Adherent Adherent Adherent Adherent Adherent	RPMI 1640-Medium with phenolred FCS (Fetal Calf Serum)	- 5 %	GIBCO GIBCO	21875-034 26010-074
DU-145 (prostata adenocc. brain met.) NCI-H460 (pulm. cc. pleur. met.) PC-3 (prostata adenocc. bone met.) HT-29 (colon adenocarcinoma)	Adherent Adherent Adherent Adherent	DMEM, Ham's F12 (1:1) (L-Glutamin 2mM) FCS (Fetal Calf Serum)	- 10 %	GIBCO GIBCO	31330-038 26010-074
A431 (epidermoid carcinoma)	Adherent	DMEM, Ham's F12 (1:1) (L-Glutamin 2mM) FCS (Fetal Calf Serum)	- 5 %	GIBCO GIBCO	31330-038 26010-074
U373 (glioblastoma) HeLa (cervix adenocarcinoma) MCF-7 (ductal adenocc. mammae)	Adherent Adherent Adherent	DMEM FBS (Fetal Bovine Serum)	- 10%	GIBCO GIBCO	41966-029 10106-169
LNZTA3WT4 (glioblastoma)	Adherent	DMEM FBS (Fetal Bovine Serum) L-Glutamin Tetraciklin	- 10% 2 mM 1µg/ml	GIBCO GIBCO GIBCO Sigma	41966-029 10106-169 25030-024 T7660
PC12 (patkány phaeochromocytoma)	Adherent	DMEM FBS (Fetal Bovine Serum) Horse serum	- 5% 10%	GIBCO GIBCO GIBCO	41966-029 10106-169 16050-122
Rat-1 (rat fibroblast) NIH3T3 (mouse fibroblast)	Adherent	DMEM Calf Serum	- 10%	GIBCO GIBCO	41966-029 26170-043
Human primary fibroblast	Adherent	DMEM FBS (Fetal Bovine Serum)	- 20%	GIBCO GIBCO	41966-029 10106-169

TUNEL Assay. For all treatments 10^5 cells were seeded in 8-well chamber slides and cultured for 24 hours, and treated with MTH-68/H.

The cells were fixed in 0.14 M phosphate-buffered saline (pH 7.4, PBS) containing 4% paraformaldehyde and 2.5% DMSO at 4°C for 60 min, washed in PBS three times for 5 min and permeabilized in PBS containing 0.1% Triton X-100, 0.1% sodium citrate at 4 °C for 2 min. Cells were washed and stained using FITC-labelled dUTP and terminal deoxynucleotide transferase at 37 °C for 60 min. TUNEL reaction was terminated by 2x SSC (0.3 M NaCl/0.03 M Na-citrate) for 10 min and cells were counterstained with propidium-iodide/RNaseA solution for 10 min at room temperature. FITC-labelled dUTP, terminal deoxynucleotide transferase and propidium-iodide/RNaseA solution were purchased from Roche Hungary Ltd. (Budapest, Hungary). Samples were washed with distilled water and covered using Vectashield H-1000 mounting solution (Vector, Burlingame, CA, USA).

Detection of Cytotoxicity Using the WST-1 Cell Proliferation Assay. The cell proliferation reagent WST-1 was purchased from Roche (Roche Hungary Ltd., Budapest, Hungary). This assay measures metabolically active mitochondria in cultured cells. Cells were grown in tissue culture grade, 24-well plates, in 1 ml culture medium as described above and infected with MTH-68/H for 72 hours. For positive apoptosis control, cells were treated for 24 hours with 1 μg/ml anisomycin, whereas for negative controls cells were treated with vehicle. For the WST-1 cell proliferation assay, treated cells were incubated for various times (from 90 to 240 minutes depending on the cell type) in culture medium containing 0.1 volume of WST-1 reagent. At the end of the treatment period 100 μl samples were transferred to a 96-well plate. The absorbance of the formazan formed was measured by a multiwell spectrophotometer at 440 nm. Measurements were performed in triplicates.

Immunocytochemistry. Cells were cultured on poly-L-lizin coated coverglasses. Following treatments cells were fixed with 4%-os freshly prepared paraformaldehyde at 4C^{O}. Immunocytochemistry was performed according to the manufacturer's instuctions of antibodies used. Slides were mounted with ProLong Antifade mounting medium (Molecular Probes, Eugene, OR, USA) and dried for 24 hours at 4C^{O}, and analysed using Olympus FV-1000 laser confocal microscope system.

Electrophoretic Detection of Internucleosomal Fragmentation of Chromosomal DNA. Analysis of DNA fragmentation was performed as described previously (22). 5x10^{6} cells were cultured in DMEM containing sera according to their requirements (see above) for 24 hours. Treatments were carried out as indicated in the figure legends. Following incubation, cells were collected by scraping them into their own medium and centrifuged at 600xg for 3 minutes. The cells were solubilized on ice in TE buffer (pH 7.4) containing 2% SDS. After centrifugation at 13500 rpm for 20 minutes at 4°C, soluble DNA in the supernatant was extracted with phenol/chloroform, and precipitated with ethanol. The dried precipitates were dissolved in TE buffer and treated with DNase free RNaseA at 37 °C for at least 1 hour. DNA fragments were separated by electrophoresis in 1.8% agarose gels, and visualized on a UV transilluminator after staining with SYBR Gold (Molecular Probes, Eugene, OR, USA).

Western Blotting. Immunoblot analysis using antibodies against proteins indicated in the figures was performed according to the manufacturer's recommendations. Cells were collected by scraping and centrifuged at 600xg for 3 min, lysed in ice-cold lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1mM EGTA, 1 mM Na-orthovanadate, 5 μM $ZnCl_2$, 100 mM NaF, and 1% Triton X-100. The lysis buffer was supplemented with 10 μl/ml phosphatase inhibitor cocktail I and 1 tablet/10 ml protease inhibitors (Complete, Mini EDTA-free tablets, Roche Hungary). Following lysis for 10 min on ice, samples were centrifuged at 13500rpm for 10 minutes at 4°C. Protein concentration was determined using the Bio-Rad Protein RC_{DC} assay system (Bio-Rad Hungary, Budapest). Thirty μg protein for each sample was resolved by SDS polyacrylamide gel electrophoresis in 10% or 18% gels. The proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech AB., Uppsala, Sweden), treated with appropriate antibodies and immune complexes were visualized using an Enhanced Chemiluminescence Detection kit (Amersham Pharmacia Biotech AB., Uppsala, Sweden) following the manufacturer's instructions. The following antibodies were used:

Antibody	*Type of antibody*	*Cat #*	*Manufacturer*
Phospho-specific Mdm2	Polyclonal, rabbit (Ser166)	#3521	Cell signaling (Beverly, MA, USA)
p53	Polyclonal, rabbit	#9282	
Phospho-specific PTEN	Polyclonal, rabbit (Ser388)	#9551	
PTEN	Polyclonal, rabbit	#9552	
Cleaved caspase-3	Polyclonal, rabbit	#9661	
Cleaved caspase-9	Polyclonal, rabbit	#9507	
Caspase-8	Polyclonal, rabbit	#9746	
eIF2α	Polyclonal, rabbit	#9722	
Phospho-specific eIF2α	Polyclonal, rabbit (Ser51)	#9721	
PERK	Polyclonal, rabbit	#sc-13073	SantaCruz Biotechnology (SantaCruz, CA, USA)
Actin (mAb, AB-1)	Monoclonal, mouse IgM	#CP01	Oncogene (Merck Ltd., Budapest, Hungary)
Rat specific PKR	Monoclonal, mouse	#P97220	Transduction laboratories (BD bioscience, San Jose, CA, USA)
Caspase-12	Polyclonal, rabbit	#BV-3182-3	MBL laboratories (Nagoya, Japan)

Electrophoretic Mobility Shift Assay. Nuclear extracts were prepared as described by Xu and Cooper. All subsequent steps were performed at 4°C. Cell pellets were washed twice in ice-cold PBS and resuspended in 10 volumes of a 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), protease inhibitors (Complete, Mini EDTA-free tablets, Roche Hungary) and phosphatase inhibitor coctail I and placed on ice for 10 min. Nuclei were collected by centrifugation and resuspended in 2 volumes of 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitors and phosphatase inhibitor coctail I and placed on ice for 20 min. After centrifugation, protein concentrations of the supernatants were determined by the Bio-Rad RC_{DC} Protein Assay kit. 5'-end labeling of the p53 oligonucleotide was performed using [γ-^{32}P] ATP (Institute of Isotopes Co., Ltd., Budapest, Hungary) and T4 polynucleotide kinase (Amersham Pharmacia Biotech AB., Uppsala, Sweden) according to the manufacturer's protocol. Double-stranded p53 oligonucleotide containing the consensus binding site for p53 (5'-TACAGAACATGTCTAAGCATGCTGGGGACT-3') was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA), double-stranded oligonucleotide containing the consensus binding site for c-Myc (5'-TGTGCGGCCACGTGTCGCGAGGCCCGG-3') was purchased from Amitof (Amitof, Boston, MA, USA).

Ten μg of nuclear proteins were mixed with 100 ng nonspecific single-stranded oligonucleotide in 4 μl buffer containing 10 mM HEPES (pH 7.5), 10% glycerol, 1 mM EDTA, 100 mM NaCl, 2 μg poly(dI-dC) for c-Myc or 3 μg poly(dI-dC) for p53 binding reactions and the final reaction volume was adjusted to 18 μl with distilled water. After a 15-min pre-incubation at room temperature 10^5 cpm of ^{32}P-labelled oligonucleotide was added and the incubation at room temperature was continued for another 30 minutes. DNA-protein complexes were electrophoresed in 5% non-denaturating polyacrylamide gels using a TRIS-Base, borate, EDTA buffer (pH 8.3) for 2.5 h at 200 V. The gel was dried and analyzed by a Cyclone PhosphorImager (Packard Instrument Co. Inc., Meriden, CT, USA).

Quantification of Infective MTH-68/H Particles. The quantitative analysis was performed as described by Lomniczi (Lomniczi, B., Studies on interferon production and interferon sensitivity of different strains of Newcastle disease virus. J Gen Virol, 1973. 21(2): p. 305-13.). $6x10^8$ primary chicken embryonic cells were infected with 0.5 ml of the supernatants of cancer cells treated with different MOI of MTH-68/H for 72-hours. After 3 days of incubation, intracellular viruses were released by sonic treatment and titrated by plaque assay.

RESULTS

MTH-68/H is cytotoxic in cancer cell cultures. According to our results the experimental vaccine MTH-68/H is cytotoxic to wild type PC12 cells and leads to the complete destruction of cell cultures after 72 hours of incubation. This cytotoxic effect is dose-dependent; 1:5-1:10 cell:virions titers lead to the loss of 50% of our PC12 cells, while cytotoxicity of MTH-68/H at the 1:800-as titer results the complete destruction of PC12 cell cultures similarly to the cytotoxicity of the protein synthesis inhibitor anisomycin. Similar results were observed in human cultures, though different cell lines showed different sensitivities to MTH-68/H. In HeLa cells the lowest titer we tested was enough to evoke complete loss of cells after 72 hours of incubation. In melanoma cell lines 50% of the cells were killed at 10:1-5:1 titer range, while death of all of the cells of our melanoma cultures was observed at 1:1-1:5 cell:virion titer. In cell cultures with colorectal cancer origins higher doses of MTH-68/H were needed to kill the cells, 5:1-1:1 and 1:5 titers to destroy 50% and 100% of the cancer cells, respectively.

Our data indicate that besides the glioblastoma and melanoma cell lines Panc-1 and HeLa cells with pancreas and cervix carcinoma origins, respectively, were the most sensitive ones to MTH-68/H. In contrast, MCF-7 breast cancer cells and our original model system, PC12 cells showed the lowest sensitivity to the experimental vaccine. Interestingly, some cell types – e.g.: U373 astrocytoma cells – show MCF-7-like MTH-68/H sensitivity in the lower cell:virion titer range, while above the 1:5 titer the cytotoxic effect of MTH-68/H was dramatically increased. At 1:50 cell:virion titer or above complete destruction of the cultures was observed similar to the cytotoxic effect of cell lines with high MTH-68/H sensitivity.

On the other hand, in experiments using rat, mouse or human primary fibroblast cultures we did not observe any cell destroying effect of MTH-68/H even at the highest cell:virion titers. Moreover, we observed the continous proliferation of these cells in the presence of the infective particles.

The main features of cell lines we tested are collected in table 3.

Cell line	Tissue origin	p53 status[2]	Genotype[2]
Primary fibroblast	Human	NA[1]	Diploid
NIH3T3	Mouse embryonic fibroblast	NA	Diploid
Rat-1	Rat embryonic fibroblast	NA	Diploid
HT-25	Human colon carcinoma	NA	Diploid
HT-29	Human colorectal adenocarcinoma	CGT/CAT mutation in codon 273	Hypertriploid c-myc, K-ras, H-ras, N-ras, Myb, sis, fos +; N-myc, abl, ros, src -; CEA+ TGFβBP +
HCT-116	Human colon carcinoma	wtp53+	Near diploid; mutation in kodon 13 of ras; Keratin + TGFβ 1/2 +
DU-145	Brain metastasis of human prostate adenocarcinoma	Both alleles are mutated: $Pro^{223}Leu$ and $Val^{274}Phe$	Hypotriploid
PC-3	Bone metastasis of human prostate adenocarcinoma	One allele is deleted; Point mutation in codon 138 resulting frameshift leading to the early termination.	Hypotriploid
PANC-1	Ductal epithelioid carcinoma of human pancreas	CGT/CAT mutation in codon 273	GGT/GAT mutation in kodon 12 of K-ras gene, Methylated p16 gene Hypertriploid
MCF-7	Human breast adenocarcinoma	wtp53+	N-ras amplification Tx-4 oncogene + Estrogen receptor + Estrogen dependent Caspase 3 -
HeLa	Human cervix adenocarcinoma	HPV16 E6+; low p53 expression	Keratin+
NCI-H460	Pleural metastasis of human large cell type lung carcinoma	Elevated p53 mRNA expression.	Hypotriploid Keratin + Vimentin +
U373	Human astrocytoma	CGT/CAT mutation in codon 273	Diploid/hypotriploid; TNFα +; Substance P rec. +; HSP28 +
LNZTA3WT4	Human glioblastoma	Endogenous p53 is inactivated. The cell line is stably transfected by wtp53 cDNA driven by CMV promoter and repressed by a tetracyclin repressor.	
A431	Human epidermoid carcinoma	CGT/CAT mutation in codon 273	Hypertriploid
HT-168-M1/9	Human melanoma	NA	NA[1]
HT199	Human melanoma	NA	NA[1]
WM983B	Human melanoma	NA	NA[1]

Table 3. *[1]NA: No data available; [2]Based on data of the American Tissue Culture Collection and publications.*

Viral replication takes place upon cell destruction induced by MTH-68/H. In order to determine the possible role of viral replication in MTH-68/H-induced cell death we infected HeLa and MCF-7 cultures with 100:1 and 10:1 titers for 24 hours. After the incubation cell culture media were collected, transferred to fresh cultures and incubated for an additional 24 hours. Following the second incubations, we observed severe cell destruction, indicating the presence some cytotoxic factor in the culture media of previously infected cultures. In order to determine if these soluble agents are newly created virions, we determined the amount of live, infective MTH-68/H particles in culture media of wtPC12, HeLa, NIH3T3 and Rat-1 cells after 72 hours of infection.

We did not find infective particles in media of NIH3T3 and Rat-1 fibroblasts, while in media of Hela and wtPC12 cells we detected infective particles after 3 days of incubation in a comparable amount to the starting titers.

Since in our preliminary experiments we observed significant loss of infectivity of MTH-68/H particles incubated at 37^{O}C for 72 hours our data indicates that active viral replication takes place in cells sensitive to MTH-68/H, while in restistant fibroblast viral replication remained restricted.

MTH-68/H-induced cell death is caused by apoptotic processes. Microscopical analysis of MTH-68/H-infected cultures revealed a high rate of vacuolization followed by shrinkage of cells with apoptotic body-like particles around them. Biochemical studies also indicated signs of apoptotic processes (e.g.: fragmentation of DNA, activation of caspases) induced in MTH-68/H-infected human cancer cells. The minimal cell:virion titer needed to cause apoptotic DNA ladder well correlated to the relative sensitivity of target cells, and the apoptosis-inducing effect of the MTH-68/H depends on the live virion content of the vaccine used. Induction of apoptosis is rapid; exposure of wtPC12 cells to MTH-68/H for 30-60 minutes was sufficient to cause full blown DNA-ladder similar to that observed in cells infected with MTH-68/H for 24 hours. In HeLa cultures treated with MTH-68/H for 12 hours, 25-30% of the cells were TUNEL positive, while 90% 24 hours post infection.

The MTH-68/H infection leads to the activation of caspase-3 and -12 in a caspase-8 and -9 independent manner. Analysis of the effector caspase-3 revealed that its cleaved form is accumulated in MTH-68/H-infected wtPC12 cells 10 hours post infection. However, similar induction of the initiator caspase-9 was not observed. Similar results were found with caspase-8 in the tested DU-145 and MCF-7 cells with prostate and breast cancer origin, respectively. In contrast, we observed activation of the endoplasmic reticulum (ER)–related caspase-12, that is clearly detectable 8 hours post infection. In addition, immunocytochemstric studies showed nuclear translocation of the ER-resident caspase-12 10 hours post infection.

MTH-68/H-evoked apoptotic processes are independent of p53. MTH-68/H induces intensive apoptosis in infected cells. Comparison of the genotype of MTH-68/H sensitive cells revealed that while many of them – e.g.: HT-29, DU145, PC3, Panc-1, HeLa, U373, LNZTA3WT4, A431cells – carry mutant p53, others – e.g.: wtPC12, MCF-7, HCT-116 – certainly express wtp53 *(see table 3)*. These data indicate that normal p53 functions are not needed to MTH-68/H-induced apoptotic cell death. In order to investigate the p53 independent fashion of the MTH-68/H induced cell death, we studied the glioblastoma cell line LNZTA3WT4, in which p53 is completely deleted, but stably transfected with a wtp53 cDNA under the control of a tetracyclin repressor. Cytotoxicity assays in this cell line showed high relative sensitivity of LNZTA3WT4 cells to MTH-68/H both in the presence and absence of tetracyclin. MTH-68/H sensitivity was similar to that of the mutant p53 alleles carrying HT-29 colorectal, PC-3 prostate or A431 epidermoid carcinoma cells. DNA binding assays performed in various cell lines showed no significant change of the DNA binding activity of p53 upon MTH-68/H infections. Thus, direct and indirect evidences suggest that p53 is not involved in the mediation of the cellular response to MTH-68/H infection in cancer cells.

MTH-68/H infection leads to the phosphorylation of PERK followed by inactivation of eIF2α. In our experiments, phosphorylation of eIF2α was observed 10-11 hours post infection, similarly to the activation of caspase-3, but few hours after the activation of caspase-12 in wtPC12 cells.

In MCF-7 cells, eIF2α phosphorylation was delayed; first signs of the eIF2α phosphorylation detected after 12 hours, but its maximum was observed only few hours later, 18 hours post infection. Several kinases are involved in the regulation of eIF2α, including the protein kinase R- like, ER-resident kinase (PERK). Immunoblot analysis of PERK showed phosphorylation of this kinase in the early stage of the MTH-68/H infection that was decreased after 10 hours of the MTH-68/H infection. Phosphorylation of PERK, thus, precedes the phosphorylation of eIF2α, and the activation of caspase-12 and -3 indicating a possible role in the MTH-68/H-induced apoptotic processes.

c-Myc and NFκB transcription factors are induced upon MTH-68/H infection. We detected increased DNA-binding activity of c-Myc 4 hours following the MTH-68/H infection of both wtPC12 and HCT-116 cells. Increased DNA-binding activity of nuclear factor kappa B (NFκB) was also observed in PC12 and HeLa cells, though in the various cell lines different specific protein complexes were found. In contrast to the course of the c-Myc activation, induction of NFκB was a bit more rapid, and the pattern of NFκB activation was very similar to the pattern of DNA-binding activity of this transcription factor upon treatments with a potent protein synthesis inhibitor anisomycin.

SUMMARY

Since many years human and non-human viruses having beneficial effects on human tumors, have been in the focus of attention of the scientific society. With the help of the investigations started in the early 1900s, virus species with oncolytic potential have been well defined. In order to introduce virotherapy in the clinical practice, research goes in two major ways: first, creating genetically modified viruses, second, using contagious agents with internal affinity to cancer cells. In our experimental programme we analyzed the effeciency of the MTH-68/H vaccine, a Newcastle disease virus strain used in small scale clinical trials. The aim of our programme was to study MTH-68/H *in vitro*, to define the MTH-68/H sensitivity of various human cancer cell lines and to analyse the intracellular events upon infection.

We proved that the MTH-68/H strain inhibits the proliferation of human cancer cell lines of different origin, but does not influence the proliferation of human, mouse and rat fibroblasts. Upon MTH-68/H infection, we detected the complete destruction of transformed human cancer cell lines, though in order to reach the highest rate of cell death, different cell:virion titers were needed in different types of cells. Analyzing data of viability assays, we defined the relative MTH-68/H sensitivity of each cell line we tested.

During the MTH-68/H induced cell death we successfully identified the morphological – shrinkage of cells, apoptotic bodies – and the biochemical – DNA fragmentation, caspase activation – hallmarks of apoptosis. Interestingly, to evoke full-blown apoptosis, short exposure (60 minutes) of PC12 cells to MTH-68/H was enough. Besides the apoptosis, typical signs of the paramyxoviral infections – vacualization, cell fusions – were also observed.

Indeed, following the infection of the transformed cell lines, active replication of the viral particles was detected, but it could not be found in our fibroblast cultures. The rate of the viral replication well correlated to the relative sensitivity of the target cell type. Since our experimental modell systems are free of any immunocompetent cells, our observations indicate that the observed cell death induced by MTH-68/H does not rely on the presence of the immune system, but must be evoked by direct cell-virus interactions.

Our data also indicate that the MTH-68/H-induced apoptotic cell death is independent of both the extrinsic caspase-8, and the intrinsic caspase-9-mediated apoptotic pathways. Data of the cytotoxicity assays support this finding. Activation of caspase-3 was detected shortly after the MTH-68/H infection in wtPC12 cells, but viability assays, based on the proper functions of the mitochondrial oxido-reductase enzyme systems, indicate cell destruction at the much later stage of the infection. Although we detected the activation of effector caspase-3 upon MTH-68/H infection, its absolute role in the mediation of apoptosis is also dubious; MTH-68/H also caused cell death in the caspase-3 deficient MCF-7 cell line. In contrast, activation of caspase-12 upon MTH-68/H infection was detected, that well correlated with caspase-3 activation. Caspase-12 is reported as an initiator and/or effector caspase, thus, activation of caspase-12 may explain the caspase-3, -8 and -9 independent fashion of MTH-68/H-induced apoptosis.

Since the primary target for current chemo- and radiotherapies is the genomic DNA and DNA damage induces p53-dependent apoptosis, the p53 status of the cancer cell has a fundamental effect on the outcome of anti-cancer treatments. According to the analysis of the genomic constellation of cell lines we tested and our own experimental results, MTH-68/H-induced cell death was identified as a p53 independent process, a finding that may have particular clinical importance. We did not observe different response to MTH-68/H in glioblastoma cells with or without p53 proteins. In addition, in p53 expressing cell lines we also did not find increased DNA-binding activity of p53 upon MTH-68/H infection.

In contrast to p53, significant changes in the activity of c-Myc and NFκB could be observed. The product of the oncogene *c-myc* is also involved in the formation of tumors including breast, lung, ovarial and colorectal cancers. It is important from the clinical point of view, that the more mutated *c-myc* the cancer tissue has, the more malignant tumor we should expect. Induction of c-Myc upon MTH-68/H treatment was detected 4 hours post infection both in wtPC12 rat phaeochromocytoma and HCT-116 human colorectal cancer cells. According to the literature, proapoptotic functions of c-Myc are generally related to the intrinsic apoptotic pathways. Upon the MTH-68/H treatment, however, we detected the activation of the effector caspase-3 much earlier than the activation of the intrinsic apoptotic pathways, thus the significance of the early c-Myc activation in the mediation of MTH-68/H-induced cell death is still in question.

NFκB, that can be both pro- and antiapoptotic, is one of the key players of the intracellular antiviral defense systems, thus, it is not surprising that we also detected the activation of NFκB during MTH-68/H infection of various cell lines.

Interestingly, in HeLa cells, showing higher relative MTH-68/H sensitivity, we observed a slower NFκB activation (8 hours post infection), while in wtPC12 cells, that are less sensitive to MTH-68/H, increased DNA-binding activity of NFκB occured rapidly, 2 hours post infection. Although the role of NFκB is not clear yet, few theoretical explanations can be provided. First, it is possible that NFκB works differently, either in pro- or antiapoptotic ways, in different cell lines upon MTH-68/H infections leading to the controversial results we observed. Second, it is also possible that NFκB is generally antiapoptotic in our systems, but in HeLa cells this defense mechanisms is somehow inhibited thereby making these cells more sensitive to MTH-68/H, while in wtPC12 cells the antiviral NFκB system may work better leading to the less MTH-68/H sensitivity of wtPC12 cells. Since the NFκB status of the target cells may influence their behavior upon MTH-68/H tretament, the role of NFκB seems to be important and requires further investigations.

According to our findings, MTH-68/H induces apoptotic cell death in cancer cells and MTH-68/H-induced processes seem to be related to the molecules involved in the regulation of the ER-stress. Upon MTH-68/H treatment rapid activation of PERK is followed by the inactivation of eIF2α and the activation of caspase-12 and caspase-3. These findings suggest that MTH-68/H infected cancer cells are eventually killed by the overload of the ER induced by the active viral replication. This vulnerability of the ER may represent the common feature that makes various cancer cell lines MTH-68/H sensitive. Distraction of the fine balance of the ER by the viral replication may lead to the activation of the ER-stress-related molecules finally leading to cell death.

ACKNOWLEDGEMENTS

I am felicitous to get all kind of support I needed for my work from *Dr. Virág Fehér MD.* Thank Her for everything!

The author is grateful to *Dr. József Szeberényi* and *Dr.László Csatáry* for all their support, and to *Mónika Vecsernyés, Györgyné Kiss, Rudolfné Schäffer, Dr. Béla Lomniczi* and *Dr. Géza Sáfrány* for their help in this work.

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PATENT APPLICATIONS

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