

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

Regulation of Apoptosis in Neural Cells: Two methods for overcoming asynchrony

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biochemistry at Massey University, Palmerston North, New Zealand.

Fleur François
2000

Abstract

Programmed cell death, or apoptosis, plays a major role in the development of the nervous system and in the pathogenesis of neurodegenerative diseases. Although many proteins that play a key role in apoptosis in other systems also appear to function in neurons, the mechanism that triggers apoptosis in neurons is unknown. Apoptosis occurs asynchronously in neural and differentiated neuronal cells, which makes biochemical studies difficult because a small number of cells are at a particular stage at any one time. Two strategies were devised to overcome asynchrony during neural cell death.

The first strategy was to separate rat pheochromocytoma (PC12) cells at different stages of commitment to cell death on the basis of cell density using equilibrium density gradient centrifugation. Three populations were defined. Cells in population 1 were the most dense and committed to cell death. They showed extensive loss of mitochondrial cytochrome c, DNA fragmentation, and chromatin condensation. Population 3 contained live cells that floated to the top of density gradients. Population 2 displayed some chromatin condensation, yet little DNA fragmentation and loss of cytochrome c. This population showed upregulation of the pro-death factor, c-Jun, and downregulation of pro-survival kinase, Akt. Importantly, these cells could be rescued from death by nerve growth factor (NGF) and thus represent an intermediate stage of apoptosis, upstream of irreversible commitment.

The second strategy was to create a cell-free system to reconstitute apoptosis. The addition of cytochrome c to human neuroblastoma (SY5Y) cell extracts activated caspase-9 and -3, and nucleolytic events in PC12 nuclei. Using this system, requirements for ATP and phosphatase activity for caspase activation and nuclear apoptosis were characterised. In addition, pro-survival molecules Akt and Creb were identified as caspase substrates during apoptosis *in vitro*.

To assess whether these events occurred *in vivo*, the kinase inhibitor staurosporine and the topoisomerase inhibitor camptothecin were used to induce apoptosis in intact SY5Y cells. The pro-survival signalling kinase Raf-1 was downregulated during both staurosporine- and camptothecin-induced apoptosis, but Akt was only downregulated by camptothecin. These studies illustrate the complex interactions of apoptosis and signalling mechanisms in neural cells.

Acknowledgments

I would like to thank my supervisors Mark Grimes and Gretchen McCaffrey for their constant support during this work and their willingness to let me pursue my own ideas. I would also like to acknowledge my collaborator Mike Dragunow (University of Auckland) who performed the immunocytochemistry on the separated PC12 populations.

Throughout my PhD I have been supported financially by a Health Research Council of New Zealand Postgraduate Scholarship, New Zealand Vice-Chancellors' Committee (NZVCC) Shirtcliffe Fellowship and NZVCC Georgetti Scholarship. In addition, a Royal Society of New Zealand Technology Award, NZVCC Claude McCarthy Fellowship, New Zealand Society of Biochemistry and Molecular Biology Young Investigator Award, and a grant from the Institute of Molecular BioSciences, Massey University, have allowed me to present this work at two international conferences. Without this level of financial support I could not have embarked on this PhD degree.

I must also thank the wonderful people who have worked alongside me in the lab during my PhD. Scott, Matt, Louise, Anna, Diane, Tanja and Shona, it was a pleasure to work with you. Thank you to Annika Haywood who has been a constant source of encouragement and support always ready to listen to my rants about my research or life in general. I'd like to make a special mention of Kathryn Stowell and John Tweedie who have nurtured and mentored my scientific development since my years as their undergraduate student.

Finally, thank you to my parents for believing in me and giving their constant unconditional support for this craziest of life experiences– the PhD.

Table of contents

Title page	i
Abstract	ii
Acknowledgments	iv
Table of contents	v
List of figures	ix
List of tables	xi
List of abbreviations	xii
Abbreviations for amino acids	xvii
List of publications arising from this thesis	xviii
Chapter 1 General Introduction	1
Chapter 2 Literature Review	7
2.1 Molecular machinery of apoptosis	8
2.11 <i>C. elegans</i>	8
2.12 <i>Drosophila</i>	13
2.13 Vertebrates	17
Bcl-2 family of proteins	17
Caspase regulation	20
2.2 Control of apoptosis	23
2.21 Role of mitochondria	23
2.22 Death receptors	25
2.23 Inhibitors of apoptosis	29
2.3 Execution events	29
2.4 Signal transduction in apoptosis	32
2.41 PI3-K and Akt signalling in survival	33
Akt substrates for survival signalling	37
2.42 Raf/Mek/Erk pathway	41
2.43 Sapk/Jnk pathway	45
2.44 p38 Mapk and Creb	47
2.45 Caspase-mediated regulation of signal transduction pathways	48
2.5 Apoptosis in neurodegenerative disease	49
2.6 Regulation of apoptosis in neurons	51
2.61 NGF signal transduction	52
2.62 Competence to die	56
2.7 Research aims	57

Chapter 3 Materials & Methods	59
3.1 Materials	60
3.2 Cell culture	60
3.3 DNA extraction	60
3.4 Internucleosomal DNA fragmentation analysis	61
3.5 Pulse field gel electrophoresis	62
3.6 Visualisation of chromatin condensation	62
3.7 Cytochrome c immunofluorescence	62
3.8 Preparation of cytosolic extracts from SY5Y cells	63
3.9 Purification of nuclei from PC12 cells	63
3.10 Preparation of total protein lysates from intact cells	64
3.11 Lowry assay for protein concentration	64
3.12 Immunoblotting	65
3.13 Stripping and reprobing of membranes	66
3.14 Image analysis	66
3.15 Calculations	66
Chapter 4 Separation of PC12 cells at different stages of apoptotic commitment	67
4.1 Introduction	68
4.11 Serum-withdrawal model of apoptosis in PC12 cells	68
4.12 Asynchrony in apoptosis	68
4.2 Experimental procedures	72
4.21 Serum-deprivation of PC12 cells	72
4.22 Differential centrifugation	72
4.23 Discontinuous density gradient centrifugation and recovery of cell populations	72
4.24 c-Jun immunocytochemistry	73
4.3 Results	74
4.31 Timetable of DNA fragmentation events in serum-withdrawn PC12 cells	74
4.32 Serum-withdrawn PC12 cells die asynchronously	77
4.33 Separation of PC12 cells at different stages of cell death	79
4.34 Population 2 but not population 1 was be rescued from cell death by NGF	83
4.35 The separated populations differ in their cytochrome c immunoreactivity	85
4.36 c-Jun protein levels were upregulated in population 2	85
4.37 Caspase activity and Akt protein downregulation	87
4.4 Discussion and future work	90
4.41 Multi-step degradation of DNA during apoptosis	90
4.42 PC12 cells were separated at different stages of cell death	91
4.43 Ordering events occurring during commitment to cell	92

death

Chapter 5 <i>In vitro</i> reconstitution of apoptosis	96
5.1 Introduction	97
5.11 <i>In vitro</i> models of apoptosis	97
5.2 Experimental procedures	99
5.21 <i>In vitro</i> reconstitution of apoptosis	99
5.22 DNA fragmentation analysis and visualisation of chromatin condensation	99
5.23 Immunoprecipitation of Bad protein	100
5.24 Immunoblotting	100
5.25 Sequence analysis	100
5.3 Results	102
5.31 Creation of a cell-free model for apoptosis in neural cells	102
5.32 Cytochrome c- activated neural cell extracts induced apoptosis in isolated PC12 nuclei	107
5.33 Caspase activation in cell-free extracts	107
5.34 Akt was cleaved during cell-free apoptosis; cleavage was prevented by phosphatase inhibitors	110
5.35 Caspase-9 and caspase-3 activation and Akt cleavage were inhibited by phosphatase inhibitors and functional depletion of ATP	112
5.36 Other proteins downstream of Akt are unaffected in the cell-free system	114
5.37 Creb is a caspase substrate during apoptosis	119
5.4 Discussion and future work	122
5.41 A neural cell-free model of apoptosis	122
5.42 Akt is cleaved by caspases during cell-free apoptosis	123
5.43 Role of phosphatases in triggering apoptosis	125
5.44 Role of ATP during apoptosis	128
5.45 Creb is a caspase substrate during cell-free apoptosis	128
Chapter 6 Staurosporine- and camptothecin- induced apoptosis in SY5Y cells	131
6.1 Introduction	132
6.11 Apoptosis in SY5Y cells	132
6.2 Experimental procedures	133
6.21 Staurosporine- and camptothecin-induced apoptosis in SY5Y cells	133
6.3 Results	134
6.31 Staurosporine-induced apoptosis in SY5Y cells	134
6.32 Raf-1 but not Akt is downregulated during STS-induced apoptosis in SY5Y cells	134

6.33 z-VAD-fmk does not prevent Raf-1 disappearance in STS- induced apoptosis	138
6.34 Akt and Raf-1 are downregulated during camptothecin- induced apoptosis in SY5Y cells	140
6.4 Discussion and future work	143
6.41 Raf-1 is downregulated during STS- and CPT- induced apoptosis	143
6.42 Akt is downregulated during CPT- induced apoptosis in SY5Y cells	146
6.43 Caspases other than caspase-3 and -9 are activated early during STS- and CPT- induced apoptosis	146
Chapter 7 General discussion and future directions	148
Chapter 8 References	151
Appendix	184
Reprints of papers	

List of Figures

Figure 1:	Deregulation of cell death underlies the pathogenesis of many disorders	3
Figure 2:	Outline of key steps in apoptotic regulation	5
Figure 3:	Model of activation of PCD in <i>C. elegans</i>	10
Figure 4:	Genetic pathway for PCD in <i>C. elegans</i>	12
Figure 5:	Model for PCD in <i>Drosophila</i>	16
Figure 6:	Bcl-2 related apoptosis regulators	18
Figure 7:	Model of caspase-9 activation	22
Figure 8:	Signalling by Fas/CD95 and TNFR1	27
Figure 9:	Multi-step chromatin degradation in apoptosis	31
Figure 10:	Growth factor-promoted activation of Akt by PI3-Kinase	34
Figure 11:	Schematic representation of 4 isoforms of Akt	36
Figure 12:	Possible downstream targets of Akt action	38
Figure 13:	Organisation of Mapk modules	42
Figure 14:	Raf/Mek/Erk pathway	44
Figure 15:	Schematic diagram of Sapk/Jnk and p38 Mapk pathways	46
Figure 16:	Asynchrony during apoptosis	69
Figure 17:	High molecular weight DNA fragmentation in serum-deprived PC12 cells	75
Figure 18:	Multi-step degradation of DNA in serum-withdrawn PC12 cells	76
Figure 19:	Quantification of apoptotic morphology in serum-withdrawn PC12 cells	78
Figure 20:	Discontinuous density gradient fractionation of apoptotic PC12 cells	80
Figure 21:	Density gradient separation of serum-deprived PC12 cells	81
Figure 22:	Pooled data for gradient separation of apoptotic PC12 cells	82
Figure 23:	Survival of NGF-recultured PC12 cell populations	84

Figure 24:	Cytochrome c staining of recultured PC12 populations	86
Figure 25:	Populations 2 and 3 differ in reactivity to c-Jun antibodies	88
Figure 26:	Immunoblotting of populations for Akt, Parp and Erk	89
Figure 27:	Model of events occurring in each of the cell populations	93
Figure 28:	Cell-free reconstitution of apoptosis	103
Figure 29:	Apoptotic DNA fragmentation in <i>in vitro</i> reactions	105
Figure 30:	Cytochrome c-activated neural cell extracts induced apoptosis in isolated nuclei	108
Figure 31:	Caspase activation in cell-free extracts	109
Figure 32:	Akt kinase cleavage and caspase-9 and caspase-3 activation are inhibited by phosphatase inhibitors in cytochrome c-activated neural cell extracts	111
Figure 33:	Phosphatase inhibitors prevented nuclear chromatin condensation	113
Figure 34:	Possible downstream targets of Akt	115
Figure 35:	Raf-1 and I κ B α are unaffected during <i>in vitro</i> apoptosis	117
Figure 36:	Gsk-3 β does not activate apoptosis in <i>in vitro</i> reactions	118
Figure 37:	Creb is cleaved during apoptosis	120
Figure 38:	Amino acid sequence of human Akt1 kinase	124
Figure 39:	Mutually antagonistic model for Akt signalling and caspase activation	126
Figure 40:	Amino acid sequence of Human Creb	129
Figure 41:	Staurosporine-induced apoptosis in SY5Y cells	135
Figure 42:	Caspase activation in staurosporine-treated SY5Y cells	136
Figure 43:	Raf-1 and phospho-Mek were downregulated during STS-induced apoptosis	137
Figure 44:	z-VAD-fmk doesn't inhibit Raf-1 downregulation or caspase activation during STS-induced apoptosis	139
Figure 45:	Akt and Raf-1 are downregulated during CPT-induced apoptosis	141
Figure 46:	Analysis of Akt and Raf-1 protein levels by densitometry	142

	during CPT-induced apoptosis	
Figure 47:	Amino acid sequence of human Raf-1	144

List of tables

Table 1:	Apoptosis in disease	4
Table 2:	Attempts to reconstitute apoptosis <i>in vitro</i>	104

List of abbreviations

AD	Alzheimer's Disease
AIDS	Acquired Immune Deficiency Syndrome
Aif	Apoptosis inducing factor
Akap	A-kinase anchoring protein
ALPS	Autoimmune lymphoproliferative syndrome
ALS	Amyotrophic lateral sclerosis
Ant	Adenine nucleotide translocator
Apaf-1	Apoptosis promoting factor 1
App	Amyloid precursor protein
ATP	Adenosine triphosphate
Bad	Bcl-X _L /Bcl-2 associated death promoter
BDNF	Brain-derived neurotrophic factor
BH	Bcl-2 homology
BIR	Baculovirus IAP repeat
BSA	Bovine serum albumin
Cad/Dff40	Caspase-activated DNase/DNA fragmentation factor 40
CamkII or IV	Ca ²⁺ /calmodulin dependent kinase II or IV
cAMP	cyclic adenosine monophosphate
CARD	Caspase recruitment domain
Caspase	Cysteine aspartic acid protease
Ced	Cell death abnormal
Ces	Cell death specification
CHEF	Clamped homogeneous electrical field
CNS	Central nervous system
CPT	Camptothecin
Creb	cAMP response element binding protein
CuZnSOD	Copper/zinc superoxide dismutase
Cyt. c	Cytochrome c

dADP	Deoxyadenosine diphosphate
ddATP	Dideoxyadenosine triphosphate
DcR	Decoy receptor
ddH ₂ O	Double distilled water
DEVD-CHO	acetyl-Asp-Glu-Val-Ala-aldehyde
Diap1	<i>Drosophila</i> inhibitor of apoptosis 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	DNA endonuclease
DR	Death receptor
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol-bis(β -aminoethyl ether N,N,N',N'-tetraacetic acid)
eNOS	Nitric oxide synthase enzyme
ER	Endoplasmic reticulum
Erk	Extracellular-signal regulated kinase (Erk1 = p44Mapk, Erk2 = p42Mapk)
Fadd/Mort	Fas-associated death domain
FasL	Fas ligand
FGF	Fibroblast growth factor
GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
Gsk-3	Glycogen synthase kinase-3
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
HD	Huntington's Disease
HSNs	Hermaphrodite specific neurons
Iap	Inhibitor of apoptosis protein
Icad	Inhibitor of caspase-activated DNase
Ice	Interleukin-1 β -converting enzyme

IGF	Insulin-like growth factor
Ikk	I κ B-kinase
Jnk	c-Jun amino-terminal kinase (Sapk)
kb	kilobase
kD	kilodalton
Mapk	Mitogen-activated protein kinase
Mek	Mapk/Erk kinase (Mek1 = Mkk1, Mek2 = Mkk2)
Mekk	Mapk/Erk kinase kinase
Mkk	Mapk kinase
Mkkk	Mkk kinase
mRNA	Messenger ribonucleic acid
Na ₃ VO ₄	Sodium orthovanadate
NF- κ B	Nuclear factor- κ B
NGF	Nerve growth factor
Nik	NF- κ B -inducing kinase
NO	Nitric oxide
NSMs	Neurosecretory motor neurons
NT	Neurotrophin
OKA	Okadaic acid
p75 ^{NTR}	p75 neurotrophin receptor
Pak	p21-activated kinase
Parp	Poly(ADP)-ribose polymerase
PBS	Phosphate-buffered saline
PC12	Rat adrenal pheochromocytoma cell line
PCD	Programmed cell death
PD	Parkinson's disease
PDGF	Platelet-derived growth factor
Pdk	Phosphoinositide-dependent kinase
PFGE	Pulse-field gel electrophoresis
PGB	PBS with glucose and BSA
PH	Pleckstrin homology domain

PI3-K	Phosphatidyl inositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PP2A	Protein phosphatase 2A
PT	Permeability transition
PtdIns	Phosphatidyl inositol
Rip	Receptor interacting protein
ROS	Reactive oxygen species
Rsk	pp90 ribosomal S6 kinases
Sapk	Stress-activated protein kinase
SAR	Scaffold attachment region
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sek1	Sapk/Erk kinase 1 (Mkk4, Jnk)
SH	Src homology
SODD	Silencer of death domains
STS	Staurosporine
SY5Y	SK-N-SH-SY5Y human neuroblastoma cell line
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TE	Tris-EDTA
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Tradd	TNFR-associated death domain
Traf-2	TNFR-associated factor 2
Trk	Tyrosine receptor kinase
tRNA	Transfer RNA
Tween-20	Polyoxyethylenesorbitan monolaurate
Vdac	Voltage-dependent anion channel

v/v	volume/volume
w/v	weight/volume
z-VAD-fmk	benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

Note on genetic nomenclature:

The conventions used for writing the names of genes and gene products is according to Murray & Hunt (1993). Gene names are always written in lower case letters and are italicised. Gene products are written with the first letter capitalised and without italics.

<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
<i>E. coli</i>	<i>Escherichia coli</i>
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>

Abbreviations for amino acids

<i>Amino acid</i>	<i>Three-letter abbreviation</i>	<i>One-letter symbol</i>
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Iso	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

(from Stryer, 1988)

List of publications arising from this thesis

François, F. and Grimes, M.L. (1998) Stages in apoptotic commitment in PC12 cells. *Mol. Biol. Cell* **9**, 368a [abstract].

François, F. and Grimes, M.L. (1999) Phosphorylation-dependent Akt cleavage in neural cell *in vitro* reconstitution of apoptosis. *J. Neurochem.* **73**, 1773-1776.