Analysis of endoplasmic reticulum stress in rat cell models

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1 INTRODUCTION

Prolonged Endoplasmic reticulum (ER) stress is a bimodal stressor. Unfavorable ER stress might lead to extensive cell loss and participates in the development of different pathological conditions, contributing to atherosclerosis, in neurodegenerative disorders, diabetes, ischaemia/reperfusion injury; on the other hand ER stress-evoked cell death is a prosperous and promising target of cancer therapy.

1.1 PROTEIN SYNTHESIS AND FOLDING IN THE ER

1.1.1 FOLDING IN THE ER

Folding of the newly synthesized polypeptide chain happens in both co- and posttranslational manner. Folding has been proven to be maintained and controlled by a strictly regulated and complex system in the ER, composed of numerous chaperons, foldases, isomerases, oxidoreductases, and their cofactors. Misfolded/unfolded proteins are restricted to enter the anterograde transport. These improperly folded proteins are identified by chaperons and aimed for degradation if the misfolded conformation persists.

Misfolded polypeptides might oligomerize, aggregate, and can be eliminated by autophagy. Single, not aggregating unfolded proteins are removed from the lumen of the ER and leave the ER through the Sec61 translocon complex, followed by their polyubiquitination and consequential degradation termed as proteasome-dependent ER-associated protein degradation (ERAD). Properly folded proteins are recognized by cargo receptors and follow the anterograde transport heading the Golgi apparatus where they undergo sorting to be secretory, lysosomal or membrane proteins.

1.2 UNFOLDED PROTEIN RESPONSE

If protein folding is disturbed, unfolded or misfolded protein molecules are retained in the ER. Physiological or pathological processes can disturb protein folding and cause retention of improperly folded proteins in the ER lumen referred to as ER stress, and a process, responding to ER stress, termed as Unfolded Protein Response (UPR) is activated. UPR promotes cellular adaptation by enhancing protein-folding capacity, reducing the load of the secretory pathways, and promoting degradation of misfolded proteins. However, when ER stress is extensive and/or prolonged, UPR can facilitate the activation of programmed cell death.

1.2.1 ER STRESS SENSORS OF THE UPR

During ER stress response there are three ER membrane-associated sensor proteins involved in the activation of signaling pathways; the activating transcription factor 6 (ATF6), the inositol requiring enzyme 1 (IRE1) and the protein kinase R (PKR)-like ER kinase (PERK). These proteins are kept in an inactive state through the direct association of their luminal domain with the ER heat shock Binding immunoglobulin Protein/Glucose-Regulated Protein 78/Heat shock protein 5 (BiP/GRP78/Hspa5) chaperon. As unfolded or misfolded proteins accumulate in the lumen of the

ER, BiP molecules become sequestrated from these three sensors resulting in the activation of the UPR-related signaling pathways.

1.2.2 The IRE1 pathway

The activated IRE1 forms homo-oligomeric complexes, which leads to its autophosphorylation and activation of its RNase activity. IRE1 splices a transcription factor coding mRNA, the X-box binding protein 1 (XBP1) mRNA, which leads to the induction of UPR-related genes. Sustained ER stress results in the interaction of IRE1 with adaptor proteins, such as tumor necrosis factor receptor-associated factor 2 (TRAF2), which leads to the activation of the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) cascades, activating the apoptosis pathways.

1.2.3 The PERK pathway

PERK is a serine/threonine (Ser/Thr) kinase, which phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) leading to the attenuation of global protein synthesis relieving the ER from the overload of proteins. Phosphorylation of eIF2α selectively increases the translation of the activating transcription factor 4 (ATF4) mRNA, which regulates UPR-related genes involved in redox homeostasis, autophagy and apoptosis, among others the transcription factor C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153). CHOP is a shared target gene of all three pathways of the UPR, as it can be induced by tATF6, XBP1 and ATF4. CHOP regulates the expression of several Bcl-2 family members contributing to cell death.

1.2.4 The ATF6 pathway

Following its dissociation from BiP, ATF6 translocates to the Golgi-apparatus and is cleaved by specific proteases. The truncated, active N-terminal fragment of ATF6 (tATF6) translocates to the nucleus and upregulates the transcription of ER chaperons (e.g. BiP) and ERAD-related genes.

1.2.5 Role of Bcl-2 family members in the ER stress

ER stress mediated apoptosis is regulated by the pro- and anti-apoptotic Bcl-2 family members. The pro-apoptotic Bax (Bcl2-associated X protein) and Bak (Bcl2-antagonist/killer) proteins have a critical role in the initiation of UPR-caused apoptosis, they form a complex with the cytosolic domain of IRE1α, which is supposed to stabilize its active form. Beside that BH3-only Bcl-2 family members (e.g. Bim [Bcl2-interacting mediator of cell death] and PUMA [p53 upregulated modulator of apoptosis]) cause the oligomerization of Bax and Bak leading to the permeabilization of the outer mitochondrial membrane leading to the release of cytochrome *c* and initiate the formation of the apoptosome.

1.3 The PI 3-K/Akt/GSK-3β axis and cellular survival

The phosphatidylinositol 3-kinase (PI 3-K)/Akt signaling pathway is a key regulator of numerous physiological and pathological processes including metabolism, development, proliferation, apoptosis and cell survival.

Stimulating cells with growth factors and with some cytokines leads to the increased activity of the lipid kinase PI 3-K, subsequently increasing the cell membrane's phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate content. These membrane-anchored

lipids cause the recruitment of proteins possessing Pleckstrin homology (PH) domain, such as Akt and 3-phosphoinositide-dependent protein kinase 1 (PDK1). Akt (referred to as protein kinase B [PKB] as well) has three isoforms (Akt1/PKBα, Akt2/PKBβ, Akt3/PKBγ) coded by distinct genes. Akt1 is widely expressed in different tissues and is mainly responsible for proliferation and survival. Akt2 participates in insulin-dependent glucose homeostasis in muscle cells and in adipocytes. Akt3 is expressed in the testes and in the brain. All three isoforms are activated upon phospholipid binding and phosphorylation on Thr308 in the activation loop and on Ser473 residues in its kinase domain by PDK1 and PDK2, respectively. Akt phosphorylates various substrates regulating a diverse set of different cellular processes including metabolism, survival and proliferation. Cell survival is thought to be regulated by Akt by phosphorylation of substrates that directly or indirectly control apoptosis pathways.

Glycogen synthase kinase-3β (GSK-3β) is a constitutively active Ser/Thr kinase that regulates important cellular processes, including glycogen metabolism, transcription, translation, cell cycle, cytoskeletal integrity and apoptosis. Activation of several signaling pathways leads to the inhibition of GSK-3β by increasing Ser9 phosphorylation (e.g. protein kinase A [PKA], Akt, protein kinase C [PKC], p90 ribosomal S6 kinase [p90RSK], p70 ribosomal S6 kinase [p70S6K]). Several substrates have been identified to be phosphorylated by GSK-3β without prior priming phosphorylation, although most of them must be prephosphorylated, "primed" four residues C-terminal to the GSK-3β phosphorylation site. Nearly 50 substrates of GSK-3β have been identified, including metabolic enzymes, translation initiation factor eIF2B, regulators of cell cycle (cyclin D1), apoptosis (Mcl-1) and several transcription factors. Among those, c-Jun , nuclear factor of activated T-cells c (NFATc), heat shock factor-1 (HSF-1) and the cAMP responsive element (CRE) binding protein (CREB) exhibit reduced DNA-binding affinity following phosphorylation by GSK-3β. GSK-3β has been identified as a key mediator in several apoptotic signaling pathways induced by growth factor withdrawal and PI 3-K inhibition, DNA damage, hypoxia and ER stress.

1.3.1 THE ROLE OF THE CREB TRANSCRIPTION FACTOR IN MAMMALIAN CELLS

CREB is a ubiquitously expressed leucin-zipper transcription factor that plays a critical role in the nervous system by regulating cell differentiation, proliferation, and seems to participate in the development of cancer and atherosclerosis as well. CREB is able to bind to the CRE sequences of DNA as a homo- or heterodimer. The kinase inducible domain contains several residues, which are responsible for the regulation of the CREB protein. The most examined residue is serine-133 (S133) which is the end-point of many kinases (PKA, Akt, PKC, Ca^{2+}/calmodulin dependent kinase II and IV, MAPK, p70 S6K). Phosphorylation of S133 allows CREB to bind to the CREB-binding protein (CBP) and to the p300 enhancing gene expression. Phosphorylation of CREB at S133 creates a consensus site for phosphorylation by GSK-3β at serine-129 (S129). The functional consequence of this phosphorylation is controversial. Some of the data in the literature support that phosphorylation of CREB at both sites is required for the full activation of CREB, meanwhile several other findings state that GSK-3β negatively regulates the DNA binding activity of CREB. Activation of the PI 3-K/Akt signaling pathway leads to the inhibition of GSK-3β in PC12 rat pheochromocytoma cells. GSK-3β phosphorylates CREB that is thought to decrease its DNA binding activity; however the consequence of the phosphorylation of S129 and S133 of the CREB is not perfectly understood yet.

1.4 ER STRESSORS

Perturbation of ER homeostasis subsequently leads to ER stress and UPR. This might be evoked *in vivo* as well as *in vitro* by a fair set of stressors including chemical compounds, viruses, ethanol exposure and hypoglycaemia. Depletion of Ca^{2+} of ER-pools can be achieved by the chelating agent EGTA, by the ionophores A23187 and ionomycin or by thapsigargin (TG) inhibiting the function of P-type sarco-endoplasmatic Ca^{2+}/ATP-ases (SERCA). Dithiothreitol (DTT) and tunicamycin (TM) evoke *in vitro* ER stress and UPR by interfering with disulfide-bond formation in the lumen of the ER and blocking the N-linked glycosylation, respectively. Chronic ethanol exposure evokes ER stress in various organs *in vivo*. In the organ damage ER stress plays a prominent role through the formation of acetaldehyde, oxidative stress, perturbation of Ca^{2+} homeostasis and homocysteine formation.

1.4.1 VIRUS-EVOKED ER STRESS

Virus infection of cells evokes a strong innate immune response and triggers ER stress as well. Viral double-stranded RNA molecules as intermediates of virus replication are recognized by the double-stranded RNA-dependent protein kinase (PKR) in host cells. PKR upon activation phosphorylates its target molecules including eIF2α, attenuating global protein synthesis and by means of this inhibits the synthesis of virus proteins. PERK is a homologue of PKR and plays a crucial role in virus replication. Overloading the ER leads to the activation of PERK phosphorylating eIF2α and attenuating global protein synthesis and shutting off virus replication. On the other hand various virus strains induce ER stress and modulate UPR in order to boost virus replication. Flaviviruses, hantaviruses and paramyxoviruses, such as simian virus 5 and respiratory syncytial virus, have been reported to induce BiP expression after infection, as viruses adopted and developed different strategies to modulate UPR pathways to enhance the ER capacity of the host cell in order to produce more effectively new virus particles. Virus-evoked ER stress might be beneficial in cancer treatment, since this type of cell death has been reported to be (partially) p53-independent.

1.5 ONCOLYTIC VIRUSES

Oncolytic viruses are tumor selective viruses and are promising tools in the therapy of cancer. Meanwhile tumor cells die after being infected with an oncolytic virus; normal cells are not affected. Possible mechanisms of the anti-tumor activity of oncolytic viruses are the following:

- transgene expression by the viral vector,
- viral replication-induced direct cell lysis,
- viral protein-induced direct cytotoxicity,
- antitumoral immune induction,
- sensitization to chemotherapy and irradiation.

The first virus which was introduced into the clinical therapy was the genetically engineered ONYX-015 adenovirus strain, selectively killing p53 negative tumor cells. Different viruses have been postulated to possess potential anti-tumor activity, as listed below, containing genetically engineered, normally occurring and attenuated virus strains: Adenoviruses (e.g.: ONYX-015, H101, CGTG-102, Ad5-Delta24GD, CV706, CV787), Reoviruses (e.g.: Reolysin), Herpes simplex virus (e.g.: Talimogene, Iaherparepvec, NV1020, H103), Poxviruses (e.g.: Vaccinia JX-594, Vaccinia GL-ONC1,

Vaccinia GM-CSF), Picornaviruses (e.g.: Seneca Valley virus), Newcastle disease virus (e.g.: MTH-68/H, PV701, NDV-HUJ), Vesicular stomatitis virus.
Newcastle disease virus (NDV) belongs to the family of Paramyxoviridae. The virus is enveloped and it is surrounded by a phospholipid bilayer. NDV is reported to enter cells both by direct membrane fusion and caveola-mediated endocytosis. Virus particles contain a negative single-stranded 15.1 kb sized RNA genome; they are pleomorphic, sized from 50 to 500 nm in diameter. In humans NDV does not cause diseases (except for mild flu-like conditions), while in natural avian host species NDV causes severe pandemics. The attenuated NDV vaccine strain MTH-68/H (more than hope-68/Hertfordshire) has a selective cytotoxic effect on transformed mammalian cells resulting in apoptosis. *In vitro* studies in our laboratory previously indicated that tumor cell lines showed a wide range of sensitivity toward MTH-68/H infection. MTH-68/H vaccine treatment prolonged survival of patients with advanced cancer resistant to conventional therapeutic protocols, thus MTH-68/H might be a promising future tool for cancer therapy considering its following features.

1. MTH-68/H is non-pathogenic in humans.
2. MTH-68/H does not show signs of antigene recombination.
3. The genome of NDV viruses does not integrate into host cells genome excluding the possibility of random integration and insertional mutagenesis.
4. MTH-68/H has been reported to induce apoptosis in a p53-independent manner.

MTH-68/H showed promising results in glioblastomas and has been thought to have immune modulation properties, direct lytic and pro-apoptotic effect, although the proper mechanism of action has not been perfectly understood so far.

2 AIMS

The experiments and findings of the current thesis aimed to characterize ER stress in rat cell cultures. For the experiments two different approaches were used.

I. TM blocks N-linked glycosylation in the ER leading to subsequent ER stress and UPR. GSK-3β has been postulated as a key molecule in the signal transduction of ER stress as well as a modulator of the activity of CREB through the phosphorylation of CREB on residue S129. Considering the possible relation between the GSK-3β-mediated ER stress and the significance of the S129 and S133 CREB phosphorylation sites, the following points were aimed to be investigated in rat cell culture models.

a) Stable transfection of PC12 cells with expression vectors coding for wtCREB and mutant CREB proteins.
b) Determination of the IC_{50} and IC_{80} concentrations of TM in wtPC12 cells using ATP assay.
c) Comparison of the survival rate of wtPC12 cells, wtCREB overexpressing and mutant CREB expressing PC12 lines using ATP and apoptosis assays after TM treatment.
d) Analysis of the activation of different ER stress/UPR related signaling pathways.
e) Studying the role of GSK-3β in the signal transduction of ER stress in wtPC12 cells, in wtCREB overexpressing and mutant CREB expressing cell lines using GSK-3β inhibitors LiCl and SB-216763 or applying GSK-3β specific knock-down technique.

f) Examination the expression of different Bcl-2 family member proteins after TM treatment in the different cell lines.
g) Observation of the association of the Bcl-2 family member Bim protein to the microtubule network using fluorescence resonance energy transfer.
h) Determination of the survival rate of primary rat vascular smooth muscle cells, Rat-1 fibroblasts and wtPC12 cells transiently transfected with wtCREB coding expression vector treated with TM with or without LiCl.

II. It has been known that the attenuated Newcastle disease virus MTH-68/H strain induces ER stress in PC12 cells. The following points were aimed to be studied after infecting wtPC12 cells with MTH-68/H particles.
a) Characterizing the gene expression alterations of wtPC12 cells with transcriptome analysis using cDNA chip after 12 hours of MTH-68/h infection.
b) Verifying the result of the cDNA chip using qRT-PCR.
c) Identifying the altered signaling pathways upon MTH-68/H infection using functional gene cluster analysis.

3 MATERIALS AND METHODS

3.1 CELL CULTURE

PC12 rat pheochromocytoma cells were cultured in Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO, USA) containing 4500 mg/l glucose, 4 mM L-glutamine and 110 mg/l sodium pyruvate and supplemented by 5% foetal bovine serum and 10% horse serum (Gibco, Carlsbad, CA, USA), referred to as high serum containing medium throughout the thesis. Cells were used between passage 5 and 20 for the experiments. Wild-type or mutant CREB expressing stable cell lines were cultured in the presence of 200 μg/ml G418-sulphate.
Rat-1 cells were cultured in RPMI-1640 medium (Sigma) containing 10% newborn calf serum (Gibco) supplemented with 90.91 U/ml penicillin, 90.91 ng/ml streptomycin and 18.18 ng/ml gentamycin (Sigma). Cells were used between passage 8 and 15 for the experiments. Primary rat vascular smooth muscle (RVSM) cells were isolated from 7 week old Sprague Dawley male animal. After the mechanic and enzymatic disruption of the aorta RVSM cells were cultured SmGM medium (Lonza, Basel, Switzerland) supplemented with SmGM-2, EGF, FGF, insulin, gentamycin/amphotericin B SingleQuotes-2 (Lonza). Smooth muscle phenotype of the culture was validated using smooth-muscle-actin specific indirect immunolabeling.

3.2 INFECTION OF PC12 CELLS WITH ATTENUATED MTH-68/H NEWCASTLE DISEASE VIRUS FOR GENE-EXPRESSION ANALYSIS

For the exon-chip analysis PC12 cell cultures (10^6 cells in 60-mm plates) were infected with highly purified batches of the NDV strain MTH-68/H. Infections were performed at the IC_{50} value for PC12 cells (12.87 particles/cell) for 12 hours.

3.3 SITE-DIRECTED MUTAGENESIS

Site-directed mutagenesis of the pcDNA3/RSV-FlagCREB vector was conducted using Stratagene's QuickChange Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instruction. Primers used for mutagenesis were synthesized by Invitrogen (Carlsbad, CA, USA). The applied sequences were the followings (mutations are indicated with bold letters): S129A forward primer: 5'- GGGAAATTCTT**GCC**AGGAGGCCTTCC-3', S129A reverse primer: 5'-GGAAGGCCTCCT**GGC**AAGAATTTCCC-3', S133A forward primer 5'-GGAGGCCT**GCC**TACAGGAAAATTTTG-3', S133A reverse primer: 5'-CAAAATTTTCCTGTA**GGC**AGGCCTCC-3'. Mutagenesis was validated by sequencing.

3.4 STABLE TRANSFECTION OF CELLS

5×10^6 PC12 cells were seeded onto 100-mm plates 24 hours prior to transfection. A day later cells were transfected with 9 μg of pcD/RSV-FlagCREB, pcD/RSV-FlagCREB S129A, pcD/RSV-FlagCREB S133A and pcD/RSV-FlagCREB S129A-S133A respectively, together with 11 μg carrier salmon sperm single stranded DNA (ssssDNA), using the calcium phosphate precipitation method. Three days after transfection, wtCREB and mutant CREB expressing cells were cultured for 4 weeks in the presence of 400 μg/ml G418-sulphate (Gibco). Separate clones were picked and subclones were cultured and checked for expression. Clones showing the highest expression of CREB in Western blot analysis were selected for further experiments.

3.5 CONFOCAL MICROSCOPY

10^4 cells/well were seeded onto poly-L-lysine coated plastic coverslips in 96-well plates in high serum containing medium, which was exchanged to a medium containing 0.5% horse serum (low serum containing medium) 24 hours later. Next day cells were treated with TM (Sigma) in 200 μl low serum containing medium. After the treatment cells were fixed in 4% paraformaldehyde in 1xPBS. Next day paraformaldehyde was removed and samples were washed with 1×PBS for 5 minutes. Non-specific antibody binding sites were blocked by adding 100 μl 10% bovine serum albumin [BSA (Sigma)] in high salt 1×PBS (1×PBS containing 23.38 g extra NaCl/l referred later as HS PBS). Samples were gently shaken at room temperature for 1 hour on a rocker. CREB and CHOP specific primary antibodies (CellSignaling Danvers, MA, USA) were added in a dilution of 1:200 dissolved in 3% BSA HS PBS, 20 μl/well. Samples were incubated overnight at 4°C. Next day samples were washed 3 times for 5 minutes in HS PBS. Secondary, fluorophore-conjugated antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were added to the samples in a final concentration of 1:200 dissolved in 3% BSA HS PBS. Samples were gently shaken overnight in the dark at 4°C on a rocker followed by washing the samples 3 times for 5 minutes in HS PBS. Samples were mounted onto coverslips using Vectashield (Vector Laboratories, Burlingame, CA, USA) anti-fading mounting medium and visualized by an Olympus FluoView 1000 confocal laser scanning fluorescence microscope (Olympus, Center Valley, PA, USA). Fluorophores were excited in photon counting and sequential modes creating single-plane images.

3.6 ATP ASSAY

Cell viability was tested by a luciferase-based ATP assay (Promega, Madison, WI). 2×10^3cells/well were seeded onto poly-L-lysine coated white-wall F-bottom 96-well plates. Next day the medium was replaced by a low serum containing medium for 1 day. Afterwards cells were treated with TM.

24 hours after the treatment 100 µl media were left in the wells. Before the measurement plates were placed to room temperature for 30 minutes. FluoStar Optima plate reader (BMG Labtech, Offenburg, Germany) measured 100 µl freshly prepared ATP assay reagent mixture into the wells. Plates were shaken for 2 minutes with 300/minute frequency. Plates were then incubated at 25°C for 10 minutes before reading and fluorescence of the wells was measured.

3.7 APOPTOSIS ASSAY

10^5 cells/well were seeded onto poly-L-lysine coated glass coverslips containing 24-well plates. Next day the medium was changed and stable transfected cells were incubated in low serum containing medium, transiently transfected cells in high serum containing medium for 24 hours. Cells were treated with TM. 24 hours later cells were fixed in 4% paraformaldehyde in 1×PBS. Cell nuclei were stained by Hoechst 33342 (Calbiochem, Darmstadt, Germany) fluorescent DNA dye in the final concentration of 0.5 µg/ml. The percentage of apoptotic nuclei was determined by counting at least 200 cells/sample in randomly chosen viewfields using Olympus BX61 fluorescence microscope (Olympus, Center Valley, PA, USA).

3.8 WESTERN BLOT ANALYSIS

5×10^6 cells were plated, kept in low serum containing medium for 24 hours and then treated with TM for 24 hours. Cells were lysed in M-Per mammalian protein extraction buffer (Thermo Scientific, Waltham, MA, USA). 40 µg of protein lysates were loaded onto 12% SDS-polyacrylamide gels and transferred onto PVDF membranes (Amersham, Buckinghamshire, UK). The following primary antibodies were used: anti-CREB, anti-actin, anti-BiP, anti-P-JNK, anti-P-p38 MAPK, anti-P-eIF2α, anti-P-GSK-3 Ser9, anti-P-CREB S133, anti-Bim, anti-Bcl-2, anti-Bcl-w, anti-Bcl-X_L, anti-Bok purchased from CellSignaling (Danvers, MA, USA, 1:1000 final dilution), anti-P-CREB S129 purchased from Thermo Scientific (final dilution 1:1000), anti-Mcl-1 purchased from Sigma (final dilution 1:500) anti-ATF6 and anti-P-IRE1 purchased from AbCam (final dilution 1:500).
Species specific horseradish peroxidase-conjugated secondary antibodies (CellSignaling) were used at 1:2000 final dilution and the immunocomplexes were visualized by Pierce ECL Western Blotting Substrate (Thermo Scientific). Protein bands were quantified with Kodak 1D software (version 3.5.5.B). Results were normalized to actin levels.

3.9 KNOCKDOWN OF GSK-3β USING SIRNA TECHNIQUE

5×10^5/well wtPC12 cells were plated for RT-qPCR into 6-well plates and 2.5×10^4 cells/well for apoptosis assay and immunocytochemistry onto Thermanox (Thermo Scientific) coverslips placed into 24 well plates. Next day transfection was performed using 1 µl/ml DharmaFECT 1 (Thermo Scientific) transfection reagent and 5 µl 20 µM ON-TARGETplus Non-targeting Pool and GSK-3β specific siGENOME SMARTpool siRNA (Thermo Scientific) according to the manufacturer`s instructions. The following day the medium was changed to low serum containing medium supplemented with 87 U/ml penicillin and 87 ng/ml streptomycin (Sigma). 24 hours later cells were treated with 5 µg/ml TM for one day or left untreated and samples were processed for further analysis**.** Knockdown of GSK-3β was validated by immunocytochemistry and qRT-PCR. For immunocytochemistry samples were incubated with anti-GSK-3β antibody (CellSignaling, 1:250)

and Cy3-conjugated anti-rabbit antibody (Jackson). Cells were visualized by Zeiss Axio Imager.M2 fluorescence upright microscope using 40× Corr M27 dry Plan-Apochromat objective (NA 0.95).

3.10 FRET ANALYSIS

Slides for immunocytochemistry were prepared using mouse anti-tubulin (Merck, Darmstadt, Germany) and rabbit anti-Bim (CellSignaling), Cy5-conjugated anti-mouse and Cy3-conjugated anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 1:200 dilution as described in the Confocal microscopy section of Materials and methods. Samples were mounted onto glass slides using 50% glycerol dissolved in 1×PBS. Imaging was conducted using Olympus FluoView 1000 confocal laser scanning fluorescence microscope. Fluorophores were excited with He and Ne lasers with 90% transmissivity. Signal of pre- and post-bleaching images was collected using 40× UPlan FLN objective (NA 0.75) creating 1024×1024 pixel single-layer images in photon count mode. Excitation of each pixel was 10 μs. Range of interest was randomly selected and samples were bleached with 4× zoom with 20 μs/pixel excitation until 90% decrease in the fluorescence of the acceptor was not reached. FRET efficiency and fluorophores' distance was calculated by Olympus FV 10-ASW Ver.01.07.01.00 software (Olympus). Experiments were repeated three times.

3.11 TRANSIENT TRANSFECTION OF CELLS WITH EXPRESSION CONSTRUCTS

5×10^4 wtPC12, Rat-1 or primary RVSM cells were plated onto 24-well plates containing poly-L-lysine coated coverslips in 500 μl final volume and cotransfected in suspension with pcDNA3 (LifeTechnologies, Grand Island, NY, USA) and pEGFP-C1 vectors (Clonetech, Mountain View, CA, USA) (mock-transfected) or with pcDNA3-FLAG-CREB and pEGFP-C1 constructs (CREB-transfected). 3.3-3.3 μg plasmid was mixed with 465 μl SmGM-2 medium followed by the addition of 20 μl FuGENE HD transfection reagent (Promega). The mixture was incubated for 10 minutes at room temperature and then cells were transfected with 37.5 μl/well transfection mixture. 24 and 48 hours after the transfection media was changed and cells were treated with TM with or without LiCl pre-treatment. Cells were fixed and stained as described in the Apoptosis assay section of Materials and methods. The efficiency of transfection was above 80%.

3.12 EXON CHIP ANALYSIS

Total cytoplasmic RNA from control and MTH-68/H-infected PC12 cell culture triplicates was isolated using Qiagen's (Hilden, Germany) RNeasy kit according to the manufacturer's instructions. Samples were analyzed on Affymetrix platform using Affymetrix GeneChip Rat Exon 1.0 ST Array chip (Santa Clara, CA, USA) by UD-Genomed Ltd (Debrecen, Hungary). Expression of specific genes was determined from raw microarray data. Gene expression data were normalized and the absolute fold-change expression (FC) was determined. At least 2-fold increase or decrease in expression was considered to be significant using unpaired T-probe with Benjamini-Hochberg correction. Functional categorization of genes with altered expression was performed by the DAVID functional annotation clustering tool using the recommended settings of the tool. Genes not recognized by the tool were excluded from analysis.

3.13 RNA EXTRACTION AND QUANTITATIVE REVERSE TRANSCRIPTASE PCR

Total RNA was isolated using the RNeasy RNA isolation kit (Qiagen) according to the manufacturer's instruction. Purity and concentration of isolated RNA was measured by NanoDrop-1000

spectrophotometer (Thermo Scientific, Waltham, MA). Two micrograms of total RNA were used for cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA) in a 20 µl final volume according to the manufacturer's instructions. SYBR Green or TaqMan analysis was conducted in duplicate using an Applied Biosystems 7500 Sequence Detector (Life Technologies). Each 10-µl SYBR Green reaction mixture contained 4 ng of cDNA, 5 µl of Fast SYBR Green Master Mix (Life Technologies) and 300 nM primer. TaqMan assay reactions contained 4 ng of cDNA, 5 µl of TaqMan Fast Universal Master Mix (Life Technologies), 300 nM primer and 150 nM probe in 10µl final volume. In cases of SYBR Green protocol melting curve analysis was performed, which resulted in single products confirming the specificity of the amplification reaction. The expression levels of the target genes were normalized to 18S ribosomal RNA levels and were calculated using the standard curve method. Primers and probes were designed using Primer Express software 3.0 (Applied Biosystems, Carlsbad, CA, USA) and were synthesized by Biotez (Berlin, Germany).

3.14 STATISTICAL ANALYSIS

Statistical analysis was conducted using GraphPad Prism 5.03 (GraphPad Software, La Jolla, CA, USA) and SPSS 13.0 (SPSS, Chicago, IL, USA). Determination of normal distribution was conducted by Kolmogorov-Smirnov test. Statistical significance was confirmed by one-way ANOVA followed by Bonferroni posthoc test if significance was observed. Data were expressed as mean ± SEM. $P<0.05$ value was considered statistically significant.

4 RESULTS

4.1 INCREASED CREB NUCLEAR OCCUPANCY

PC12 cells expressing the wild-type CREB, Ser129Ala-CREB, Ser133Ala-CREB and the double mutant Ser129Ala-Ser133Ala-CREB (referred throughout as wtCREB, S129A CREB, S133A CREB and S129A-133A CREB, respectively) constructs showed a substantially higher CREB expression compared to wtPC12 cells in Western blot experiments. Clones selected for further studies expressed a comparable amount of the CREB protein and were further analyzed by immunocytochemistry. Compared to wtPC12 cells the wt or mutant CREB expressing clones showed considerably higher nuclear CREB occupancy mostly in euchromatin regions indicated by single-layer confocal images using indirect CREB-specific immunocytochemistry.

4.2 CREB DECREASES THE TM-INDUCED APOPTOSIS IN PC12 CELLS

To determine the toxic concentration of TM wtPC12 cells were exposed to different concentrations of the agent for 72 hours and viability of the cells was determined using ATP assay. The concentration resulting in a 50% decrease in viability was found 0.01 µg/ml (referred through the thesis as low concentration TM). The 5 µg/ml concentration of TM (referred through the thesis as high concentration TM) was completely toxic for the cells. Further wtPC12, wtCREB and mutant CREB expressing cells were treated with high concentration of TM and assayed for ATP content. The wt and all mutant CREB overexpressing cells showed higher survival rate compared to wtPC12 cells. Survival rate of the S133A-CREB overexpressing cells was statistically different from the wtPC12 results.

These results were confirmed by apoptosis assays. Control and TM-treated cells were studied by fluorescence microscopy and apoptotic cells were counted by nuclear morphology after staining with Hoechst 33342 dye. High TM concentration induced apoptosis in approximately 35% of wtPC12 cells during the 24 hour treatment period. In contrast, overexpression of the wtCREB and the S133A mutant CREB rescued cells from apoptosis, in these cultures approximately 15% of cells underwent apoptosis and this result was significantly different form the TM-treated wtPC12 cells. Expression of the S129A-CREB and the double S129A-S133A mutant CREB induced 22 and 23% apoptotic cells.

4.3 TM PROVOKES ER STRESS IN THE DIFFERENT PC12 CELL LINES

24 hours of TM treatment increased the amount of BiP protein in wtPC12 and in all types of CREB-transfected cell lines in a dose-dependent manner as a hallmark of UPR. Then specific target molecules for each of the three pathways of UPR were chosen. The amount of ATF6 was increased in wtPC12 and in all CREB overexpressing clones in comparison with their untreated control samples. The quantity of tATF6 did not increase in wtPC12 cells; however overexpression of wtCREB decreased the tATF6 level, furthermore all mutant CREB expressing cell lines showed a slight diminution in the level of tATF6. In wtPC12 cells TM treatment induced the phosphorylation of IRE1 and the same rate of the phosphorylation in all CREB overexpressing cell lines was observed. JNK is activated similarly by high concentration of TM in wtPC12 and in the different CREB construct transfected cells. The level of p38 MAPK phosphorylation was reduced in the CREB transfected cell lines in comparison to the wtPC12 cells. The activation of the PERK arm of ER stress is shown by phospho-PERK dependent eIF2α phosphorylation. As a result of TM treatment, a slightly increased phosphorylation level of the eIF2α protein can be detected in wtPC12, S129A-CREB and S129A-S133A-CREB expressing cell lines. In wtCREB and S133A-CREB expressing cell lines the eIF2α phosphorylation level decreased in comparison with the control samples.

Upregulation of the transcription factor CHOP is a common point of convergence in all three pathways of the UPR. Therefore the CHOP induction in the TM-treated cell lines by immunocytochemistry was examined. The induction of CHOP can be detected after TM treatment in wtPC12 and in all CREB-transfected cell lines.

4.4 TM-INDUCED APOPTOSIS CAN BE PREVENTED BY THE INHIBITION OF GSK-3β

WtPC12 and the different CREB construct expressing cell lines were pre-treated with the widely used GSK-3-inhibitor LiCl for 60 minutes prior to the 24 hours of TM treatment and cells with apoptotic nuclear morphology were scored in a fluorescence microscope. LiCl treatment reduced the TM-induced apoptosis in wtPC12 and in all CREB construct-transfected cell lines. In wtPC12 and in the double mutant S129A-S133A CREB expressing cell lines this inhibition was statistically significant.

Similar results were obtained when another selective GSK-3 inhibitor, SB-216763 was used. Cell lines were pre-treated with SB-216763 for 60 minutes prior to 24-hour TM treatment and apoptotic nuclei were scored in a fluorescence microscope. As LiCl, the SB-216763 treatment reduced the TM-induced apoptosis in wtPC12 and in all CREB construct-transfected cell lines. In these experiments the decrease in the rate of apoptosis as a result of the inhibition of GSK-3 was significant in the wtPC12, in the S129A CREB and in the double mutant S129A-S133A CREB expressing cell lines.

Inhibition of GSK-3β by LiCl and SB-216763 was confirmed using GSK-3β specific siRNA-mediated knockdown.

It is known, that Akt phosphorylation of GSK-3β on Ser9 inhibits its kinase activity. To examine these effects wtPC12 and CREB-transfected cells were treated with LiCl and TM alone as well as with the combination of both. As it was expected the level of GSK-3β phosphorylation was increased in the LiCl-treated samples in all cell lines. TM treatment induced the activation of GSK-3β in wtPC12 and in all CREB construct expressing cell lines; the level of phosphorylation decreased in comparison with the control samples. LiCl treatment in combination with the TM treatment decreased GSK-3β activity in all cell lines.

To check the phosphorylation state of S129 and S133 residues on CREB protein in PC12 cell lines, Western blot experiments were performed using phospho-CREB-specific antibodies. The phosphorylation of S129 residue on CREB protein was detected in all of the cell lines except in the S129A-S133A CREB double mutant cells. The slight increase in the phosphorylation of the S129A CREB mutant after TM treatment could be caused by the phosphorylation of the endogenous CREB protein. Increased CREB phosphorylation was detected on residue S133 in the wtPC12, in the wtCREB and in the S129A CREB expressing cell lines after TM treatment. The endogenous CREB protein expression level did not change significantly after TM treatment in our PC12 cell lines.

4.5 CREB OVEREXPRESSION ALTERS THE EXPRESSION OF BCL-2 FAMILY MEMBERS

Since the activation of the pro-apoptotic BH3-only family member Bim protein is essential for ER stress-induced apoptosis in several different cell types, we wanted to determine the Bim expression in our CREB expressing cell lines. As a result of alternative splicing, there are three splice variants of Bim expressed [Bim-extra-long (Bim_{EL}), Bim-long, (Bim_{L}) and Bim-short (Bim_{S})]. Bim_{EL} is the most abundant and it is sequestered on microtubules in a complex with dynein light chain. In wtPC12 cells TM treatment caused a slight increase of Bim_{EL} protein expression, while in the different CREB construct expressing cells the amount of Bim_{EL} decreased as a result of the TM treatment. The Bim_{L} and Bim_{S} levels showed increased expression of these splice variants after 24 hours of high concentration of TM treatment in wtPC12 cells and the level of both of them decreased in all CREB overexpressing clones. The expression of the Bcl-2 protein decreased in wtPC12 cells and in all CREB overexpressing cell lines. In the wtCREB and in the S133A CREB expressing cells a marked decrease was observable in the control samples in comparison to the wtPC12 and the other mutant CREB expressing cells. The expression of Mcl-1 decreased in wtPC12, in wtCREB and in the S133A CREB expressing cells as a result of the TM treatment. The Bcl-X_{L} and Bcl-w levels showed a slight decrease only in the S133A CREB expressing cells.

Bok is a ubiquitously expressed member of the pro-apoptotic multidomain Bcl-2 family, which activates the intrinsic apoptosis pathway. In wtPC12 cells TM induced the Bok expression up to 10 hours and it was completely abolished by 24 hour. Interestingly, the expressions were very strong in the wtCREB and in the S133A CREB control cells and no significant change could be detected in the level of expression even after 24 hours of TM treatment. In S129A and S129A-S133A CREB expressing cells the Bok expression was lower in the control samples and a slight increase could be detected due to TM treatment.

4.6 CREB INFLUENCES THE ASSOCIATION OF BIM TO THE MICROTUBULE NETWORK

Bim has been reported to bind to the actin and microtubule cytoskeletal network in resting cells. Upon different stress stimuli JNK and p38 MAPK stress kinases are able to induce dissociation of Bim from the cytoskeleton and activate the intrinsic pathway of apoptosis. Fluorescence resonance energy transfer (FRET) in confocal microscopy was used to characterize the interaction between Bim and the microtubule network. Since Bim does not directly bind to the cytoskeleton, but for instance binds through the dynein complex to the microtubule system, indirect labeling was aimed to be strong enough to study energy transfer between the labeled Bim and tubulin molecules. For the energy transfer acceptor photobleach method was used with Cy3-labeled rabbit anti-rat specific antibody as a donor and donkey Cy5-labeled anti-mouse antibody as the acceptor recognizing Bim and β-tubulin, respectively. In control cells a clear association of Bim and the microtubule labeling could be observed. In wtPC12 cells high concentration of TM treatment induced the diminution of FRET between the fluorophores indicating the separation of Bim from the microtubule complex. In wtCREB overexpressing and CREB mutant expressing cell lines the FRET could be evoked after 24 hours of TM treatment in the cells representing the close localization of the two fluorophores, implicating the association of Bim to the microtubule complex.

4.7 CREB DECREASES THE TM-INDUCED APOPTOSIS IN VARIOUS TYPES OF RAT CELLS

WtPC12, Rat-1 and primary RVSM cells were transiently transfected with pcDNA3 and pEGFP-C1 constructs (mock-transfected) or with pcDNA3-CREB and pEGFP-C1 constructs (CREB-transfected). 48 hours after transfection cells were treated with TM or pre-treated with LiCl prior to the 24 hours of TM treatment. Transfected, EGFP positive cells were scored for apoptosis according to their nuclear morphology in a fluorescence microscope. Overexpression of CREB significantly decreased the TM-induced apoptosis in wtPC12 and in Rat-1 in comparison to the mock-transfected samples. LiCl decreased the cytotoxicity of TM in primary RVSM cells. Parallel to LiCl treatment transient overexpression of CREB clearly reduced TM-evoked apoptosis in wtPC12, Rat-1 and in primary RVSM cells.

4.8 GENE EXPRESSION ALTERATIONS IN WTPC12 CELLS AFTER MTH-68/H INFECTION

In order to analyze genome-wide gene expression changes caused by an oncolytic NDV strain in a tumor cell line, total RNA was isolated from uninfected wtPC12 cells and from cultures infected with MTH-68/H for 12 hours at a multiplicity of infection corresponding to the IC_{50} value. Samples were analyzed as described in the according section previously. 729 genes (corresponding to 773 exons) were found to be induced and 612 genes (631 exons) to be repressed by virus-infection, at least two-fold. Transcriptional regulation of 5 of the up-regulated genes and 4 of the down-regulated genes were validated by qRT-PCR. Genes with significantly altered expression were categorized into clusters by the DAVID functional annotation clustering tool. According to the functional annotation the following pathways were identified from the functional clusters formed.

1. Pathways for innate immunity
    a. RIG-I-like receptor signaling pathways
    b. Toll-like receptor signaling pathways
    c. Interferon-stimulated pathways

2. Pathways of growth factor signaling and cell cycle regulation
3. Pathways of cellular stress and apoptosis
    a. Intrinsic apoptosis pathway
    b. Extrinsic apoptosis pathway
    c. ER stress pathway

5 DISCUSSION

5.1 THE ROLE OF GSK-3β IN ER STRESS

GSK-3β has been proved to be an important modulator of apoptosis and ER stress. There are multiple mechanisms that can regulate the inactivation of GSK-3β via Ser9 phosphorylation, among those the PI 3-K/Akt pathway is one of the best characterized cell survival signaling pathways. GSK-3β regulates its downstream targets, among them a number of transcription factors directly through phosphorylation, one of them is the nuclear phosphoprotein CREB, which has been described as an important factor in the survival of various cell types. The mechanism by which ER stress activates GSK-3β activity is not completely clear.

5.2 THE SIGNIFICANCE OF S129 AND S133 RESIDUES OF CREB IN THE ER STRESS

The three arms of ER stress signaling were stimulated during prolonged TM treatment in the PC12 cell lines used. Increase in the level of chaperon BiP was observed in all cell types indicating the propagation of induced adaptation mechanisms. The level of the tATF6 showed decrease, meanwhile a prominent rise of the full length uncleaved ATF6 could be observed in the S129A and S129A-S133A double mutant clones. Triggering of the IRE1 pathway and subsequent activation of the stress kinases JNK and p38 MAPK was evoked in all cell lines. PERK-dependent attenuation of the global translation by the phosphorylation of eIF2α was decreased in wtCREB and in S133A cell lines. The ATF4/ATF6-dependent CHOP expression could be detected in the nuclei of all cell types, which clearly indicates the active ER stress response.

Although ER stress was evoked, the present study shows that wt and different phosphorylation site mutant CREB proteins were able to protect PC12 cells from TM-induced apoptosis. Different mechanisms can be considered to be responsible for the increased survival of these cell lines.

One possible explanation for the increased cell survival is the absence of S129 and/or S133 phosphorylation sites. It was demonstrated that GSK-3β has a central role in the ER stress signaling pathway in our cell lines, since inhibitors of GSK-3β (LiCl and SB-216763) partially protected PC12 cells from TM-induced apoptosis.

It has been described, that phosphorylation of CREB at S133 is required for CREB to be transcriptionally active. It was further demonstrated that phosphorylation of CREB at S133 created a consensus site for phosphorylation by GSK-3β at S129. Few studies have addressed the functional consequences of this secondary phosphorylation of CREB by GSK-3β.

We showed that GSK-3β phosphorylates CREB protein on S129 during TM-induced ER stress. As this phosphorylation was reported to decrease the DNA binding activity of CREB, this could lead to the decreased expression of the CREB-regulated genes (e.g. Bcl-2). Using computational analysis several thousand genes were found to be CRE-dependent. Assuming that GSK-3-dependent CREB

phosphorylation highly regulates cell death in PC12 cells, hypothetically the S129 mutants, the S133 mutant cell lines, just like the S129-S133 double mutant cells should show lower apoptotic response to TM, which was confirmed by apoptosis assay experiments.

Another mechanism which could contribute to CREB-dependent survival of these cell lines is the "stealing" of CRE sequences by CREB from ATF6 and ATF4. CREB has been reported to constitutively bind to its target sequences in both unstimulated and stimulated cells. Cotransfection experiments showed that CREB inhibits the CRE binding ability of ATF4 *in vitro*. Overexpression of wt and all the mutant CREB proteins decreased the expression of ATF4 in control cells in comparison to the wtPC12 cells. TM treatment induced the expression of ATF4 protein but it did not colocalize with the CREB protein in the CREB expressing clones. The increased attendance of either wt or mutant CREB could interfere with ATF4 and ATF6 transcription factors and could account for the increased survival rate by competing with the apoptosis-inducing ATF6 and ATF4 proteins for CRE, regardless of the presence or phosphorylation state of S129 and S133 sites on the CREB protein.

5.3 THE ASSOCIATION OF CREB AND THE BCL-2 FAMILY RHEOSTAT

Interestingly, wtCREB overexpressing cells show a reduced amount of Bcl-2, even though several groups identified Bcl-2 expression to be CREB-dependent in different models. Cells were preincubated in low serum containing medium for 24 hours and treated with TM for 24 hours as well in low serum containing medium throughout the experiments, GSK-3β assumed to be subsequently active and able to phosphorylate the S129 residue on CREB which leads to decreased Bcl-2 expression. In those cell lines in which the S129 residue is intact on CREB protein (wtCREB and S133A CREB clones) Bcl-2 expression is much weaker compared to wtPC12 cells.

Using a computational approach previously showed that the *bok* promoter contains a half site CRE sequence, although CREB binding in SACO (serial analysis of chromatin occupancy) library was not confirmed. This could answer the enhanced level of the proapoptotic Bok in CREB overexpressing and mutant CREB expressing cells, however, the TM-induced survival of these cell lines is better compared to wtPC12 cells. Bok selectively binds to the Mcl-1 protein but not to Bcl-2 or Bcl-X_L. Increased Bok expression in wtCREB and S133A CREB expressing cells might contribute to the sequestration of Mcl-1 after TM treatment. According to our experiments expression of different CREB constructs prolongs the expression of Bok protein since in wtPC12 cells it is not expressed after 24 hours. The presence of S129 residue in the CREB protein enhances the level of its expression. Bok expression did not change significantly after TM treatment of the different clones.

The BH3-only Bcl-2 family member Bim is expressed as three splice variants, Bim_{EL}, Bim_L and Bim_S. Bim_{EL} and Bim_L have been reported to be sequestered to dynein light chain in resting cells and may be dissociated upon phosphorylation by JNK and p38 MAPK. All three splice variants are reported to be able to bind to anti-apoptotic Bcl-2 family members blocking their function and promote cell death.

5.4 CREB-DEPENDENT CYTOSKELETAL REARRANGEMENT

Alteration of the cytoskeletal system might also be responsible for the enhanced survival of the CREB expressing cell lines. It was demonstrated by FRET analysis that Bim is located in the vicinity of the tubulin complex under resting conditions and, as a result of TM-induced ER stress, this

association visibly diminished in wtPC12 cells. In wtCREB and mutant CREB expressing cell lines the interaction remained stable even after prolonged ER stress evoked by TM.
Compared to wtPC12 cells all the other cell lines showed a remarkable lower level of the active P-p38 MAPK, nevertheless lower level of active P-JNK level was detected in wtCREB overexpressing cells. Lower activity of these stress kinases could account for decreased phosphorylation of Bim and prolong its binding to the microtubule complex interfering with apoptosis.
Bim_S with its shortest lifespan out of all three splice variants is synthesized upon different stress stimuli *de novo* and possesses the most toxic effect. Wt and S133A CREB cells show noticeably lower Bim_{EL} and Bim_S expression in response to high concentration of TM compared to the other cell lines, which observation can be parallel to the improved survival rate of these cell lines.
Increased survival of wt and mutant CREB expressing cells could be interpreted by the altered Bim rheostat which might be affected at different levels. It is also possible that CREB regulates cytoskeletal rearrangement since several dynein-related genes contain CRE promoter regions.

5.5 General aspects of the GSK-3β/CREB axis; overexpression of CREB decreases the toxicity of TM in Rat-1, wtPC12 and RVSM cells

Previous studies demonstrated the principal role of GSK-3β and CREB in the survival of Rat-1, PC12 and RVSM cells. Transient transfection experiments indicated that overexpression of CREB may potentiate the survival of different cell types used in the present study under prolonged ER stress. As cells were cultured in low serum containing media during transient CREB-expression experiments a remarkable cell loss was observed within 36 hours in the primary RVSM culture. Due to this observation the TM-treatment protocol was modified and wtPC12, Rat-1 and RVSM cells were cultured and treated in high serum containing media. Despite of the high serum conditions TM was able to evoke apoptosis in all three cell lines investigated, but interestingly the primary RVSM cells showed the lowest vulnerability towards TM among the cells investigated. This phenomenon might be explained by the high level of growth factors being present in the culture medium of primary RVSM cells, likely to stimulate the growth factor-dependent PI 3-K/Akt pathway leading to the subsequent inactivation of GSK-3β.

5.6 MTH-68/H induces innate immune response and cell death in wtPC12 cells

WtPC12 cells were infected with the oncolytic MTH-68/H attenuated NDV strain. Cells were infected for 12 hours at a multiplicity of infection according to the IC_{50} (12.87 virus particle/cell) value. Due to the infection 729 and 612 genes were up- or down-regulated, respectively, at least 2-fold. In the case of up-regulated genes more than 50 genes were induced 50-fold or higher and another 70 genes were expressed 10- to 50-fold of control cells. The prominent induction of these genes is an expected effect of the strong antiviral response while the virus replicates and triggers apoptotic signaling. The up- and down-regulated genes were classified into 176 and 146 overlapping clusters, respectively by the DAVID functional annotation tool. Several up-regulated gene clusters are involved in the innate immune response, inflammation and death signaling of the infected cells. Genes of cell cycle regulation and cellular metabolism were typically down-regulated.

5.7 MTH-68/H INFECTION STIMULATES INTERFERON-RELATED PATHWAYS

A wide-range of tumor cells lack IFN expression and are thought to be highly selective to oncolytic viruses. Meanwhile normal cells are capable to express different IFNs upon oncolytic virus infection, in tumor cells this protective mechanism is missing and are killed by other oncolytic activity of the virus. In wtPC12 cells as suggested by the microarray experiment the lack of IFN response cannot be responsible of the cytotoxicity of MTH-68/H in these cells. Although IFNα, IFNβ (Type I, viral), IFNγ (Type II, immune) and IFNλ (Type III) associated signaling pathways are strongly induced in wtPC12 cells with the subsequent induction of IFN stimulated genes (ISGs) upon NDV infection, this is not sufficient to prevent cells from apoptosis.

5.8 MTH-68/H INFECTION INDUCES CELL CYCLE ARREST

MTH-68/H infection of PC12 cells affected the expression of a number of cell cycle regulatory genes coding for both stimulators and inhibitors of cell cycle. Genes were up- and down-regulated in both categories, but the overall balance of gene expression changes favours cell cycle arrest contributing to the anti-tumor effect of MTH-68/H.

5.9 INDUCTION OF APOPTOSIS BY MTH-68/H INFECTION

A possible mechanism of cytotoxic activity of MTH-68/H in wtPC12 cells might be the induction of the different apoptotic pathways. Alterations at the level of mRNA were seen in the intrinsic, extrinsic apoptotic pathways as well as in the ER stress-related pathways in the gene expression pattern. As it was seen, MTH-68/H only induces a few genes according to the intrinsic pathway of apoptosis (*Apaf1, Bid, Casp7*). This is not surprising as considered that the intrinsic pathway of apoptosis is highly regulated at the posttranslational level (e.g. phosphorylation, dephosphorylation, oligomerization and proteolytic cleavage).

Furthermore the extrinsic pathway of apoptosis was induced in wtPC12 cells upon NDV infection. Due to MTH-68/H infection wtPC12 cells express a high level of certain death ligands (*Tnf, Tnfsf10 [TRAIL], Faslg*) and related signaling molecules (e.g. *Ripk2, Ripk3, Birc2, Birc3, Fas. Tnf-r1*). Considering that FasL and its receptor were only poorly induced by MTH-68/H (*Faslg, Fas,* 2.2, 2.3-fold increase, respectively), TRAIL appears to be the most important autocrine/paracrine mediator of virus cytotoxicity parallel to the previous findings found in the literature.

NDV has been postulated to kill tumor cells according to several studies by the stimulation of intrinsic and/or extrinsic pathways of programmed cell death or through ER stress-related apoptosis. The data from the microarray experiments suggest that all three apoptotic pathways might be responsible for the MTH-68/H mediated oncolysis. As described previously MTH-68/H is able to replicate in wtPC12 cells and as a sign of evoked ER stress activation. As indicated by the microarray experiments MTH-68/H strongly induced several genes, such as *Casp4, Casp12, Ddit3, Atf3* that code for proteins of the pro-apoptotic solution of ER stress.

6 SUMMARY

I. It was demonstrated that in addition to wtCREB overexpression, the expression of dominant mutant S129A, S133A and S129A-S133A CREB enhances the survival of PC12 cells under prolonged ER-stress.

II. In the signal transduction of ER stress GSK-3β and CREB seem to play a characteristic role. Pharmacological inhibition of GSK-3β enhanced survival in these cells and confirms GSK-3β to be a promising therapeutical target in ER stress related diseases.

III. CREB phosphorylation sites might regulate not only survival through altering the expression of Bcl-2 family members, but likely to promote changes in the cytoskeletal structure through the regulation of expression of microtubule-associated dynein-related proteins.

IV. In addition expression of any CREB construct used throughout the study might "steal" the CRE binding sites from ATF4 and ATF6 may temper the outcome of ER stress.

V. The present findings ascertain CREB as a potent inhibitor of the ER stress-signaling cascade. Identifying the possible mechanisms leading to increased viability might contribute to the understanding of the pathology of ER stress related disorders and could help to characterize ER stress related therapeutic targets.

VI. MTH-68/H induces a strong IFN response in wtPC12 cells, but this phenomenon alone cannot be responsible for the oncolysis of wtPC12 cells.

VII. MTH-68/H infection arrests cell cycle and pushes cells to cytostatic and cytocidal stage.

VIII. MTH-68/H induces the expression of genes being responsible for the intrinsic, extrinsic programmed cell death and ER stress related apoptosis.

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Balogh, A., M. Nemeth, I. Koloszar, L. Marko, L. Przybyl, K. Jinno, C. Szigeti, M. Heffer, M. Gebhardt, J. Szeberenyi, D. N. Muller, G. Setalo, Jr., and M. Pap. "Overexpression of Creb Protein Protects from Tunicamycin-Induced Apoptosis in Various Rat Cell Types." *Apoptosis* 19, no. 7 (2014): 1080-98.

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