PROCEEDINGS AND ABSTRACTS OF INVITED SPEAKERS
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S-1
REGULATION OF RIPKS IN CELL SURVIVAL AND CELL DEATH BY APOPTOSIS AND NECROPTOSIS, INSIGHTS AND THERAPEUTIC POTENTIAL

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Necroptosis was initially identified as a backup cell death program when apoptosis is blocked. However, it is now recognized as a cellular defense mechanism against infections and is presumed to be a detrimental factor in several pathologies driven by cell death. Necroptosis is a prototypic form of regulated necrosis that depends on activation of the necrosome, which is a protein complex in which receptor interacting protein kinase (RIPK) 3 is activated. The RIP homotypic interaction motif (RHIM) is the core domain that regulates activation of the necrosome. To date, three RHIM-containing proteins have been reported to activate the kinase activity of RIPK3 within the necrosome: RIPK1, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), and DNA-dependent activator of interferon regulatory factors (DAI).

RIPK1 is a key molecule determining cellular fate downstream of several innate immune receptors. It is a serine/threonine kinase consisting of an N-terminal kinase domain linked by a largely unstructured intermediate domain to a C-terminal death domain. In the TNF signaling pathway, RIPK1 paradoxically promotes cell survival as well as cell death. These opposite cellular functions are mediated by 2 distinct faces of RIPK1. Upon binding of TNF to TNFR1, RIPK1 is recruited to the receptor complex I where it acts as a scaffold protein promoting cell survival as well as cell death. These opposite cellular functions are mediated by 2 distinct faces of RIPK1. Upon binding of TNF to TNFR1, RIPK1 is recruited to the receptor complex I where it acts as a scaffold protein promoting cell survival, in part, by activating the canonical NF-kB pathway. Specific conditions can however activate RIPK1, and its kinase activity then regulates assembly of 2 possible cytosolic death-inducing complexes, namely complex IIb (RIPK1-FADD-Caspase-8) and the necrosome (RIPK1-RIPK3-MLKL). These complexes respectively drive TNF-mediated apoptosis or necroptosis. The precise molecular mechanism(s) controlling RIPK1 activation is (are) currently unknown. Similarly, how RIPK1 kinase activity contributes to both cell death processes still remains unclear. Despite this lack of understanding, it is evident that RIPK1 can play a dual role downstream of TNFR1 and that its kinase activity needs tight repression to avoid unnecessary damage to the organism.

Targeting necroptosis can occur at three levels: blocking RIPK1 and RIPK3 kinase activity, and blocking MLKL. Novel drugs and known drugs have been identified in cellular screening which block necroptosis. In vivo they are effective in blocking inflammatory, degenerative and infectious diseases. On the other hand induction of necroptosis has been found effective in inducing immunogenic cell death.
TYPE 2 TRANSGLUTAMINASE: A KEY REGULATOR OF PROTEOSTASIS UNDER CELLULAR STRESSFUL CONDITIONS

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Eukaryotic cells are equipped with an efficient quality control system to selectively eliminate misfolded and damaged proteins, and organelles. Abnormal polypeptides that escape from proteasome-dependent degradation and aggregate in the cytosol can be transported via microtubules to inclusion bodies called ‘aggresomes’, where misfolded proteins are confined and degraded by autophagy. We showed that Type 2 transglutaminase (TG2) knockout mice display impaired autophagy and accumulate ubiquitinated protein aggregates upon starvation. Furthermore, p62-dependent peroxisome degradation is also impaired in the absence of TG2. We also demonstrate that, under cellular stressful conditions, TG2 physically interacts with p62 and they are localized in cytosolic protein aggregates, which are then recruited into autophagosomes, where TG2 is degraded. Interestingly, the enzyme’s crosslinking activity is activated during autophagy and its inhibition leads to the accumulation of ubiquitinated proteins. Taken together, these data indicate that the TG2 transamidating activity has an important role in the assembly of protein aggregates, as well as in the clearance of damaged mitochondria by macroautophagy.

Recently, we have isolated and characterized exosomes derived from cells either expressing or not TG2, under stressful conditions (i.e. proteasome impairment or expressing a mutated form of huntingtin (mHtt) containing 84 polyglutamine repeats). Our results show that TG2 is present in the exosomes only upon proteasome blockade, a condition in which TG2 interacts with TSG101 and ALIX, two key proteins involved in exosome biogenesis. Interestingly, we found that TG2 favours the assembly of a protein complex including mHtt, ALIX, TSG101 and BAG3, a co-chaperone involved in the clearance of mHtt. The formation of this complex is paralleled by the selective recruitment of mHtt and BAG3 in the exosomes derived from TG2 proficient cells only. Overall, our data indicate that TG2 is an important player in the biogenesis of exosomes controlling the selectivity of their cargo under stressful cellular conditions. Taken together these data indicate that TG2 plays a key role in the regulation of proteostasis under stressful cellular conditions.
S-3

AUTOPHAGY AT THE INTERSECTION BETWEEN CELL SURVIVAL AND CELL DEATH: ROLES IN INFLAMMATION AND LYSOSOMAL HOMEOSTASIS

Vojo DERETIC

Departments of Molecular Genetics and Microbiology, Cell Biology and Physiology and Neurology, University of New Mexico Health Sciences Center, USA

Autophagy is a fundamental biological process that fulfills general and specialized roles in cytoplasmic homeostasis, and is at the intersection between cell survival and cell death. This talk will cover the subsystems in autophagy and the recent progress in our understanding of how they come together in the contest of immunity and inflammation. We will also give an update on organizers of precision autophagy in the context of immune and other functions. Furthermore, the role of TRIM proteins in autophagy-based lysosomal homeostasis and their role in lysosomal cell death will be presented.
NOVEL REGULATORS OF AUTOPHAGY*

Devrim GÖZÜAÇIK

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Autophagy is a key biological event that occurs at low basal levels in all cell types from yeast to mammals under non-deprived conditions, performing homeostatic functions such as protein degradation and organelle (e.g. mitochondria) turnover. It is rapidly upregulated during cellular stress, providing cells with recycled intracellular building blocks and substrates for energy generation, hence allowing them to survive unfavorable conditions. Autophagy dysregulations play a critical role in the pathogenesis and progress of several human health problems, including neurodegenerative disorders (i.e. Alzheimer’s, Parkinson’s and Huntington’s diseases), degenerative syndromes (i.e. Dystrophies and dystrophic syndromes), lysosomal storage disorders (i.e. Gaucher’s disease), inflammation and cancer.

In Gozuacik Laboratory in Sabanci University, we mainly focus on the discovery of novel autophagy regulators: RNAs, proteins and pathways (basic research). Moreover in close collaboration with clinicians, we study implications of our findings in human disease formation (pathology and pathogenesis research) and diagnosis (marker research). In collaboration with chemists and pharmacologists, we search for means to modulate autophagy for treatment purposes (drug research). In this speech, results from our basic and medical studies on autophagy will be discussed. *This work was supported by The Scientific and Technological Research Council of Turkey (TUBITAK) 1001 Grant numbers 112T272 and 110T405 and Sabanci University.

Selected References:
INTRODUCING THE TURKISH JOURNAL OF BIOLOGY – PAST ACHIEVEMENTS AND FUTURE EXPECTATIONS

Ekrem GÜREL

Abant Izzet Baysal University, Department of Biology, Turkey

The Turkish Journal of Biology, an international scientific journal, is published by the Scientific and Technological Research Council of Turkey (TÜBİTAK) and accepts English-language manuscripts only (research or review articles, short communications, letters to the editor) concerning all kinds of biological processes including biochemistry and biosynthesis, physiology and metabolism, molecular genetics, molecular biology, genomics, proteomics, molecular farming, genetic transformation, bioinformatics, systems biology, cell and developmental biology, stem cell biology, and reproductive biology. The submitted (online only) manuscript is first checked for format and plagiarism. If the manuscript contains language errors, it is then returned for language editing. Following the format and language checks of the manuscript, the Editor-in-Chief evaluates the manuscripts in terms of the journal’s scope, and then assigns a Manuscript Editor, who reads the manuscript and assigns at least 3 reviewers. The Manuscript Editor and Editor-in-Chief may reject the manuscript without peer review if they do not comply with the Instructions for Authors or if they are beyond the scope of the journal. It normally takes 4-6 weeks to collect the reports of the reviewers and Manuscript Editor’s recommendation to the Editor-in-Chief. If a revision is required, it may take for another 4-6 weeks to complete the evaluation process. The acceptance rate is about 9%, and published articles are freely available online since 1996. The Turkish Journal of Biology has an editorial board of international members from several key disciplines of biology, and has been indexed in Web of Science since 2009, with a gradually increasing impact factor reaching 1.34 the year 2014. The journal keeps expanding its submission geography, currently receiving manuscripts from more than 60 countries around the globe. Its five-year progress expectation is to achieve an impact factor of 2.00 or more and receiving manuscripts from researchers of more than 100 countries.
MITOCHONDRIAL SUBSTRATES: A TOOL TO COMBAT CANCER

Boris ZHIVOTOVSKY

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Mitochondria play an important role in regulation of various cell death modalities. Outer mitochondrial membrane permeabilization and release of several proteins, such as cytochrome c, SMAC, AIF, etc. from the intermembrane space of mitochondria are regarded as a “point of no return” in many models of apoptosis. Accumulating evidence suggested that mitochondria-generated reactive oxygen species are involved in this process. However, depending on their overall concentration at steady state levels, and efficiency of mitochondrial antioxidant enzymes cell death can be prevented.

Cancer cells demonstrate dramatically increased glycolysis even under air-saturated conditions (Warburg effect), whereas mitochondrial contribution to ATP supply is restrained. Consequently, drugs that can perturb glycolysis, might display beneficial therapeutic effects. Our recent observations revealed that among agents that can modulate tumor cell death are members of the Krebs cycle, succinate and citrate. The later can directly suppress glycolysis via inhibition of phosphofructokinase. Importantly, mutations of succinate dehydrogenase (SDH) characterize several tumors. Inhibition of SDH results in accumulation of succinate in cytosol and subsequent activation of hypoxia-inducible factor, which is responsible for upregulation of glycolytic pathway and mitochondrial silencing. We found that in addition to this pathway succinate might suppress apoptosis at mitochondrial level. A link between mitochondrial metabolic changes and cell death as well as how alteration of energy producing pathways can sensitize tumor cells to treatment will be discussed.
S-7
AMBRA1 NEGATIVE CONTROL AT THE CROSSROAD AMONG AUTOPHAGY, CELL PROLIFERATION AND CELL DEATH

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Cell Stress and Survival Unit, Danish Cancer Society Research Centre, Copenhagen, Denmark

The activating molecule in Beclin 1-regulated autophagy (AMBRA1), also known as autophagy/beclin-1 regulator 1, is a highly intrinsically disordered and vertebrate-conserved adapter protein that is part of the autophagy signalling network. AMBRA1 is an important regulator of embryonic development, and both its mutation or inactivation have been shown to impact several pathologies of the nervous system, and to be involved in carcinogenesis. Recent studies have revealed that AMBRA1 can coordinate a cell response to starvation or other stresses by integrated functions that include translocation of the autophagosome core complex to the ER, regulative ubiquitylation and stabilization of the kinase ULK1, selective mitochondria removal and cell cycle down-regulation. On the other side, AMBRA1 itself appears to be targeted by a number of regulations, such as Cullin-dependent degradation, caspase cleavage and several modifications, ranging from phosphorylation to ubiquitylation.

Here we will discuss two relevant novel pathways of AMBRA1 down-regulation: i) AMBRA1 targeting by miR7, an autophagy- and cell-cycle-related microRNA, in a signalling loop we have identified that involves the oncogene c-myc and the phosphatase PP2A; ii) AMBRA1 proteolytic cleavage to generate a novel positive mediator of mitochondrial apoptosis. Indeed, the C-Terminal part of AMBRA1, generated by caspase cleavage upon apoptosis induction, is able to inhibit the anti-apoptotic factor BCL2 by a direct binding through its BH3-like domain. Altogether, both mitochondrial AMBRA1-BCL2 networking and AMBRA1 regulation by miRNAs may represent novel targets in development of therapeutic approaches in human diseases.
IN VITRO AND IN VIVO CHARACTERIZATION OF CELL SURVIVAL GENES USING DESTABILIZED CAS9

Şerif ŞENTÜRK

Dokuz Eylul University - Izmir International Biomedicine & Genome Institute

One of the problems limiting the use of current CRISPR systems is the constitutive endonuclease activity when Cas9 and its sgRNA are co-expressed. Here, we sought to improve upon existing CRISPR/Cas9 techniques to generate a system that (1) would provide potent, robust and temporally controlled gene editing, (2) be applicable to a broad spectrum of cell types and tissues, (3) facilitate high throughput manipulation and (4) be traceable. To this end, we exploited recently developed strategies in which a cell-permeable ligand is used in conjugation with a single genetically encoded destabilizing domain (DD) to regulate the expression of any protein of interest. By fusing the FKBP12-derived destabilizing domain to Cas9 (i.e., DD-Cas9) we demonstrated that this method of conditional regulation of protein stability could be exploited for rapid and reversible Cas9 expression in vitro. We validated the efficiency of this new platform by conditionally targeting a variety of genes controlling diverse biological processes. By targeting the RPA3 gene and EGFR in the “EGFR-addicted” cells, in particular, we demonstrated the ability of the system to identify genes that are essential for sustained cell growth and survival. The unique aspect of this method is the conditional regulation of Cas9 protein expression independently of its mRNA expression. When coupled with a conditional Cre allele (Cre-ER²), DD-Cas9 could be utilized to facilitate the analysis of genes that modulate disease onset and progression in a variety of pre-existing mouse models of human disease based on Cre-lox system. In summary, our data indicate that fusing Cas9 to a destabilizing domain provides a highly efficient and potent, easy scalable, robust and tunable new modality for temporal control of gene editing that can be applicable to a broad spectrum of in vitro and in vivo models.
STAMPING PROLIFERATION AND CELL DEATH IN PROSTATE CANCER

Fahri SAATÇİOĞLU

University of Oslo, The Faculty of Mathematics and Natural Sciences, Department of Biosciences, Norway

The six transmembrane protein of prostate (STAMP) family, also known as six transmembrane epithelial antigen of prostate (STEAP), have been implicated in prostate cancer (PCa). STAMP1 and STAMP2 protein expression is increased in human PCa compared with benign prostate and they regulate central proliferative signaling pathways in PCa cells in vitro and in vivo. In addition, STAMP1 and STAMP2 expression inhibits cell death resulting in robust tumor growth. Consistent with these findings, drug-induced therapeutic silencing of STAMPS by systemic nanoliposomal-siRNA delivery profoundly inhibits tumor growth in preclinical mouse PCa models. These data suggest that STAMPS have a key role in determining life and death decisions in PCa and thus may serve as novel therapeutic targets.
ELK-1 – A CRITICAL REGULATOR OF BRAIN TUMOR PROLIFERATION VS NEURODEGENERATION?

Eray ŞAHİN1,2, Melis SAVAŞAN SÖĞÜT1,2, Başak KANDEMİR1,2, Işıl AKSAN KURNAZ2

1Gebze Technical University, Department of Molecular Biology and Genetics, Molecular Neurobiology Lab (AxanLab), Gebze, Kocaeli
2Yeditepe University, Biotechnology Graduate Program, Kayisdagi, Istanbul
3Anadolu Saglık Merkezi, Tıbbi Hizmetler Direktörlüğü, Gebze, Kocaeli

Although Ternary Complex Factor Elk-1, a member of the ETS superfamily of transcription factors, has traditionally been studied as a transcriptional regulator of immediate-early gene induction upon mitogenic stimulation, its presence in neurons has long generated great interest in its role in these largely post-mitotic group of cells. In this review, we will discuss work from our laboratory and others’, and try to illustrate various roles of Elk-1 in learning, memory, neuronal survival vs death, as well as tumorigenesis.

Key words: ETS, Elk-1, survival, brain tumor, neurodegeneration, tumorigenesis

Introduction

The prototype member of the ETS domain transcription factor superfamily is the c-ets-1, the cellular isoform of the v-ets, the viral E-twenty-six (E26) protein, from which the family derives its name (Buchwalter et al, 2004). The Ternary Complex Factor (TCF) subfamily of this group consists of Elk-1, Net, Sap-1a and Sap-1b, and is named as such due to a three-way complex formed between TCF, Serum Response Factor (SRF) protein, and the Serum Response Element (SRE) on the target promoters (Buchwalter et al, 2004; Sharrocks, 2001). The TCF Elk-1 has long been studied as a mitogen-activated transcription factor that regulates expression of immediate-early gene (IEG) induction, with ERK MAPK transcriptional activation (Yang et al, 1999), therefore presence of Elk-1 in axons and dendrites of neurons was a conundrum (Sgambato et al, 1998; Besnard et al, 2011).

Induction of long-term potentiation (LTP) in the dentate gyrus, contextual fear conditioning and visual light stimulation were all shown to result in rapid phosphorylation of ERK MAP and Elk-1 in different parts of the brain (Davis et al, 2000). One mechanism for the function of Elk-1 in neurons could be regulating survival of neurons actively involved in learning activity (Vickers et al, 2004; Dermir et al, 2011). Yet, other researchers have argued a pro-apoptotic role for Elk-1 in neurons (Barrett et al, 2006a).

Elk-1 in brain tumors and proliferation

ETS proteins include many oncogenes, tumor suppressors, regulators of apoptosis or survival, and as such are usually implicated in a large spectrum of tumors (Breunig et al, 2015). It must also be noted that while normal brain tissue showed almost no Ets-1 expression, different grade gliomas, as well as recurrent astrocytomas, were shown to significantly express Ets-1 and this expression was correlated with state of malignancy.

ETS proteins have therefore been studied as potential targets of cancer therapy; an inhibitor of Ras/Erk activation of Elk3, for instance, was shown to inhibit tumor growth, as well as restrict metastasis, in mice. Similarly, Elk4/Sap-1 was shown to upregulate pro-survival gene mcl-1 in glioblastomas, and downregulation of Elk4 decreased Mcl-1 levels and sensitized the tumor cells to apoptosis (Day et al, 2011).

Our laboratory had previously shown that wildtype Elk-1 repressed egr-1 in SH-SY5Y neuroblastoma cells, in which egr-1 was shown to exert pro-apoptotic effects, and that this downregulation of egr-1 was dependent on SUMOylation of Elk-1 (Demir and Kurnaz, 2008). Hematopoietic factor GATA2 was shown to be upregulated in glioblastoma multiforme (GBM), and promote Elk-1 expression, which was indicative of tumor progression. Indeed, Elk-1 was previously identified as a downstream target for PKC-g and Akt, in addition to ERK MAPK, in mitogenic response in GBM (Mut et al, 2008; Mut et al, 2011), and to colocalize and interact with mitotic kinase Aurora-A in GBM (Demir and Kurnaz, 2013). Therefore Elk-1, as with some other ETS proteins, appear to be involved in tumor initiation and progression.
Elk-1 and hypoxia

As oxygen is a key component of energy metabolism in aerobic organisms, inadequate oxygen supply, called hypoxia, leads to a stress in the cell and if persists may lead to dysfunction and even death. Tumors, to circumvent the problem of hypoxia that results from their hyperproliferative state, secrete angiogenic factors such as VEGF and result in new vessel formation into the tumor mass. Hypoxia-inducible factors (HIFs), composed of HIF-1α, HIF-2α (EPAS1) and HIF-3α (EPAS2), are the critical elements in this regulation (Kaelin and Ratcliffe, 2008). HIF-1α was shown to regulate the cells initial response to acute hypoxia, whereas HIF-2α was involved in chronic hypoxia. HIF-3α, on the other hand, was recently been shown to have different developmentally-regulated isoforms, some of which have opposite actions to hypoxia, but commonly act as inhibitors of HIF-1/2α (Duan, 2016).

Under normal oxygen levels (normoxia), HIF-1α is hydroxylated by the prolyl hydroxylases (PHD) such as HIF-prolyl hydroxylase 2 / Egln1 or HIF-prolyl hydroxylase 3 / Egln3. This hydroxylation results in the interaction of HIF-1α with von Hippel Lindau protein (pVHL), which recruits E3 ubiquitin ligase to HIF-1α and results in labeling of HIF1α for degradation by proteasome (Semenza, 2012). Once accumulated and translocated to the nucleus, HIF-1α then forms a dimer with HIF-1β, and drives expression of hypoxia-inducible genes such as vascular endothelial growth factor (VEGF) (Kaelin and Ratcliffe, 2008; Yucel and Aksan Kurnaz, 2007).

Previous studies have shown that ERK MAPK and its substrate Elk-1 were both activated upon hypoxia, which was argued to be important in tumor growth and metastasis. Knockdown of Elk-1 in tumors was shown to significantly decrease the expression of HIF-2α-dependent hypoxia-inducible genes, and Elk-1 was observed to physically interact with HIF2α (Aprelikova et al, 2006). On the other hand, CD133, a cell surface protein and a cancer stem cell marker, was regulated by both HIF-1α and HIF-2α, in addition to Elk-1, although in this promoter Elk-1 interacted with HIF-1α but not HIF2α, indicating that different modes of regulation exist for different targets (Ohnishi et al, 2013). Interestingly, E2-EPF ubiquitin carrier protein (UCP), which is highly expressed in many tumors and targets pVHL for degradation thereby stabilizing HIF-1α in cells, was shown to be regulated by Egr-1 as well as SRF, the transcriptional partner for Elk-1.

In both the hippocampus and frontal cortex of mice, in normal tissue, Elk-1 was found to be phosphorylated at Serine 383 upon hypoxic preconditioning. Therefore, it was interesting that in microarray analysis carried out in SH-SY5Y cell lines under normal oxygen tension, overexpression of a constitutively active Elk-1-VP16 fusion protein resulted in up- or down-regulation in a number of hypoxia signaling pathway elements (unpublished data; Table 1; summarized in Fig.1).

Table 1. Hypoxia signaling pathway elements up- or down-regulated in response to exogenous Elk-1-VP16 overexpression in SH-SY5Y cells under normoxic conditions

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<td>HIF3A</td>
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</tr>
<tr>
<td>HIGD1B</td>
<td>2.57</td>
<td>0.001</td>
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<td>HIGD1A</td>
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<td>SOD3</td>
<td>2.49</td>
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<td>EGLN3</td>
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<td>HIF1AN</td>
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<td>SOD2</td>
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<td>EGLN1</td>
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<td>HYOU1</td>
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</tr>
<tr>
<td>VHL</td>
<td>-3.43</td>
<td>0.0066</td>
</tr>
<tr>
<td>SIRT6</td>
<td>-21.17</td>
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Since HIF-2α-target promoters were found to overlap with Elk-1 targets in a previous study (Aprelikova et al, 2006), we carried out a preliminary study on promoter sequences of a subset of genes regulated by Elk-1-VP16 in aforementioned microarray study. HRE motif was detected in 74 of 393 promoters analyzed, 73 of which contained both ets and HRE motifs. For 55 promoters among those, the proximity between these two motifs were found to be less than 500 bp, which strengthens a possible transcriptional interaction of Elk-1 and HIF-1α on these promoters (Fig. 2).
erythropoietin, EPO, through HIF-1α transcriptional activity, which was shown to be regulated closely by SOD3 (Suliman et al, 2004). Additionally, in pVHL-deficient renal cell carcinoma, HIF-1α was found to repress mitochondrial SOD2 expression (Gao et al, 2013). Furthermore, EPO was shown to protect SOD2-deficient astrocytes from oxidative stress-induced damage and increased their viability. It is therefore important to note that in Elk-1-VP16 expressing SH-SY5Y cells, SOD3 and EPO are upregulated, while SOD2 and VHL are both downregulated (Table 1).

Anti-aging protein and histone deacetylase SIRT6 was shown to regulate glucose homeostasis as a co-repressor of HIF-1α (Zhong et al, 2010). In the brain, SIRT6 was found to be downregulated during maturation, and overexpression in cortical and hippocampal neurons was shown to reduce cell viability under oxidative stress conditions, but not in normal cells. More importantly, in Alzheimers patients as well as transgenic Alzheimer model mice, SIRT6 levels were recently found to be decreased due to Ab42 accumulation, and overexpression of SIRT6 can overcome Ab42-induced DNA damage in these cells. Therefore, it is interesting to note that Elk-1 repressed SIRT6 expression significantly in SH-SY5Y microarray (Table 1; Fig. 1).

This brings about the question of whether Elk-1 may hijack these pathways in establishing a critical balance of cell death vs survival vs proliferation in the brain, thereby regulating neurodegeneration – survival-brain tumor proliferation axis (Fig. 3).

Fig. 2. Venn diagram illustrating the number of promoters having putative binding sites of Elk-1 alone, both Elk-1 and Hif-1α or Hif-1α alone. In mammalian kidney, one of the adaptations to hypoxia is the upregulation of erythropoietin, EPO, through HIF-1α transcriptional activity, which was shown to be regulated closely by SOD3 (Suliman et al, 2004). Additionally, in pVHL-deficient renal cell carcinoma, HIF-1α was found to repress mitochondrial SOD2 expression (Gao et al, 2013). Furthermore, EPO was shown to protect SOD2-deficient astrocytes from oxidative stress-induced damage and increased their viability. It is therefore important to note that in Elk-1-VP16 expressing SH-SY5Y cells, SOD3 and EPO are upregulated, while SOD2 and VHL are both downregulated (Table 1).

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Fig. 3. A cartoon summary of our working model for Elk-1 function. Based on the expression level, phosphorylation status or subcellular localization of Elk-1, the cell can be tipped off to either overproliferation, leading to brain tumorigenesis, or cell death, resulting in neurodegeneration. When in balance, the system maintains survival of the cell.
Elk-1 in neurodegeneration

In cotransfection experiments with α-synuclein, a protein commonly mutated in Parkinsons Disease, particularly the A53T mutation of α-synuclein was found to attenuate MAPK signaling, interact with Elk-1, and result in its decreased phosphorylation (Iwata et al, 2001). Phototransfection of Elk-1 mRNA to dendrites, but not soma or axons, of neurons produced cell death in a DNA binding-dependent manner (Barrett et al, 2006). It was further shown that upon proapoptotic stimuli, Elk-1 localized to mitochondria, and overexpression of Elk-1 in neurons resulted in decreased viability. In organotypic hippocampal slices of rat brain, amyloid beta plaque (Aβ) accumulation was shown to increase expression of actin-binding protein centaurin-a1 (CentA1) in neurons, which in turn leads to increased association of Elk-1 with mitochondria, resulting in synaptic dysfunction (Szentmari et al, 2013). In the light of various evidences showing proliferative effect of Elk-1 in brain tumors, it is unclear why Elk-1 should cause cell death in neurons.

One possibility is that the mode of Elk-1 mRNA delivery, namely phototransfection, creates a stress to the cell, thereby parallel apoptotic pathways are upregulated and crosstalk with Elk-1; another possibility is that dendritic translation from Elk-1 mRNA results in mislocalization of this protein, which alarms the cell and triggers cell death. Indeed, presence of large number of mitochondria in dendrites and pro-apoptotic effect of mitochondrial Elk-1 protein supports this hypothesis (Barrett et al, 2006).

ERK MAPK-dependent phosphorylation of Elk-1 is mostly concentrated to Serine 383 – Serine 389 phosphorylations, which result in enhanced DNA binding and transcriptional activity (Yang et al, 1999). However, Sharma and colleagues have identified a specific phosphorylation of Elk-1 on Threonine 417 to be closely correlated with neurodegeneration and the P-T417-Elk-1 phosphoform to associate with inclusion bodies in human Lewy Body Disease, Alzheimer’s Disease, and Huntington’s Disease samples, which they argued to be evidence for a causative function of Elk-1 in neurodegeneration (Sharma et al, 2010). We, however, had identified this phosphorylation of Elk-1 to be exclusively localized to DNA in mitosing cells (Demir and Kurnaz, 2013), therefore similar to mitochondrial localization it is possible to assume once again that not the presence of Elk-1 per se, but the mislocalization of particularly the P-T417-Elk-1 phosphoform to inclusion bodies instead of nucleus could be a stress call for the cell, initiating cell death.

Supporting this hypothesis are the observations that RNAi mediated knockdown of Elk-1 levels in Huntington’s cells promoted caspase activation and cell death (Anglada-Huduet et al, 2012), that activation of ERK MAPK and subsequent phosphorylation of Elk-1 on Ser 383 residue resulted in increased SOD1 expression, and that in microarray analyses Elk-1-VP16 HIF prolyl hydroxylases Egln1 and Egln3 were repressed by Elk-1-VP16 (Table 1; Fig. 1).

Conclusion

In spite of observations and reports summarized above, Elk-1-deficient mice were reported to be viable, with no major phenotype abnormalities other than sterility of males, and exhibited no changes in proteomes of brain or spleen (Cesari et al, 2004). The only change was a reduction in kainic acid-induced expression of c-fos in hippocampus of Elk-1-deficient mice when compared to wildtype littermates (Cesari et al, 2004). High level of redundancy between TCF members is thought to dampen the negative effects on cell viability in most types of neurons in Elk-1 knockout studies (Sharrocks, 2001). More recently, behavioural deficits such as open field, spatial object recognition and elevated maze performance, as well as spatial and associative memory upon blast exposure, were observed in Elk-1-deficient mice (Patel et al, 2014).

Therefore, in the context of nervous system our working model is one where “normal” levels of Elk-1 phosphorylation and activity, as well as nuclear localization, are likely to maintain survival, however if the balance is somehow disturbed, be it through mitochondrial and dendritic localization, or over phosphorylation, either hyperproliferation and brain tumor progression or cell death and neurodegeneration is observed. Further analyses to indeed question and challenge such a model, and to establish the exact mechanisms underlying such a balance in detail, are required.

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DIMINISHED CYCLIN DEPENDENT KINASE ACTIVITY AND
MTOR ARE CRITICAL IN THE CELL DEATH DECISION THROUGH
AFFECTING STAT SIGNALLING DIFFERENTLY IN PROSTATE
CANCER CELLS

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Prostate cancer is the second most frequently diagnosed cancer as well as the
sixth leading cause of death in males with cancer worldwide. Androgens play a
critical role in prostate cancer development. However prostate cancer cells may
progress androgen-independently that causes higher mortality rates. Therefore,
new therapeutic targets and clarification of their signaling pathways is critical in
treatment of aggressive prostate cancer cases. One of the promising anti-cancer
strategy is the inhibition of proliferating cancer cells via targeting cyclins and
cyclin-dependent kinases (CDKs) complexes, which causes supression of cell
survival signalling routes. New generation CDK inhibitors roscovitine (CYC202,
seliciclib) or purvalanol inhibits specific CDK targets and thus prevents cell
proliferation and induces apoptosis. In this study, purvalanol and roscovitine
was used to expose the mechanism underlying mTOR-related apoptotic and/or
autophagic response and to understand roles of mTOR depending on its signal
cascades in the cell death processes via mTOR silenced androgen receptor (AR)
negative PC3, DU145 and AR positive LNCaP prostate cancer cell lines. In
PC3 and LNCaP cells, CDK inhibitors were used alone and with mTOR siRNA
combination to scan and analyze differentiation upstream and downstream
targets of mTOR using Pathscan ELISA Assay. CDK inhibitors purvalanol and
roscovitine affects activation of mTOR and mTOR-related kinases in a CDK-
independent manner. However purvalanol exerts more potent inhibitory function
than roscovitine. In PC3 and LNCaP cell lines, mTOR deficiency causes blockage
of apoptotic processes induced by CDK inhibitors. On the other hand, regulation of
STAT1 and STAT3 proteins by mTOR seems to determine apoptotic effects of those
drugs. Increased STAT3 Ser727 phosphorylation levels by CDK inhibitors leads
to decrease STAT3-FoXO1 and CDK5 activity. Diminished CDK5 activity then
causes AR-STAT3 dissociation. Therefore, especially differentiation in STAT3
eexpression and phosphorylation status play a vital role in the manner of regulation
of cell survival and cell death pathways signalling. However, CDK inhibitors and
their combination with mTOR siRNA leads to diverse STAT3 expression profiles
in DU145 cell line. In conclusion, CDK inhibitors are promising drug candidates
in the treatment of aggressive prostate cancer cells through modulating different
molecular targets depends on the cell type.
THE BRIGHT AND THE DARK SIDES OF REPROGRAMMING TO PLURIPOTENCY

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Somatic cell reprogramming with a few defined transcription factors to pluripotency is a several weeks long process. The driving forces behind this phenomenon and the cascade of events are very poorly understood. It is however crucial to uncover the fine details of this process in order to comprehend the true property of these induced pluripotent stem cells (iPSCs) and so better tailor their future therapeutic and disease study use.

Several years ago, we developed a reprogramming method utilizing a piggyBac (PB) transposon-mediated delivery of the reprogramming transgenes. Beyond the ability of seamless removal of the transgenes once pluripotent stem cells have been generated, this system has additional unique features. For example, when combined with the doxycycline inducible transgene expression system, we found that these transgenes are very efficiently regulatable by adding or withdrawing doxycycline. *In vivo* differentiated somatic cells derived from these iPSCs can be reprogrammed to “secondary” iPSCs (2ºiPSC) by simply adding doxycycline to the culture medium. Somatic cell lines produced with this method frequently return to 2ºiPSC in a “population” manner, which allows us to study the cascade of molecular events during the entire process of reprogramming. This unexpected and unique property of the PB reprogramming system allowed the characterization of the three week reprogramming process at an almost daily resolution at multiple omics levels, leading to a better understanding the molecular events associated with generating pluripotent cells from somatic cells.

In parallel, we have investigated the genetic changes associated with the reprogramming process. We identified de novo generated copy number variations at the early phase of reprogramming, which created a high level of genetic mosaicism. Intriguingly, the genome damage load is decreasing when the cells are cultured for an intermediate period of time. Our studies led us to conclude that this phenomenon is due to selection against mutated cells.
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STEM CELL THERAPY APPROACHES TO ISCHEMIC CARDIOMYOPATHY

Human Umbilical Cord Mesenchymal Stem Cell Applications Contributing To The Progression Of Ischemic Cardiomyopathy In Animal Models

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Abstract: Cellular therapies comprise the current and encouraging approach for preventing/curing heart failure, one of the main sequels of myocardial infarction (MI) causing deaths and numerous health problems almost all over the world. Varying types of stem cells from different sources having different characteristics have been tested in several experimental and clinical studies for the healing process of myocardial damage following acute MI. Many factors such as obtaining conditions, expansion capabilities, banking and route of delivery are all considered to designate the most appropriate type of cell(s) in cardiomyogenic damage repair. In recent years, human umbilical cord stroma-derived mesenchymal stromal cells (hUCS-MSC) revealed promising results in various tissue damage models. In this mini review, experimental MI studies in laboratory animal models are discussed regarding their therapeutic effects. Collectively, allogeneic use of hUCS-MSCs, a promising alternative to the usage of many autologous cell types, seem to be safe and reveal a significant regenerative effect on damaged heart tissue as well as many other tissue types tested so far.

Key Words: Myocardial infarction, human umbilical cord stroma-derived mesenchymal stem cells, cardiac repair, regenerative medicine

Introduction

Myocardial infarction (MI) is still one of the most frequent causes of deaths around the world and is often results with scar formation in the infarcted heart (1-4). Since the discovery of the cardiac stem cells (CSC) and revealing of the cardiomyogenic differentiation capacities of these cells, the notion that the heart is not a self-renewal organ has started to change considerably (1, 3). Except for the fact that the CSCs being relatively few, the regeneration ability of heart is still very limited in spite of the factors contributing to the regeneration of the heart, like migration of the bone marrow stem cells to the injured regions with the influence of many signaling molecules that come into play as a result of the injury (1). Limited benefits of the pharmacological and interventional methods for preventing/reducing scar formation and the heart’s limited intrinsic regeneration ability cannot prevent the problem of ischemic cardiomyopathy at changeable levels after MI. Thus, experimental and clinical studies have intensively focused on the therapies using stem cells and their derivatives to support the regeneration ability of heart and more importantly, to prevent the functional tissue loss by directly differentiating to the cardiomyocytes (2, 5, 6).

Bone marrow-derived mononuclear cells, mesenchymal stem cells (MSCs), skeletal myoblasts, peripheral blood endothelial progenitor cells and CSCs were the main cell types that were taken in consideration in clinical studies so far, after their positive effects on the cardiac regeneration were revealed in experimental studies (1, 2, 4, 6, 7). As for the embryonic stem cells (ESC) which are superior to all these aforementioned cells in terms of potency, they have limited usage in clinic studies because of certain ethical problems and their tumorigenic potential (1-3, 7). Induced pluripotent stem (iPS) cells, on the other hand, eliminate the ethical problems and owing a high transdifferentiation potency just like the ESCs can also cause rejection response although they are syngeneic (2) and can form tumors (1, 3, 7). The human umbilical cord stroma-derived mesenchymal stromal cells (hUCS-MSCs), although tested in lesser number of studies so far compared to the ones above, took place in experimental and clinical studies (8) in which healing effects are investigated during the regeneration of infarcted heart after MI. The UCS-MSCs can differentiate to cardiomyocyte-like cells as well as they can display adipogenic, osteogenic, condrogenic, neurogenic and myogenic differentiation under suitable in vitro conditions (9-12). The UCS-MSCs express the well-established MSC markers like CD73, CD90 and CD105, whereas they do not contain the hematopoietic or endothelial cell markers such as CD45, CD34 and CD31 (10, 12, 13). Moreover, as these cells express MHC class I molecules, they do not express HLA-DR, a class II MHC molecule directly related to the immune
4 weeks after the transplantation (15). Dayan et al. applied 2 × 10⁶ hUC-PVCs to the periinfarct zone 2 weeks after MI and, then they sacrificed the animals. Besides having multipotent nature, they cause neither teratoma formation nor rejection response, all of which specify them as a proper cell type to be tested in cell therapies. In this mini review, experimental studies examining the regeneration potential of the hUCS-MSCs in myocardial repair thus the therapeutic effects in MI cases are discussed.

**MI Modelling in Animals**

Left coronary artery (15, 16) or more frequently left anterior descending (LAD) coronary artery (11, 17-24) ligations were performed in order to generate an experimental MI, in NIH nude immunodeficient (21, 22) or healthy rats (15), SCID (24), NOD/SCID gamma null immunodeficient (19, 20) or C57BL/6 mouses (17, 23), rabbits (11, 16) and miniswines (18). It is also necessary to indicate that cyclosporine was applied to the animals for immune suppression after cell transplantation where the healthy rat MI model (15) or the rabbit MI model (11) was used. The necessity of applying an immune suppression prior to hUCS-MSC transplantation or preferring immunodeficient animals could cause a dilemma. In one of these experimental studies, Dayan et al. determined that the hUCS-MSC application after MI increases the number of the anti-inflammatory monocytes/macrophages in the circulation and furthermore infiltrate the cardiac tissue (19). In relation to this, the anti-inflammatory cytokines also increase in the infarcted tissue whereas the pro-inflammatory cytokines decrease. This is an important finding that supports the immune modulator features of the hUCS-MSC.

**Cell Preparing and Delivery**

Besides the variety of the animals used in those experiments, different approaches in terms of the cell doses, time(s) and route(s) of cell application, and laboratory procedures before cell application were reported in these studies. For instance, Wu et al. suspended 5 × 10⁶ CMDiI-labeled hUCS-MSCs in 200 µL PBS and enjected them to the periinfarct zone 2 weeks after MI and, then they sacrificed the animals 4 weeks after the transplantation (15). Dayan et al. applied 2 × 10⁶ hUC-PVCs (human umbilical cord-perivascular cells) suspended in 200 µL DMEM via the tail vein 48 hours after MI and then sacrificed the animals 16 weeks after MI (19). One hour after performing MI in rabbits, Latifpour et al. applied 5 × 10⁶ BrdU-labeled hUCS-MSCs in 50 µL PBS to the perinfarct area by a subepicardial enjection and harvested the hearts by the sacrifice 30 days after cell transplantation (11). After MI induction in mini swines Zhang et al. applied CMDiI-labeled 4 × 10⁶ hUCS-MSC suspended in 2 mL PBS into 9-10 perinfaarc areas by enjecting 0.2 mL per area and then sacrificed the animals 6 weeks after intervention (18). Yannarelli et al. injected GFP labeled 0.5 × 10⁶ hUC-PVCs in 50 µL PBS to the infarct border just 15 minutes after MI induction in a group whereas applying the same number of hUC-PVCs in 200 µL PBS via the tail vein in another group and then sacrificed all animals 14 days after MI (20). Nascimento et al. applied 2 × 10⁶ hUC-MSCs in 20 µL 5% BSA/PBS by intramyocardial enjection into four areas after LAD ligation then sacrificed animals 14 days after surgery (17). Since Li et al. investigated the effects of the hUCS-MSC cotransplanted with human cord blood CD34+ cells on MI, they applied 5 × 10⁶ hUCS-MSCs in PBS to a group of rabbit MI models 4 weeks after MI. They injected 5 × 10⁶ hUCS-MSC and 5 × 10⁷/kg CD34+ cell combination in PBS to 4 areas at the infarct border in another group. They sacrificed the animals 4 weeks after the transplantation (16). Zhang et al. examined the effects of the hUCS-MSCs preconditioned with H₂O₂ (hydrogene peroxide) in MI treatment. For this purpose, three hours after the LAD ligation, they applied 1 × 10⁷ preconditioned hUCS-MSCs in a group. They applied the same dose hUCS-MSCs without preconditioning via the tail vein to another group of animals and they sacrificed all animals 28 days later (23). Alternative route of cell delivery was the graft application tested in two studies (21, 22). Liliyonna et al. used GFP and firefly luciferase-labeled subamnion cord lining (CL)-MSCs, and they applied 2 × 10⁶ cells-enriched grafts onto the scar areas 2 weeks after MI (22). Martinez et al. produced 1.5 × 10⁶ CL-MSC-GFP-Fluc containing angiogenic spheroids and they coated each spheroids with 2 × 10⁶ HUVECs. Later, 150 angiogenic spheroids, in other words, grafts containing 2.25 × 10⁶ CL-MSC-GFP-Fluc and 3 × 10⁶ HUVECs were produced and were implanted onto the scar tissue 2 weeks after MI (21). Winters et al. divided the 5 × 10⁶ hUCS-MSCs produced under hypoxic conditions into 10 µL volumes in PBS and injected into four perinfarct areas intramyocardially following the stabilization of the heart rate in MI induced mice. Then they sacrificed the animals 4 or 12 weeks after the application (24).

**Functional Recovery in Cardiac Functions**

Changes in the cardiac functions were evaluated after the cell administrations using...
parameters that reflect myocardial damage and functions such as left ventricular fractional shortening (LVES), left ventricular ejection fraction (LVEF), left ventricular end diastolic diameter (LVEDD), left ventricular end systolic diameter (LVESD) and left ventricular wall thickness. Shortly, these analyses revealed that hUC-PVCs improved cardiac functions in a short term and reduced the ventricular remodeling in a long term (19). The results of the other experimental studies similarly indicate that the hUCS-MSCs delivered after MI improve left ventricle functions (11, 15-18, 20-24).

**Histopathological Outcomes**

It was suggested that the stem cells exert their contributions to the cardiac regeneration by directly transforming into cardiomyocytes or participating the vessel formation, and by inhibiting the apoptosis while stimulating the endogenic CSC and new vessel formation and regulating the extracellular matrix organisation with their paracrine effects (3, 5). In histological analyses of the cell-transplanted myocardium, the existence of the donor cells and their de novo protein synthesis, apoptosis and apoptotic cells, vascularization levels and the size and dissemination of the scar area were carefully evaluated. Transplantation of pre-labeled donor cells made their demonstration possible in the infarcted myocardium (11, 15, 18, 20, 21) using histopathological analyses. The donor cells occasionally encountered in the infarcted regions were positive for a cardiomyocyte marker cTnT (cardiac troponin T) as well as troponin I, F-actin (filamentous actin) or an endothelial marker vWF (von Willebrand factor) (11, 15, 18, 24). Upon positive observation for αSMA (α-smooth muscle actin) (15, 21, 24) it was suggested that these cells had a capacity of differentiation into the cardiomyocyte lineage and consequently participate in cardiac regeneration by directly or indirectly supporting the smooth muscle and/or endothelial cell differentiation (15, 24). Cardiomyocytes detected in the infarcted heart reions were found to differentiate from the hUCS-MSC and formed gap junctions with the host cardiomyocytes thus synthesizing connexin 43 protein (11, 18, 24). In addition to the differentiation towards the cardiac direction, donor cells were also suggested to stimulate the CSCs by regulating their proliferation and differentiation (18). Following cell transplantation, the vessel density increases at significant level both in infarcted and perifarct areas (15-18, 20-22). Moreover, some transplanted hUCS-MSCs were localized close to the vascular structures and possibly participated in the angiogenesis (15, 21, 24). Interestingly, it was revealed that the transplanted hUCS-MSCs secreted VEGF in the ischemic myocardium and participated in the therapeutic revascularization (15). It was also determined that hUCS-MSC transplantation after MI reduced the scar size (11, 16, 18, 21, 22, 24) and decreased the number of the apoptotic cells in the areas neighboring the infarct zone (15, 17-19).

**Different Approaches of hUC-MSC Therapy**

Li et al. compared the pure application of hUCS-MSCs with the cotransplantation of these cells with CD34+ cells obtained from the human cord blood in a rabbit MI model. They determined that the recovery in the heart functions, vascularization and the reduction in the infarct area became more distinct when the hUCS-MSC was applied with CD34+ cells (16). Moreover, after the observation that the cTnI (cardiac troponin I) positive cells were exclusively localized in the infarct zone only in the cotransplantation group, they suggested that this approach could stimulate the cardiomyogenic differentiation (16). Zhang et al. transplanted the H2O2 preconditioned hUCS-MSCs to a group of mice after MI (23) as compared to non-preconditioned cells. Cumulating data of these two groups showed that the cardiac functions and the microvascular density were significantly better and the fibrosis was significantly lesser in the mice treated with preconditioned hUCS-MSC (23). When compared to the pure applications of hUCS-MSCs, contranplantation modalities like in the study conducted by Li et al. (16) or applications of adapting the cells into the infarct microenvironment as in the study conducted by Zhang et al. (23) seem to increase the effectiveness of the treatment.

Another group of approach was the graft application containing angiogenic sphereoids consisting of CL-MSC coated with HUVEC (21) or grafts produced from CL-MSC supported with flaps (22).

**Conclusion**

Overall results of these experimental studies for determining the curative effects of the hUCS-MSC after MI revealed that these cells improve cardiac functions and regulate a reconstruction at the histopathologic level either by transdifferentiation or via their paracrine effects and their contributions to the immune modulation. Although the positive effects of the cells have been proven, different routes of cell delivery is still controvertial. In this sense, more experimental studies are needed to enable comparisons between different aspects of the treatment process. Furthermore, expanding the accumulation of knowledge and practical experiences with clinical studies will be useful for developing more effective treatment methods.

**References**

TARGETING INFLAMMATORY AND APOPTOTIC PATHWAYS BY AGENTS DESIGNED BY MOTHER NATURE FOR PREVENTION AND TREATMENT OF CANCER

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Chronic infections, obesity, alcohol, tobacco, radiation, environmental pollutants, and high-calorie diet have been recognized as major risk factors for the most common types of cancer. All these risk factors are linked to cancer through inflammation and apoptosis. While acute inflammation that persists for short-term mediates host defense against infections, chronic inflammation that lasts for long-term can predispose the host to various chronic illnesses, including cancer. Linkage between cancer and inflammation is indicated by numerous lines of evidence; first, transcription factors NF-κB and STAT3, two major pathways for inflammation, are activated by most cancer risk factors; second, an inflammatory condition precedes most cancers; third, NF-κB and STAT3 are constitutively active in most cancers; fourth, hypoxia and acidic conditions found in solid tumors activate NF-κB; fifth, chemotherapeutic agents and gamma irradiation activate NF-κB and lead to chemoresistance and radioresistance; sixth, most gene products linked to inflammation, survival, proliferation, invasion, angiogenesis, and metastasis are regulated by NF-κB and STAT3; seventh, suppression of NF-B and STAT3 inhibits the proliferation and invasion of tumors; and eighth, most chemopreventive agents mediate their effects through inhibition of NF-κB and STAT3 activation pathways. Thus suppression of these proinflammatory pathways may provide opportunities for both prevention and treatment of cancer. We will discuss the potential of nutraceuticals derived from spices and from traditional medicine in suppression of inflammatory pathways and their role in prevention and therapy of cancer.
Quercetin is the most abundant flavonoid present in the diet and its disease preventing properties have been largely investigated. Among these, quercetin is able to modulate several hallmarks of cancer, including resistance to apoptosis. Previous studies from our group demonstrated the capacity of quercetin to sensitize several leukemia cell lines and B-cells isolated from patients affected by chronic lymphocytic leukemia (CLL) to death ligand agonists (e.g., anti-CD95 and rTRAIL). Moreover, in association with canonical and innovative chemotherapeutic drugs (fludarabine, ABT-737 and BH3-mimetics), quercetin synergistically enhances the drug response against CLL. This effect is mediated by changes in the expression and activity of anti-apoptotic proteins belonging to the Bcl-2 family. Among these, Mcl-1 has been associated to apoptotic resistance in CLL. We reviewed the apoptotic-enhancing activity of quercetin in vitro (leukemic cell lines) and ex vivo (B-cells from CLL patients) depending upon the down-regulation of Mcl-1. We hypothesize that quercetin inhibits key kinases, such as PI3K and CK2, down-regulating the PI3K/Akt pathway and, consequently, reducing resistance to apoptosis in CLL.

**Key Words:** quercetin; protein kinases; antioxidant; chronic lymphocytic leukemia

**Introduction**

Quercetin (3,3’,4’,5,7-pentahydroxyflavone) (Fig. 1) is the most abundant dietary flavonol in food. It is present in fruits, vegetables and beverages (black tea, green tea, red wine) [1]. We and others largely reviewed the antioxidant, anti-inflammatory, anti-angiogenic, anti-proliferative and pro-apoptotic effects of quercetin in cellular models [2]; however, it mechanism of action is not fully understood with several mechanisms that have been postulated, but, to our knowledge, no direct binding to specific cellular targets have been identified. Despite this apparent contradiction, quercetin is among the most studied dietary polyphenols, similarly to resveratrol, green tea catechins, curcumin and others. We attribute the interest for this molecule in cancer to its “pleiotropic” nature, meaning the capacity to hit simultaneously different cellular targets leading to cell death [3].

In the present short review, we briefly analyze the case of quercetin in Chronic Lymphocytic Leukemia (CLL), where we hypothesize the existence of specific and directs targets of the molecule.

![Fig. 1. Structure of quercetin aglycone (retrieved from PubChem Compound)](http://www.TurkJBiochem.com)
Fig. 2. DPBA staining of HPB-ALL cells. Cells (1.0 x 10^6/ml), derived from a human tymanoma [23], were incubated with quercetin (50 mM) (panels a and b) or control solvent (dimethyl sulfoxide, DMSO) (panels c and d) for 60 minutes at 37°C, washed in phosphate buffer saline solution and subsequently suspended in a solution of DPBA, (2-aminoethyl diphenylborinate, 2.5 mg/ml in formalin 10%), a highly selective stain for flavonols [30]. After staining, images were captured using fluorescence microscopy (Axiovert 200 Zeiss) and photographed using phase-contrast (panels b and d) and FICT filters (panels a and c) with 400X magnification.

These scientific evidence raise the paradox of the functional link between the chemical structure of quercetin (and other dietary phytochemicals) and their biological activities. Being molecules possessing both antioxidant and pro-oxidant actions, doubts have been expressed by several authors on the real existence of a scavenging activity in vivo, under biological conditions, considering their low intracellular concentrations and the existence of alternative and very efficient antioxidant defense mechanism, such as the Keap1/Nrf2/EpRE system [3, 7]. Therefore, it is plausible that the molecular explanation of the efficacy of quercetin against cancer is not related to its antioxidant properties, but must be searched elsewhere in the multiple biological features of the molecule.

Quercetin behaves as a “not specific” kinase inhibitor

Quercetin has also been investigated as inhibitor of tyrosine and serine/threonine kinases. As reported in dated and more recent screenings using kinases purified from mammalian tissues or expressed in prokaryotic or eukaryotic systems, quercetin can behave as kinase inhibitor on more than 100 different kinases. It was generally tested at micromolar concentrations (2-30 mM) with a decrease of enzymatic activity ranging between 30 and 95% [8, 9]. As expected, flavonoids, other than quercetin, share this feature, as recently reviewed [10], and this explains the definition of “not specific” kinase inhibitor. However, some discrepancies emerged from these studies being works performed in vitro and largely based on the use of recombinant enzymes. To confirm the existence of a real inhibitory effects of quercetin on specific kinases, it is mandatory to prove it in the cellular context where the kinase is physiologically expressed. Important issues must be considered such as: i. intracellular concentration and stability of the inhibitor; ii. sub-cellular localization of the target kinase(s); iii. their regulation by other factors (e.g. presence of regulatory subunits, post-translation modifications, etc.); iv. level of expression and degradation of the target kinase(s) [5]. Among the different kinases which can be inhibited by quercetin with relatively low Kᵢ, we concentrate our interest on two of them, Phosphatidyl-Inositol-4,5-bisphosphate 3-kinase (PI₃K) and Casein Kinase 2 (CK2), for their role in CLL (see next paragraph). Using the X-ray crystallographic structures of porcine PI₃K bound to wortmannin, quercetin, LY294002 and myricetin Walker et al. [11] demonstrated that all these molecules fit into the ATP binding site. The Kᵢ value for quercetin was of 0.28 mM, comparable with the IC₅₀ of 3.8 mM, previously measured for bovine brain PI₃K. In addition, this study reports the interesting observation that the well-known PI₃K inhibitor, LY294002, that was designed using quercetin as lead compound, and myricetin, a quercetin analog, bind PI₃K in different orientations related to each other by 180° [11]. This result confirms the differences existing among different inhibitors in competitively inhibit the same protein kinase. Overall, the demonstration of a direct binding of quercetin to PI₃K supports the large amount of data on the inhibitory effect of quercetin on the PI₃K/Akt pathway, leading to cell growth inhibition [12].

CK2 is a constitutively active dual specificity kinase possessing multiple functions in normal and malignant cells [13]. More recently, an active role of CK2 has been described in different forms of leukemia, including CLL. In fact, CK2 resulted as a potential activator of PI₃K/Akt pathway due to its capacity to phosphorylate and inactivate PTEN (phosphatase and tensin homolog), a negative regulator of PI₃K/Akt pathway [14]. Structural evidence, obtained from in vitro and in silico data, clearly indicate that quercetin competes with the ATP binding site of CK2 [15, 16]. Using a different approach, it has reported that a biotinylated version of quercetin binds and inhibits both the pure enzyme and the kinase pulled-down from cellular extracts [17]. As for PI₃K, these in vitro data represent the theoretical base to believe that quercetin directly interferes with CK2 in vivo, as suggested by an our previous study [18].
Quercetin in CLL

CLL represents the most frequent form of leukemia in adults in the Western world (3.5-6.15 cases per 100,000 subjects) and is characterized by an asymptomatic phase that may last for decades without the need of treatments for patients. Unfortunately, in others, CLL evolves rapidly to advanced clinical stages (Rai or Binet) becoming fatal also in the presence of chemotherapy [19]. In the recent years, the efficacy of treatments against CLL significantly improved with the introduction in clinics of the immunochemotherapy protocol which includes the combination of fludarabine, cyclophosphamide and rituximab (the anti-CD20 monoclonal antibody) [20]. Unfortunately, despite these important improvements, complete remission is rare and CLL remains an incurable disease. In CLL patients refractory to chemotherapy, a resistance to apoptotic induction has been often documented in their B-cells (B-CLL), due to the up-regulated activity of anti-apoptotic factors belonging to the Bcl-2 family [21]. This latter evidence stimulated our interest for quercetin in CLL. In fact, we firstly reported that quercetin associated with the agonistic antibody anti-CD95, which binds and activates the death receptor CD95/Fas/Apo-1, or to rTRAIL (recombinant TRAIL) able to bind the TRAIL (TNF-related apoptosis-inducing ligand) induced apoptosis in several human leukemia cell lines of lymphoid or myeloid origin [6, 22, 23]. It is worthwhile to note that in these group of papers, we demonstrated that quercetin per se was neither cytotoxic, nor apoptotic, but only when associated with pro-apoptotic agents enhanced apoptosis in leukemic cells.

Subsequently, we moved to B-cells isolated from CLL patients undergoing chemotherapy and selected based on their apoptotic resistance. In this ex vivo model, we reported that both combined treatments, i.e.: 1. quercetin plus anti-CD95 or rTRAIL; 2. quercetin plus fludarabine, a first-line drug in CLL, significantly increased apoptosis [24]. Finally, we explored the capacity of quercetin to potentiate the effect of ABT-737, a BH3 mimetic agent, which binds with high affinity Bcl-2 and Bcl-X\textsubscript{L}, whose over-expression is associated with apoptotic resistance in CLL [25]. It is important to mention that ABT-737 shows no affinity for Mcl-1 (Myeloid cell leukaemia-1), a further pro-survival member of the Bcl-2 family) [21]. In both B-CLL and leukemic cell lines, quercetin and ABT-737 synergistically induced apoptosis [26].

When we investigated the molecular mechanism(s) triggered by quercetin to enhance cell death, we discovered that they converged on Mcl-1 expression and activity. In fact, quercetin reduced Mcl-1 anti-apoptotic activity by the following mechanisms: i. destabilization of its mRNA and induction of proteasome-dependent degradation [27]; ii. inactivation of PI\textsubscript{3}K/Akt pathway [26], which up-regulates the anti-apoptotic activity of Mcl-1 [28]. As discussed above, in the latter case, it is plausible that the effect of quercetin involves the direct inhibition of PI\textsubscript{3}K and CK2 kinases, both positive regulators of Akt activity. In fact, when active, PI\textsubscript{3}K phosphorylates PIP\textsubscript{2} (phosphatidylinositol-4,5-biphosphate) to PIP\textsubscript{3} (phosphatidylinositol-3,4,5-triphosphate) which binds the PH domain of Akt stimulating a conformational that activate the kinase [29]. In parallel, CK2 phosphorylates and inactivates PTEN, the phosphatase that convert PIP\textsubscript{3} into PIP\textsubscript{2}, inhibiting the activation of Akt [14].

In conclusion, we hypothesize that in CLL quercetin contributes to lower the threshold of apoptotic resistance due to Mcl-1 over-expression, acting on multiple and independent pathways: i. inhibition of PI\textsubscript{3}K; ii. inhibition of CK2; iii. destabilization of Mcl-1 [27]. To support this view, we have evidence that quercetin easily cross the membrane of cells and becomes bioavailable within 1 h from treatment (Fig. 2). As a result of a functional Mcl-1 down-regulation, the efficacy of ABT-737 (and other BH3-mimetics) increases due to their effect on inhibiting the anti-apoptotic Bcl-2 and Bcl-X\textsubscript{L}. Studies are in progress to experimentally confirm these hypotheses which may represent the bases to design specific clinical trials in patients with recurrent or aggressive forms of CLL.

References

S-17
MER RECEPTOR TYROSINE KINASE AND MACROPHAGE PHAGOCYTOSIS OF APOPTOTIC CELLS

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One of the hallmark changes associated with apoptosis is the exposure of anionic phospholipids such as phosphatidylserine (PtdSer) and phosphatidylethanolamine on the outer leaflet of the plasma membrane. Phagocytic cells express a variety of receptors that confer the capacity for recognition of this membrane alteration, including BAI-1 and Tim4. In addition, phagocytes express receptors that interact with PtdSer-binding molecules that act as a bridge between the phagocyte and the apoptotic cell. Mer (gene name Mertk) is a receptor tyrosine kinase expressed by a variety of different cell types, including macrophages derived from most tissues. Mer serves as a receptor for Protein S and Gas6, PtdSer binding proteins that rapidly opsonise apoptotic cells. Mer can mediate tethering of Protein S-opsonised apoptotic cells and activation of the intrinsic kinase activity of Mer signals apoptotic cell internalisation. In addition, TLR-dependent pro-inflammatory cytokine production by macrophages is influenced by Mer-dependent signalling. We have examined Mer expression during human monocyte differentiation to macrophages in vitro and explore the relationship between monocyte expression of Mer and the capacity to bind the Mer ligand, Protein S. Together, our new data reveal a novel role for Mer in the control of monocyte function.
T lymphocytes play a pivotal role in the immune response as a key regulatory and effector cells. Upon antigenic stimulation, naïve CD4 T cells can active, proliferate and differentiate into unique signature cytokine expressing effector T helper (Th) cells for instance Th1, Th2 and Th17. Recently discovered Th17 cells are known to play a critical role in various inflammatory pathologies including Multiple sclerosis (MS), Rheumatoid arthritis (RA) and cancer. Th1 cells are involved in cell-mediated immune response and are responsible for the clearance of intracellular pathogens. Th2 cells are involved in humoral immune responses, allergies and responds to some parasites. They secrete IL-4, IL-13, IL-10 and IL-5. Th17 cells are linked to several autoimmune diseases and mediate immune response to bacterial and fungal infections. They secrete IL-17, IL-21 and IL-22 cytokines.

Apoptosis is a process that involves sequential activation of a series of proteins such as caspases after an appropriate stimulation. Apoptosis can be carried out by external (receptor-ligand interaction) or internal (mitochondria) mediated pathways. Fas, FasL, DR5 and TRAIL are apoptotic proteins whereas FLIP and Bcl-xL are anti-apoptotic proteins, which involve in apoptotic pathways. It is known in the literature that Activation Induced Cell Death (AICD) is important in eliminating activated T cells. The Fas/FasL pathway is very important in T cell death. TCR signaling can lead to the deletion of activated peripheral T cells through apoptosis. The survival or death of effector T cells is affected on IFNγ, IL-4 and IL-2.

T helper cells show different susceptibility to apoptosis. For instance, Th17 cells are less sensitive than Th1 cells to Fas mediated apoptosis because they have a higher expression of FLIP. Th17 are more sensitive to Fas mediated apoptosis than Th2 cells due to their higher expression of FasL. Re-stimulated Th17 cells undergo AICD through a Fas/FasL mediated pathway that is unaffected by IFNγ. Human Th17 cells are phenotypically resembles differentiated memory T cells but they are distinct from central memory, exhausted and senescent T cells. Human Th17 cells are long lived cells. Overall data showed that there is an unequal cell death in differentiating T helper subsets. The further investigation of cell survival and death signal networks in T helper cells will help us better understand the molecular mechanisms of various pathologies such as autoimmunity, cancer and infection diseases. This work was supported by a grant from the Scientific and Technological Research Council of Turkey (TUBITAK). Project grant number is 110T412 and awarded to Dr. Ayten Nalbant.

Key Words: T Cells, T Helpers, Th1, Th2, Th17, Aicd And Apoptosis
S-20

FASL-MEDIATED APOPTOSIS AS AN EFFECTIVE MEANS OF INDUCING TOLERANCE TO ALLOGENEIC PANCREATIC ISLETS

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Activation induced cell death (AICD) is a process integral to the maintenance of immune tolerance. Fas ligand (FasL)-induced apoptosis plays a critical role in AICD. While the tolerogenic nature of FasL has long been appreciated, previous attempts to harness its immunomodulatory potential for the treatment of autoimmunity and allograft rejection have proven unsuccessful. This is primarily due to the diverse functions of FasL and the observed toxicity associated with the use of agonistic antibodies to Fas receptor.

Conclusion: We overcame these obstacles by generating a novel apoptotic form of FasL and transiently displaying this molecule on the surface of pancreatic islets for immunomodulation. FasL-engineered allogeneic islets overcame rejection and treated diabetes without toxicity to the graft or recipient.

Key Words: FasL, Apoptosis, diabetes, tolerance, ProtEx™, SA-FasL, Apoptosis, Islet transplantation

Background: The adaptive immune system is able to respond to a wide variety of antigens due to the combinatorial generation of T and B cell receptors. The semi-random nature of gene rearrangements that allows for this diversity leads to the creation of some receptors which respond to host antigens. Left unchecked, these autoreactive cells will initiate autoimmune diseases focusing on tissues expressing their cognate antigen. To avoid autoimmunity, tolerance to self-antigen is induced centrally during T and B cell development, and enforced peripherally by several mechanisms.

One crucial mechanism in maintaining tolerance is the induction of apoptosis. The apoptotic deletion of cells bearing a T cell receptor (TCR) or B cell receptor (BCR) recognizing self-antigens is an important step in lymphocyte development and a vital part of maintaining tolerance in the periphery 1. In the periphery, apoptosis of self- and hyper-reactive cells is often initiated by signaling from Fas 2,3.

Fas is a TNF superfamily receptor expressed on the surface of cells of both the innate and adaptive immune system, as well as on cells comprising other tissues such as the liver. Upon binding its ligand FasL, a death-inducing signaling complex (DISC) forms at the cytoplasmic side of the Fas receptor. The DISC is composed of proteins interacting either through death domains (DD) or death effector domains (DED), and is composed of FADD, Caspase 8, and cFLIP 4. The FADD-mediated interaction of procaspase molecules through their DEDs allows for their cleavage and activation. The now active caspase 8 can then initiate two distinct apoptotic pathways: the extrinsic and intrinsic pathways 5,6. In the extrinsic pathway of Fas-mediated apoptosis, caspase 8 activates downstream “executioner” caspases, such as caspase 3, leading to proteolysis, DNA degradation, and cell death 5. In the intrinsic pathway, caspase 8 cleaves and activates BID, a member of the BH3-only group of the Bcl-2 family 5,7. Unless inhibited by Bcl-2, proapoptotic members of the Bcl-2 family trigger the release of cytochrome C from mitochondria and subsequent cell death. In general, Fas-mediated apoptosis in lymphocytes depends primarily on caspase 8 activating caspase 3, whereas other cell types depend on BID-mediated apoptosis 5,6.

The degree to which a cell is susceptible to Fas-mediated apoptosis is dependent on both cell-intrinsic and cell-extrinsic factors. Signaling pathways can modulate the expression of Fas and FasL on the cell surface and up- or down-regulate proteins that inhibit caspase activation 8-12. Due to this, Fas is a key receptor for activation induced cell death (AICD). AICD allows for the selective removal of hyperreactive and/or autoreactive cells 1,13. Upon activation, T helper cells upregulate the expression of Fas and FasL, increasing their sensitivity to Fas-mediated AICD.
AICD can result either from Fas binding to FasL on the same cell, resulting in suicide, or binding to FasL on a nearby cell (often termed ‘fratricide’). Effector CD4+ T cells responding to abundant self-antigen are more sensitive to apoptosis than a similar effector responding to less abundant non-self-antigen, allowing for the selective deletion of autoreactive cells.

Mice lacking either Fas or Fas ligand demonstrate pathogenic proliferation of lymphocytes, development of abnormal T cells, and infiltration of T and B cells into solid organs. These mice also have anti-DNA antibodies in their serum and demonstrate an increased susceptibility to Lupus-like disease. Importantly, the deletion of autoreactive T cell clones in the thymus was found to be unimpaired, but clonal deletion of T cells reacting to super-antigens in the periphery was defective 14,15. These findings highlight the importance of Fas signaling in peripheral regulation of self-reactive T cells.

Early in the study of the Fas/FasL signaling pathway, it was observed that Fas-FasL interaction could induce proliferative signals 16,17. This led to the search for non-apoptotic signaling through Fas. The DISC component c-FLIP can heterodimerize with caspase 8, altering its substrate specificity. Caspase 8 can also cleave c-FLIP, releasing N-terminal fragments that can directly or indirectly activate both MAPK and NF-κB signaling pathways leading to pro-inflammatory, non-apoptotic signaling 17,18. Finally, it has been observed that Fas can suppress TCR signaling in naïve T cells without triggering apoptosis in certain settings 17. Taken together these data indicate that the immunomodulatory effects of Fas/FasL signaling extend beyond the induction of apoptosis in strongly activated cells. The effects of Fas signaling depend greatly upon the dosage of Fas and FasL, as well as the larger context (i.e. TCR signaling and local cytokine milieu) in which the signaling occurs. The issues of tolerance to self-antigen can become complicated further by the use of transplants to remediate autoimmune damage, as in the case of islet transplants in patients with type 1 diabetes. Beyond dealing with non-self antigens, such grafts also have a population of adaptive immune cells primed to reinitiate the destruction of the islets 19. Because of this, islet transplants require systemic immunosuppression and recipients often return to insulin injections within five years 20. An immunomodulatory approach which reduces or eliminates the need for immunosuppressive drugs would be of immense benefit.

While Fas-FasL signaling is a potent mechanism of AICD and critical for tolerance to self-antigens, attempts to translate this immunomodulatory aspect of Fas signaling into clinical gains have been unsuccessful. One early approach tried using an agonistic anti-Fas antibody, which resulted in fatal liver failure when used in mice 21. The use of FasL as a biologic has also been problematic thus far, as soluble FasL has been shown to be ineffective, or even to inhibit Fas-mediated apoptosis 22. Work to leverage Fas signaling for therapeutic purposes remains ongoing.

FasL has been shown to be a significant driver of localized immunomodulation that confers an immunoprivileged status for various vital organs 3. Our group has developed a technology, ProtEx™, that allows simulation of this physiologic form of localized immunomodulation. ProtEx™ technology involves generation of novel immunological ligands by fusing the extracellular domain of molecule of interest with a streptavidin core, allowing the efficient and transient display of these molecules on biotin-modified biological surfaces 23. Our lead immunomodulatory molecule is SA-FasL. SA-FasL has effectively been displayed on the surface of various cells, tissues, and grafts 23,24. The engineering process is rapid, effective, and does not negatively impact the function of FasL or engineered cells, tissues, or organs, and the SA-FasL persists on the cell surface in vivo for days to weeks without detectable toxicity 24.

Work from our group, utilizing a model in which allogenic BALB/c islets were transplanted into chemically diabetic C57BL/6 mice showed robust engraftment of islets engineered with SA-FasL, but not islets engineered with streptavidin alone, or un-engineered islets, with only transient rapamycin treatment 24. Notably, SA-FasL-engineered islet grafts had a greatly increased proportion of Foxp3+ regulatory T cells (Treg) compared to control grafts, and further work demonstrated that these Tregs were essential for the maintenance of tolerance to the allograft 24. Additional work in a mouse model of cardiac allograft demonstrated a significant increase in graft survival when transplantation was combined with the transfer of SA-FasL-engineered splenocytes 25. This demonstrates the feasibility of immunomodulation in situations where direct engineering of a tissue may not be practical.

In conclusion, Fas-driven apoptosis is a central mechanism in creating and maintaining immune tolerance in the periphery. However, the dose and context dependent effects of Fas-FasL interaction have made therapeutic uses of this pathway problematic. The use of ProtEx™ technology combined with the SA-FasL fusion protein allows for a graft-specific, membrane-associated display of FasL that prevents immune-mediated tissue destruction. Further work in this area may eventually allow for more durable islet transplants, and potentially even remove the need for immunosuppressive drug regimens.

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References
ABNORMAL BRAIN GANGLIOSIDE ACCUMULATION TRIGGERS APOPTOSIS IN EARLY ONSET TAY-SACHS DISEASE MOUSE MODEL

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Tay-Sachs disease is a severe lysosomal storage disorder caused by mutations in the HEXA gene coding for α subunit of lysosomal β-Hexosaminidase A enzyme, which converts GM2 to GM3 ganglioside. Unexpectedly, the HexA−/− mice have a normal lifespan and show no obvious neurological impairments until at least 1 year of age, owing to the ability of these mice to catabolize stored GM2 ganglioside via sialidase(s) removing sialic acid into glycolipid GA2 which further processed by β-Hexosaminidase B, thereby bypassing the HexA defect. To elucidate whether sialidase Neu3 can contribute to GM2 ganglioside degradation, we generated mice model with combined deficiencies of β-Hexosaminidase A and Sialidase Neu3. HexA−/−Neu3−/− mice are healthy at birth but died at 1.5-4.5 months of age. Thin layer chromatography and mass spectrometry analysis of brain from HexA−/−Neu3−/− mice showed abnormally accumulated GM2 ganglioside level. Slow movement, ataxia and tremor are among neurological abnormalities. In the current study, we delineate whether there is apoptosis in Tay-Sachs mice model’s brain. In order to profile the expression of 84 key genes related to apoptosis in the cerebellum and cortex from 4.5 months old mice, we used RT2 Profiler PCR Array system specific for apoptosis. We found that mRNA levels of pro-apoptotic genes such as TNF and caspase 4 increased 5.8X and 2.1X respectively in HexA−/−Neu3−/−. On the other hand, mRNA levels of anti-apoptotic genes such as Bag1 and Bcl2 (1.6 X), Cd40lg (1.8X) and Naip (7.1X) decreased in cerebellum of the same mice as compared to HexA−/− mice revealing the apoptosis in early-onset Tay-Sachs mice model. We suggest that once a critical threshold of GM2 ganglioside storage is reached in the cerebellum, a signaling cascade is triggered which activates neuronal death.