Name: Jacob Baus

Title: Identification of Novel Cell Death Inducers

Running title: Identification of Novel Cell Death Inducers

Abstract

Cell death caused by cardiac infarction events commonly result in the development of heart disease. The identification and classification of genes in these cell death pathways improves medical professionals understanding of these mechanisms and helps in the development of gene therapy techniques which can be used to impede the onset of heart disease. The goal of this research was to identify novel genes in cell death pathways. We amplified cDNA for 10 genes which were screened for high death potential. This amplified cDNA was then added into an inducible vector and inserted into an adenovirus. This adenovirus was then introduced to cells with the doxycycline (DOX) inducer and examined for cell death. We expected to see cells that had virus applied without DOX to be healthy and living, and cells with virus and DOX to have significant cell death from the induced gene. Instead, we observed substantial cell death in both cases of virus applied to cells and healthy, living control cells. We concluded that the induced gene could not be responsible for this death and rather viral toxicity caused the increased death. Further testing these viruses, with consideration of recent literature, could demonstrate if these genes are active in a cell death pathway.

Keywords: adenovirus, apoptosis, doxycycline, necroptosis, necrosis

Introduction

The goal of this research project was to identify 10 novel genes as being involved in cell death processes as well as the death pathway which each gene acts. The three forms of cell death which each gene could act are apoptosis, necrosis, and necroptosis. Apoptosis is the controlled, self-initiated cell death process which occurs in organisms during development and normal day to day functions. The mechanism for apoptosis (Fig. 1) is controlled by the Bcl-2 family of genes which can be classified into proapoptotic and antiapoptotic (Vaux et al. 1992). *Bax* and *Bak* are two highly studied proapoptotic Bcl-2 family genes which remain largely inactive in a cell until an apoptotic signal is received (Chipnuk et al. 2006). Once the signal for cell death is received, *Bax/Bak* then releases a molecule that initiates mitochondrial outer membrane permeability (MOMP). MOMP forms large pores in the outer membrane of the mitochondria and allows for the release of cytochrome c (cyt c) into the cytoplasm. Cyt c then triggers the activation of caspases who are ultimately responsible for the apoptotic destruction of the cell by (Liu et al. 1996). The mitochondria maintains the ability to produce ATP until the cell is on the verge of perishing.

Necrosis is a regulated cell death triggered by high stress, particularly ischemia (loss of blood supply to a region) in the case of myocardial infarction. Unlike apoptosis, necrotic cell death includes the rupture of the plasma membrane, organelle swelling, and inflammation to the surrounding area resulting in an unorganized and chaotic death. Similar to apoptosis, this pathway is regulated by *Bax/Bak* and cells lacking both these genes have been shown to be resistant to cellular swelling and necrotic death (Karch et al. 2013). The defining event of necrosis (Fig. 2) is the formation of a mitochondrial permeability transition pore (MPTP) which is regulated by the *ppif* gene and its protein product cyclophilin D (Baines et al. 2005). Calcium

build up in a cell from ischemia causes the cyclophilin D to be produced, triggering the formation of the MPTP. The breach in the mitochondrial membrane results in loss of ATP production and the cell is unable to maintain itself (Karch and Molkentin 2015). Since the cell no longer has the energy means to activate the apoptosis cascade events, apoptotic death cannot occur.

Finally, necroptosis a relatively newly discovered form of cell death which utilizes components of both apoptosis and necrosis. Necroptosis (Fig 3) occurs when the signal for apoptotic death is received by a death receptor but necrotic activities ensue instead. Caspase 8 is an initial activator of apoptosis, and its activity with RIP1/RIP3 complex initiates apoptosis (Feltham et al. 2017). When its activity is inhibited, necroptotic death occurs and the RIP1/RIP3 complex activates the mixed lineage kinase like complex (MLKL) (Karch and Molkentin 2015). This protein group is then believed to guide RIP1/RIP3 to other portions of the cell, including the mitochondria, to continue a necrotic like death process (Sun et al. 2012). MLKL activity is essential for only this necroptosis process as null mice were resistant to the necroptotic death but still susceptible to apoptosis (Wu et al. 2013). RIP3 is also needed for necroptosis since RIP3 knockout cells are resistant show strong resistance to cell death with caspase 8 inhibited (He et al. 2009).

Prior the initiation of the project, the Molkentin lab had already identified a previously novel gene involved in the prevention of cell death. The gene TGN91 when overexpressed in mice resulted in increased protection against ischemic injury (Karch et al. 2016). The discovery led the team to continue investigating other unknown genes with intention of better understanding the mechanisms of cell death. My task in the study was to amplify the selected cDNA samples from the same cDNA library and prepare them for insertion to an adenovirus.

From there, the virus would be used to deliver the selected genes to cells which can then be observed and assayed for cell death. The research goal was to identify the roles of which a select group of novel genes in heart cells that play during cell death. The identification of these mechanisms could further aid in the battle against heart disease by targeting and preventing cardiac cell death which occurs immediately following an ischemic event such as a heart attack.

Materials and Methods

Ten genes cDNA were chosen for experimentation based on their death potential as selected through a screening process. These genes included *PEMT*, *TMEM40*, and *TEX261*. The ten cDNA were then amplified through polymerase chain reaction (PCR) and purified. The purified genes were then inserted into an inducible AdenoX vector (Fig. 4) and the desired recombinant AdenoX test gene plasmids were identified using standard recombinant DNA techniques. The AdenoX plasmids were then used to construct a recombinant virus for the delivery of the test gene to human embryonic kidney (HEK) cells. We would then assay the cells looking for protein markers through a Western Blot to determine cell death classification. The protein markers were as follows: cyclophilin D for necrosis, cytochrome c for apoptosis, and MLKL for necroptosis.

Results

Recombinant virus was produced successfully from the amplified cDNA samples of *PEMT*, *TEX261*, and *TMEM40*. The *TMEM40* recombinant virus was tested on HEK cells. We expected to see cell death in the culture with both virus and the inducer (DOX) and no cell death in plates without virus (our control) or with virus but without DOX (Table 1). We observed

living HEK cells in the control plates as expected (Table 2). In the virus plates, we observed significant cell death in both induced and uninduced conditions.

Discussion

While we were able to successfully transfer several genes into the viral vector, it was very concerning to see that all cells with virus present perished while we expected non-induced virus cultures to show no effect. We had expected only the DOX induced cells to die (expected visual of death shown in Fig. 5). This result is likely not due to the gene itself since our vector was not activated in the wells without DOX applied. Since control cells with DOX applied remained healthy as expected, the death must be due to the presence of non-induced virus itself. We conclude that the death of the cells is likely due to viral toxicity meaning there was too much virus present, though not activated, for the cells to survive. To support this idea, we would need to create a recombinant virus with an empty vector. If the virus is the cause of the cell death in these experiments, then we would again expect the application of virus (without a gene) to result in cell death.

We must also consider the concentration of the applied virus in future experiments to conclude this result was due to viral toxicity. A similar experiment involving adenovirus application by Xie et al. (2014) used 100 MOI (100 viral particles per 1 cell) as the lowest concentration and 800 MOI as the highest. We did not determine the MOI in our experiment, therefore it is possible that the MOI used exceeds that reported by Xia and colleagues. This offers us an explanation for our results and further tests should follow procedures outlined in these successful experiments.

Since this research, these and other novel cell death genes have been partially identified in their role of cell death. ATAD3A, a gene whose mechanism is unknown, has recently been observed as a prominent effector in cancer cells. Its overexpression in breast and colon cancer cells suggest that it is required for maintenance of anti-apoptotic characteristics. (Teng et al. 2016). Additionally, knockouts of ATAD3A have shown to inhibit the development and proliferation of breast cancer cells. Another gene shown to have significant cell death potential is FAM176A which has been shown to trigger apoptotic cell death when overexpressed (Xie et al. 2014). This beneficial cell death was shown in human lung cancer cells raising possibilities on its future use in cancer treatments and gene therapy. Similar to our methods, this project utilized an adenovirus as a vehicle for overexpression of a gene, and their results offer further incentive to continue this investigation.

Acknowledgements

I would like to thank Cincinnati Children's Hospital for providing me this research opportunity and its funding through the Summer Undergraduate Research Fellowship (SURF) program. I would like to thank Dr. Jason Karch for acting as my mentor through the 10-week program and providing me with the knowledge and training to attack this project, as well as Dr. Jeffery D. Molkentin for inviting me into his lab and providing equipment and materials. I would also like to thank the Ripon College Biology Department, especially Dr. Mark Kainz, for preparing me for this research experience as well as mentoring me through the process of drafting this thesis.

Literature Cited

Baines, C.P., R.A. Kaiser, N.H. Purcell, N.S. Blair, H. Osinska, M.A. Hambleton, E.W. Brunskill, M.R. Sayen, R.A. Gottlieb, G.W. Dorn II, J. Robbins, and J.D. Molkentin. 2005. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434: 658-662.

Bouchier-Hayes, L., L. Lartigue, and D.D Newmeyer. 2005. Mitochondria: pharmacological manipulation of cell death. Journal of Clinical Investigation 115: 2640-2647.

Chipuk, J.E., L. Bouchier-Hayes, and D.R. Green. 2006. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death and Differentiation 13: 1396–1402.

Feltham, R., J.E. Vince, and K.E. Lawlor. 2017. Caspase-8: not so silently deadly. Clinical and Translational Immunology 6: e124. doi: 10.1038/cti.2016.83

He, S., L. Wang, L. Miao, T. Wang, F. Du, L. Zhao, and X. Wang. 2009. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-α. Cell 137: 1100–1111.

Karch, J., and J.D. Molkentin. 2015. Regulated Necrotic Cell Death. Circulation Research 116: 1800–1809.

Karch, J., J.Q. Kwong, A.R. Burr, M.A. Sargent, J.W. Elrod, P.M. Peixoto, S. Martinez-Caballero, H. Osinska, E.H. Cheng, J. Robbins, K.W. Kinnally, and J.D. Molkentin. 2013. Bax and Bak function as the outer membrane component of the mitochondrial permeability pore in regulating necrotic cell death in mice. eLife 2: e00772. doi: 10.7554/eLife.00772

Karch, J., M.J. Brody, O. Kanisicak, M.A. Sargent, J.D. Molkentin. 2016. Identification of Novel Anti-necrotic Factor TGN91. Circulation Research 119: 119.

Liu, X., C.N. Kim, J. Yang, R. Jemmerson, X. Wang. 1996. Induction of Apoptotic Program in

Cell-Free Extracts: Requirement for dATP and Cytochrome c. Cell 86: 147-157

Sun, L., H. Wang, Z. Wang, S. He, S. Chen, D. Liao, L. Wang, J. Yan, W. Liu, X. Lei, X. Wang. 2012. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 148: 213–227.

Vaux, D., I.L.Weissman, and S.K. Kim. 1992. Prevention of programmed cell death in *Caenorhabditis elegan* by human bcl-2. Science 258: 1955.

Xie, H., J. Hu, H. Pan, Y. Lou, P. Lv, and Y. Chen. 2014. Adenovirus vector-mediated FAM176A overexpression induces cell death in human H1299 non-small lung cancer cells. BMB Reports 47: 104-109

Teng, Y., X. Ren, H. Li, A. Shull, J.Kim, and J.K. Cowell. 2016. Mitochondrial ATAD3A combines with GRP78 to regulate the WASF3 metastasis-promoting protein. Oncogene 35: 333-343.

Wu, J., Z. Huang, J. Ren, Z. Zhang, P. He, Y. Li, J. Ma, W. Chen,Y. Zhang, X. Zhou, Z. Yang, S. Wu, L. Chen, and J. Han. 2013. Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Research 23: 994–1006.

Tables and Figures

Table 1. Expected results from application of *TMEM40* recombinant virus.

	Control	Virus
No Dox	+	+
DOX	+	-

(+) represents living, healthy cells while a (-) represents significant cell death.

Table 2. Results from application of *TMEM40* recombinant virus.

	Control	Virus
No Dox	+	-
DOX	+	-

(+) represents living, healthy cells while a (-) represents significant cell death.

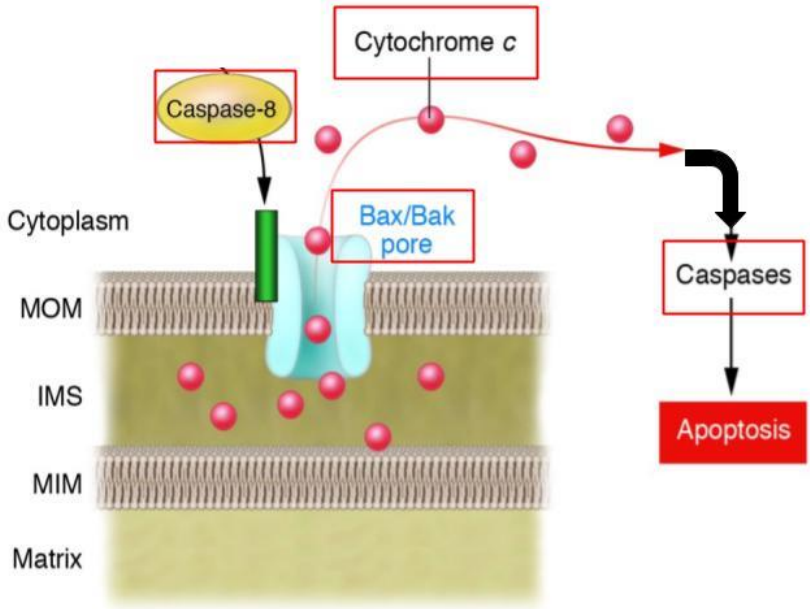

Figure 1. Representation of apoptosis focusing on the mitochondrial membranes. Modified from image by Bouchier-Hayes et al. (2005). The releasing of Cytochrome c through the Bax/Bak pore triggers the organized caspase cascade and results in efficient cell death. MOM stands for the mitochondrial outer membrane, IMS for the intermembrane space, and MIM for the mitochondrial inner membrane.

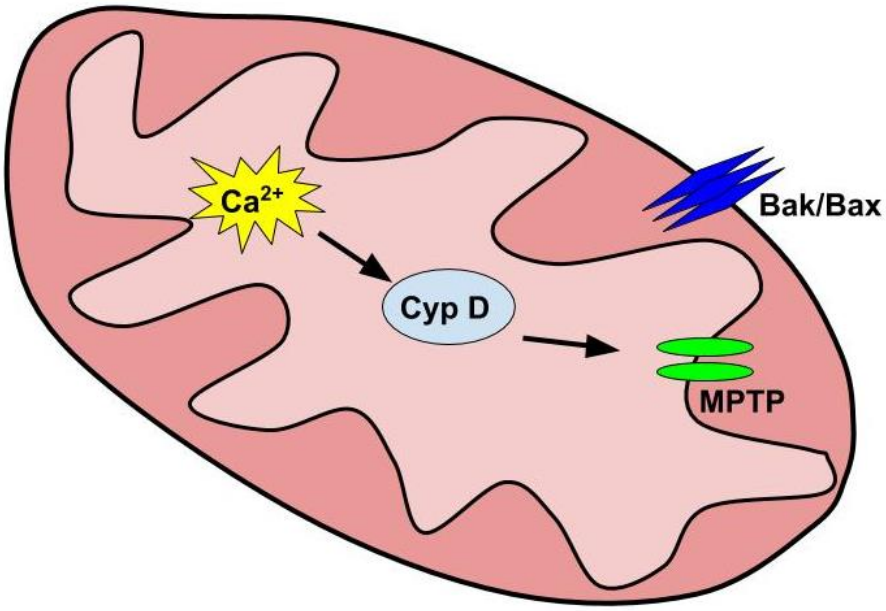

Figure 2. Representation of necrosis caused by calcium overload in mitochondria. This sequence of events results in mitochondrial dysfunction and adverse cell death. MPTP is the mitochondrial permeability transition pore and Cyp D represents activated cyclophilin D.

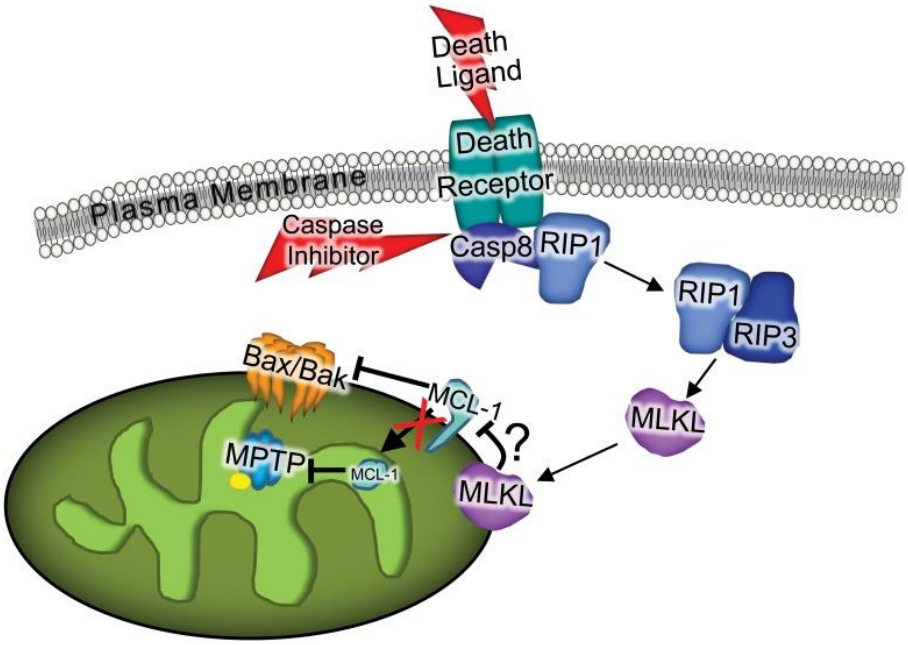

Figure 3. Representation of necroptosis as modified from image from Karch et al. (2015). This pathway is triggered by inhibition of the Caspase 8 molecule which is needed for apoptosis. The mitochondria then undergoes MPTP formation and necrosis ensues. MLKL is the mixed lineage kinase like complex

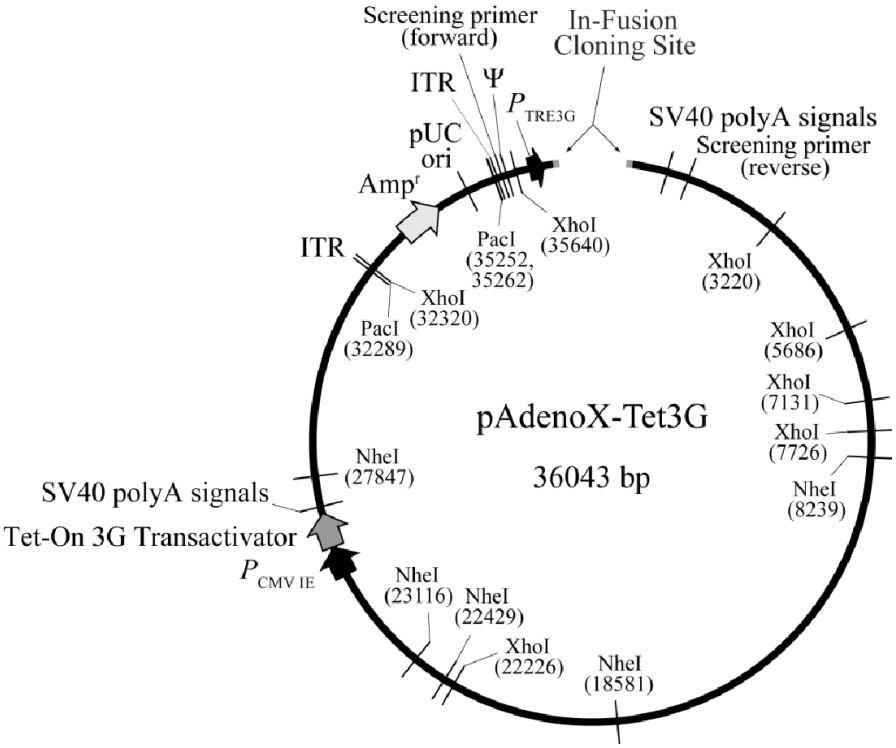

Figure 4. Diagram of the inducible vector which the genes were inserted into. The Tet-On 3G Transactivator is the region where doxycycline (or other tetracycline derivatives) act to induce transcription of the gene place in the In-Fusion Cloning Site.

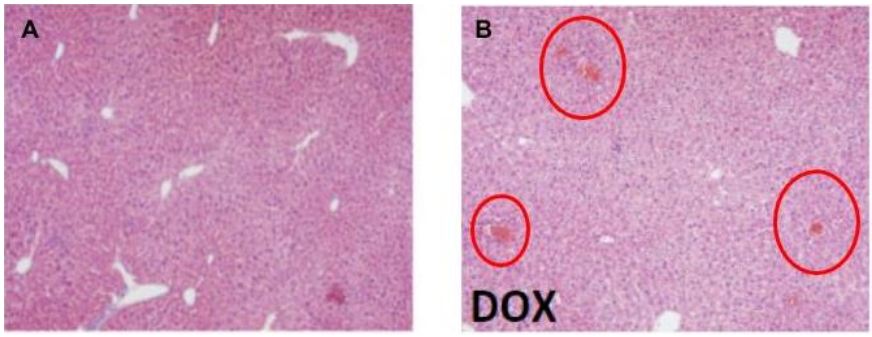

Figure 5. Example of appearance of induced cell death. A) Healthy HEK cells with uninduced adenovirus. B) Early HEK cells receiving recombinant adenovirus and induced overexpression by DOX. Circles indicate notable regions of cell death.