Death on the slopes

Symposium on Cell Death Pathways

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in cancer cells has led to the emergence of new drug targets, and recent studies have identified novel cell-death pathways. These exciting developments were discussed at the recent Keystone Symposium on Cell Death Pathways in Whistler, Canada. Many elegant studies were presented at the meeting; unfortunately, we cover only a few key topics here owing to space limitations. This report highlights presentations on the connections between cell death and mitochondrial dynamics, metabolism and cancer, and non-apoptotic cell death.

Fission, fusion and the MOMP confusion

Mitochondrial outer membrane permeabilization (MOMP) is an important regulator of apoptosis that results in cyt c and Smac release, and in caspase activation. Many models for MOMP have been put forward, often involving pro-apoptotic Bcl-2 family members such as Bax and Bak. Recent findings show that Bcl-2 family members also influence mitochondrial fission and fusion (reviewed in Suen *et al*, 2008). Fission and fusion occur during normal cellular homeostasis; however, evidence suggests that these organelle interactions could also affect apoptosis signalling.

Several models of the interplay between MOMP and fission/fusion were discussed at the meeting. R. Youle (Bethesda, MD, USA) suggested that the mitochondrial fission/fusion machinery is important for cyt c release (Suen *et al*, 2008). Bax and the pro-fission GTPase Drp1 co-localize at foci on the mitochondrial membrane, and these foci subsequently turn into fission sites. His group detected Bax translocation, mitochondrial fragmentation and cyt c release almost concomitantly. Interestingly, Bax/Bak-knockout cells contain short mitochondria and have lower rates of mitochondrial fusion (Karbowksi *et al*, 2006). J.C. Martinou (Geneva, Switzerland) hypothesized that Drp1 facilitates Bax aggregation and oligomerization at mitochondrial constriction sites, thereby facilitating pore formation. The overexpression of a mutant Drp1 that fails to stimulate Bax oligomerization delays cyt c release and apoptosis induction (Parone *et al*, 2006). Martinou further suggested that MOMP is the outcome of dysfunctional membrane fission. By contrast, S. Martin (Dublin, Ireland) showed that although Bax and Bak can modulate fission and fusion, these events are not required for cyt c release and apoptosis (Sheridan *et al*, 2008), as

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See Glossary for abbreviations used in this article.

Introduction

The field of cell-death research has matured since pioneering discoveries showed that apoptosis is regulated genetically. Although many of the core caspase-signalling pathways are known, some of the most basic aspects of the biochemistry of mitochondrial apoptosis induction have remained elusive and are, in certain cases, controversial. Research on the metabolic dysfunction that occurs

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Glossary

AML	acute myeloid leukaemia
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BetA	betulinic acid
BH3	Bcl-2 homology 3
BH3-only	Bcl-2 proteins that have only one of the Bcl-2 homology regions, BH3
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 interacting mediator
Bmf	Bcl-2 modifying factor
cyt c	cytochrome c
DHEA	dehydroepiandrosterone
Dronc	<i>Drosophila</i> Nedd2-like caspase
Drp1	dynamamin-related protein 1
E μ -Myc	transgenic mice in which the immunoglobulin heavy chain enhancer (E μ) drives the expression of c-myc in cells of the B lineage
ErbB2	erythroblastic leukemia viral oncogene homologue 2
FADD	Fas-associated protein with death domain
Glut1	glucose transporter 1
HDAC	histone deacetylase
Hsp70	heat-shock protein 70
Mcl-1	myeloid cell leukaemia sequence 1
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NF- κ B	nuclear factor- κ B
Puma	p53 upregulated modulator of apoptosis
RIP	receptor interacting protein
ROS	reactive oxygen species
Smac	second mitochondria-derived activator of caspase

the expression of a dominant-negative Drp1 does not inhibit either event. Furthermore, although various anti-apoptotic Bcl-2 family members inhibit cell death, they do not block mitochondrial fission or cristae rearrangements.

Although many groups are investigating the mechanism of MOMP, and the relationship between Bax and Bak and mitochondrial fission/fusion, the precise mechanisms are still unclear and there are many conflicting results. However, the idea that Bcl-2 family proteins can have dual effects in cell death and mitochondrial fission/fusion is convincing. Martin suggested that the switch for these different roles is determined by BH3-only proteins. Some BH3 domains show significant structural plasticity upon protein binding (Smits *et al*, 2008), which might result in a change of protein function. For example, Bcl-2 switches from protector to killer upon binding a short peptide (Kolluri *et al*, 2008).

It is not surprising that the specific mechanisms of MOMP remain a mystery; mitochondria are heterogeneous and sub-compartmentalized, and interact with each other and with other organelles. A 'dirty little secret' is that cell-death researchers often use crude heavy-membrane fractions as 'mitochondria'. This fraction contains other structures that might influence apoptosis signalling. D. Green (Memphis, TN, USA) showed that using crude mitochondria can lead to new insights into an old apoptosis-signalling concept: the induction of apoptosis by the sphingomyelin pathway. The involvement of sphingomyelin-derived metabolites in pro-apoptotic mitochondrial alterations identified by the Green laboratory is different from what has been proposed in previous

studies on the putative involvement of ceramide or sphingomyelin receptor-mediated apoptosis. Green showed that sphingolipids could regulate the Bcl-2 family effector proteins Bak and Bax, in order to promote their activation, oligomerization and MOMP. This was also demonstrated in protein-free liposomes, in which sphingolipid metabolites markedly increased permeabilization by Bax and cleaved Bid. Interestingly, the removal of endoplasmic reticulum desensitized mitochondria to BH3-only proteins owing to a lack of sphingomyelinase activity. Green speculated that the endoplasmic reticulum might affect apoptosis signalling by providing a lipid milieu for mitochondria that assists the activation of Bax/Bak. As the most commonly used mitochondrial isolation protocols result in a heavy-membrane fraction containing both mitochondria and the endoplasmic reticulum—not to mention lysosomes—this is something to keep in mind in further studies.

Metabolism, cell death and glutamine addiction

Although mitochondria are a keystone of apoptotic events, they are also essential for cell survival because they provide energy to cells through respiration. Crosstalk occurs between the metabolism and apoptosis pathways in response to changes in nutrient availability. For example, T cells require growth factors to stimulate glucose uptake, and undergo p53-dependent apoptosis after growth-factor withdrawal. As discussed by J. Rathmell (Durham, NC, USA), the expression of the glucose transporter Glut1 induces glucose uptake and inhibits Bax activation in activated T cells (Zhao *et al*, 2008). This is regulated by the anti-apoptotic Bcl-2 protein Mcl-1, which is stabilized in response to glucose uptake. After growth-factor withdrawal, glycogen synthase kinase 3 targets Mcl-1 for degradation, p53 signalling induces the expression of Bim and Puma, and apoptosis is initiated. Glut1 expression is sufficient to suppress p53 activity after growth-factor withdrawal, and cell death can be inhibited by knocking out both Bim and Puma. Rathmell suggested that Bim, which is downstream of the endoplasmic reticulum-stress pathway, and Puma, which is downstream of the metabolic-stress pathway, work in parallel to initiate apoptosis in response to glucose deprivation.

Apoptosis induction can also be triggered by metabolic changes downstream of glucose uptake. S. Kornbluth's (Durham, NC, USA) work in oocytes provided insight into how cells sense metabolic changes and initiate apoptosis during development. NADPH produced by the pentose phosphate pathway inhibits apoptosis in *Xenopus* oocytes (Nutt *et al*, 2005). This is mediated by the phosphorylation of caspase 2. Kornbluth proposed that a similar mechanism occurs in *Drosophila* S2 cells. She showed that a cell-permeable malate analogue—predicted to increase NADPH production—rescued DHEA-induced cell death, which is caused by reduced NADPH levels and increased caspase activity. Kornbluth suggested that malate inhibits apoptosis by preventing DHEA-mediated dephosphorylation of the Dronc prodomain. Although it is usually considered to be a caspase 9 homologue, Kornbluth proposed that Dronc is more similar to caspase 2 and—like *Xenopus* caspase 2—might act as a metabolic sensor of apoptosis.

Tumours have high metabolic rates in order to sustain rapid cell growth and division, and there is a renewed focus on determining how tumour cells can continue to proliferate as they deplete essential macromolecules. In addition to glucose, many cells also require glutamine for survival. Glutamine is metabolized at a tenfold higher rate by cancer cells than by normal cells, although only 10% is used for

protein synthesis (DeBerardinis *et al*, 2007). Like glucose, glutamine is an energy source, and Myc-transformed tumour cells use glutaminolysis to produce NADPH to drive lipid and nucleotide biosynthesis. C. Thompson (Philadelphia, PA, USA) described glutamine addiction in oncogenic Myc-transformed cells, in which the expression of Myc is sufficient to induce glutamine dependence. Glutamine—but not glucose—is required in cells with inducible Myc activation, as they undergo apoptosis after serum withdrawal. M. Yuneva (San Francisco, CA, USA) showed that glutamine is required for the activity of the Krebs cycle, and that the inhibition of glutamine metabolism through the Krebs cycle induces Myc-dependent apoptosis (Yuneva *et al*, 2007). Furthermore, the Krebs cycle substrates pyruvate and oxaloacetate can protect Myc-transformed fibroblasts from apoptosis induced by glutamine deprivation. Similarly, Thompson showed that the replacement of glutamine with α -ketoglutarate allows cells to survive in the absence of serum.

Future studies will elucidate the complex relationship between metabolism and cell death in more detail. It is hoped that an increased understanding of metabolic regulation will lead to *in vitro* models that better mimic the *in vivo* tumour microenvironment.

On the horizon: cancer treatment and prevention

Understanding metabolic-pathway function during tumour development is leading directly to new ideas for therapeutic intervention. J. Brugge (Boston, MA, USA) uses three-dimensional mammary cell-culture models to investigate mammary morphogenesis. Mammary-lumen formation requires Bim-mediated apoptosis; however, in the absence of Bim, inner cells undergo rampant autophagy and eventually die through non-apoptotic cell death (Mailleux *et al*, 2007). Cells that are deprived of extracellular matrix *in vitro* have reduced ATP levels and high levels of ROS owing to defects in glucose uptake. However, oncogenes such as ErbB2 can elevate ATP levels by rescuing glucose metabolism. Interestingly, antioxidants can also elevate ATP levels by rescuing the ROS inhibition of fatty-acid oxidation. Therefore, Brugge suggests that the survival of tumour cells outside their natural matrix niches requires alterations that prevent apoptosis and rescue metabolic defects caused by a loss of glucose uptake. Furthermore, the elevation of tumour-cell antioxidant activity could suppress the natural mechanisms that eliminate aberrantly proliferating cells and promote tumour-cell survival.

Research into cancer-cell metabolism is also leading to new ideas for cancer prevention. The monoallelic loss of the autophagy gene *beclin1* is prevalent in human cancers, suggesting that autophagy is tumour suppressive even though it also occurs in tumour cells as a survival mechanism. E. White (New Brunswick, NJ, USA) showed that autophagy-defective cells accumulate p62-protein aggregates in response to metabolic stress (Mathew *et al*, 2009), which is accompanied by elevated ROS production, accumulation of damaged mitochondria and activation of the DNA-damage response. The suppression of p62 accumulation in autophagy-defective tumour cells rescues oxidative stress and prevents the activation of the DNA-damage response. Importantly, this aberrant and persistent p62 accumulation in autophagy-defective cells promotes altered gene expression and tumorigenesis. Therefore, the autophagic degradation of p62 protects cells from damage that can lead to tumour formation; this led White to propose that autophagy promoters could be used to prevent cancer in some circumstances.

Several candidate anticancer therapeutics have recently emerged from cell-death research. J.P. Medema (Amsterdam, the

Netherlands) showed that BetA induces autophagy and cell death, in addition to a mitochondrial morphology similar to that of patients with Barth syndrome. BetA-mediated cell death is dependent on mitochondrial permeability transition, and specifically induces death in tumour cells while untransformed cells remain healthy (Mullauer *et al*, 2009). BetA variants with a higher cell permeability could be interesting therapeutics.

New drug strategies that target Bcl-2 family proteins have also emerged. A. Strasser (Parkville, Australia) found that Bcl-xL—but not Bcl-2—is required for lymphoma development in E μ -Myc transgenic mice (Kelly *et al*, 2007). A loss of Bcl-xL reduced the abnormal accumulation of pre-B cells caused by the overexpression of Myc, and accelerated the apoptosis of pre-leukaemic cells. The monoallelic loss of Bim restored the ability of Myc to induce pre-B/B-cell lymphoma development in the absence of Bcl-xL. Treatment with the BH3-targeting drug ABT-737 notably blocked the development of E μ -Myc lymphoma, and reduced the number and accelerated the apoptosis of pre-leukaemic E μ -Myc B cells. Therefore, BH3-targeting drugs have the potential to be effective in the treatment of lymphoma. Furthermore, BH3-targeting drugs can be made more effective through combinatorial treatment. HDAC inhibitors—such as vorinostat—selectively kill tumour cells through the mitochondrial pathway (Ellis *et al*, 2009), although the overexpression of pro-survival Bcl-2 proteins, which are common in cancers, prevents the effect of HDAC inhibitors. By using an E μ -Myc mouse model, R. Johnstone (Melbourne, Australia) demonstrated that the efficacy of HDAC inhibitors in cancer therapeutics could be increased by the combinatorial treatment of ABT-737 with vorinostat. Bmf is a transcriptional target of vorinostat, which induces histone hyperacetylation within the Bmf promoter region. The vorinostat/ABT-737 synergistic effect is lost if Bmf is knocked down, indicating that Bmf is functionally important in this drug combination.

Genetic heterogeneity affects tumour progression and response to chemotherapy, thereby creating serious obstacles to cancer treatment. By using mouse models for AML, S. Lowe (Cold Spring Harbor, NY, USA) recapitulated the differences in response to chemotherapy that are associated with various genotypes. A retroviral approach that conditionally suppresses gene function *in vivo* led to the discovery of genes that are required for leukaemic maintenance (Zuber *et al*, 2009). Mouse models of AML with common human AML mutations were used to correlate different mutations with disease prognosis. Poorly responding leukaemias showed a marked attenuation of the p53 response, which might explain their drug resistance. Therefore, Lowe suggests that genetic information and assessment of p53 activation should be used to guide human AML treatment.

Although surprisingly few cancer drugs have resulted from cell-death research so far, recent advances are promising. Furthermore, new developments from the field of death receptor-mediated pathways, although not the focus of this meeting, are also promising and might lead to additional drug candidates. It will be exciting to follow the development of combinatorial treatments, as well as those that take advantage of genetic information.

New frontiers in the world of cell death

We have long known about multiple forms of cell death; however, relatively little is known about non-apoptotic death mechanisms. The depletion of Hsp70—a survival protein that is highly expressed in many cancers—induces a caspase-independent cell death

Fig 1 | Cells light up like Christmas trees during lysosomal rupture. BJ fibroblasts, loaded with acridine orange (green cytosolic and red lysosomal staining) (A) before and (B–E) during continuous photo-oxidation (at 489 nm). Blue light causes lysosomal membrane oxidation and subsequent membrane rupture. Confocal images were captured every 500 ms in two channels (BP505–550 and LP650) by a Zeiss LSM DUO. Courtesy of T. Kirkegaard and M. Jäättelä.

that requires the release of lysosomal cathepsin into the cytosol (Nylandsted *et al*, 2004). M. Jäättelä (Copenhagen, Denmark) described changes in lysosomal composition, trafficking and function that occur during cancer transformation and progression (Fig 1; supplementary Movie S1 online). Jäättelä found that Hsp70 binds to lysosomal lipids, thereby stabilizing lysosomes in cancer cells and suggesting that Hsp70-expressing cancer cells might be resistant to lysosome-targeting drugs. Non-apoptotic cell death might also occur during development. K. McCall (Boston, MA, USA) reported that the lysosomal proteins Spinster and Deep Orange are required for nurse-cell death in the developing *Drosophila* egg chamber (Peterson *et al*, 2007), which is caspase independent and requires cell-autonomous DNase-II activity. Her results suggest that lysosomes surround nurse-cell nuclei and rupture during the late stages of nurse-cell death. S. Shaham (New York, NY, USA) described a new form of cell death

in *Caenorhabditis elegans* development—the cell-autonomous death of linker cells—that is also observed during mammalian development. Interestingly, a genetic screen identified a requirement for several developmental-timing genes in this pathway, whereas apoptosis and autophagy genes did not seem to be involved (Abraham *et al*, 2007). Finally, F. Chan (Worcester, MA, USA) described a type of caspase-independent cell death known as programmed necrosis. This process requires RIP1, which also mediates NF- κ B activation. Chan showed that RIP3 is required for the phosphorylation and stable binding of RIP1 to FADD, allowing the formation of a RIP1–FADD–RIP3 complex. The RIP3-mediated activation of RIP1 results in a transient induction of necrosis-associated kinase activity. Vaccinia virus-infected RIP3^{−/−} mice do not have necrotic tissue injury or virus-induced inflammation. These mice fail to control viral infection, suggesting that programmed necrosis could be a potential antiviral-defence mechanism.

Although we have long known about alternative forms of cell death, the mechanisms of these types of cell suicide are still elusive. In addition, little is known about how alternative death mechanisms are regulated. Undiscovered cell-death regulators might exist and/or known death regulators could function in a manner that is cell context-specific. Ultimately, we cannot exclude the possibility that the regulation of apoptotic and non-apoptotic mechanisms might be similar. Further research will be necessary to resolve these questions.

Summary

The 2009 Keystone Symposium on Cell Death Pathways showcased exciting research on many aspects of cell death. Here we have highlighted a few of these discoveries. Although much is known about mitochondrial dynamics, metabolism and cell death, we are just beginning to understand the relationships among these processes. These discoveries, together with new findings about non-apoptotic cell death, have the potential to influence cancer treatment. We can therefore proclaim that cell-death research is not dead—fundamental discoveries will be made about how cells die as new models and methods emerge.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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