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Meeting Report

# Apoptosis and disease: a matter of cell fate

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Bristol's first apoptosis conference was heralded by the dramatic return of Concorde to its home. However, it went over our heads as we focussed on the life or death decisions made by our cells and how apoptosis defects in tissue development and homeostasis can contribute to human diseases. Aberrations in apoptotic responses to death signals contribute to cancer development and resistance to treatment, and to autoimmune diseases, whereas enhanced cell death contributes to degenerative and immune deficient diseases. Understanding how cell fate decisions are made *in vivo* and how cell death pathways are executed or held in check will be central to our future success in treating these diseases.

## A complex decision of life or death

When a cell is damaged, a quick decision must be made: cell cycle arrest and repair, or death of the cell by apoptosis?[1] X Lu (Ludwig Institute, London, UK) asked: what determines which genes p53 transactivates in response to damage – the apoptosis genes (e.g. *Bax*, *Noxa*, *Pidd* and *Puma*) or the cell cycle arrest genes (*p21*, *cyclin G*, *14-3-3σ*)? Wild-type p53 is lost in 50% of human tumours, but in breast cancer, for example, 75% of tumours retain wild-type p53. Other important changes in carcinogenesis can result in defects in p53 function. Lu's group has discovered an important family of p53 regulators, the ASPP family of proteins (human **A**nkyrin repeat, **S**H3 domain and **P**roline-rich sequence **P**roteins that are **A**poptosis **S**timulating **P**roteins of **p**53), which determine whether p53 activates apoptosis or growth arrest genes.[2]

ASPP1 and ASPP2 bind to the DNA binding domain of p53 to stimulate p53-induced apoptosis. The DNA binding domain is the most conserved and important region of p53. To address whether ASPP proteins were also conserved, Lu turned to our old friend, the nematode 'worm' *Caenorhabditis elegans*, an excellent tool to dissect DNA damage response pathways.[3] There was only one ASPP-related protein in *C. elegans*: iASPP. By contrast to ASPP1 and 2, iASPP inhibited p53-induced apoptosis.[4] Consistent with an antiapoptotic role, depletion of human iASPP by RNAi or antisense RNA in MCF-7 breast carcinoma cells or U2OS osteosarcoma cells, led to p53-induced apoptosis. Furthermore, *C. elegans* iASPP could inhibit p53-induced apoptosis in human cells, illustrating the functional homology of the conserved human and *C. elegans* iASPP proteins. Hence, in human cells ASPP1 and 2 stimulate p53 function, whereas iASPP inhibits p53 function.

So how does the cell decide whether p53 induces apoptosis or growth arrest? ASPP1 and 2 can potentiate p53-induced apoptosis, but they do not potentiate the G1 arrest: ASPP 1 and 2 increased transactivation of the *Bax* promoter and the *PIG3* promoter, but not the *Mdm2*, $p21^{WAF-1}$ or *cyclin G* promoters. When does p53 induce apoptosis genes? Lu suggested that the ASPP proteins might decide.[2]

G Melino (Tor Vergata, Rome and Leicester, UK) illustrated the role played by the p53 homologue, p73, in apoptosis signalling in response to DNA damage. The p73 pathway is independent and parallel to that of p53, and is relevant for cancer development, progression, and therapy. The *p73* gene is subject to complex regulation. It has two distinct promoters; one produces the longer TAp73 protein, which is proapoptotic, and the other the shorter ΔNp73 protein, which is antiapoptotic.[5] Both p53 and TAp73 can transactivate the second promoter to induce ΔNp73 (Figure 1). ΔNp73 lacks the transactivation domain, but functionally inactivates p53 and TAp73, creating a regulatory dominant negative feedback loop. Hence, the life of the cell hangs in the balance.

sing an inducible system for TAp73, Melino demonstrated that, like p53, TAp73 induces apoptosis by upregulating many apoptosis genes, with *Bax*, *Puma*, and *Scotin* identified as TAp73 targets. The translocation of Bax to the mitochondrion, facilitated by Puma, allows the exit of cytochrome *c* and activation of the apoptosome[6] (Figure 2). In addition, TAp73 induces ER stress mediated by the direct transactivation of *Scotin*,[7] a p53-inducible gene cloned by David Lane's group.[8] So how does ΔNp73 perform its survival function? The ΔNp73 isoform, by tetramerisation with TAp73 or p53, was shown to reduce the transcription of *Bax* and *Puma* and inhibit apoptosis.

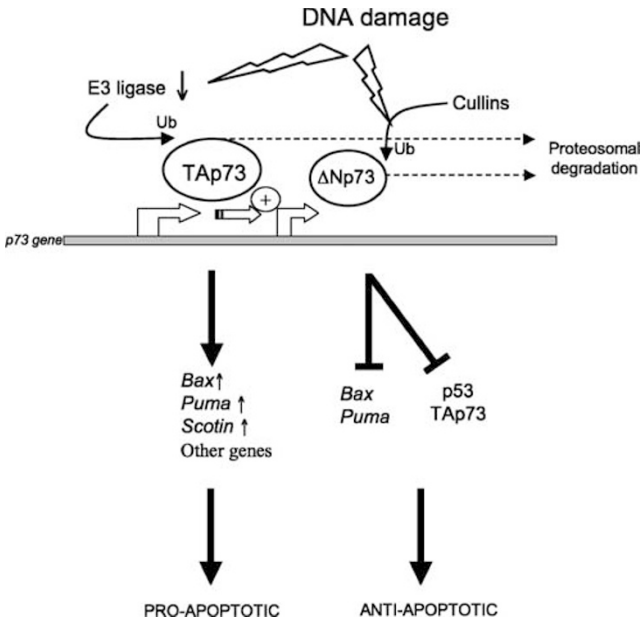


**Figure 1** p73 regulation of apoptosis in response to DNA damage. *p73* gene has two distinct promoters for TAp73 (proapoptotic) and ΔNp73 (antiapoptotic) proteins. TAp73 transactivates the promoter for ΔNp73, and ΔNp73 functionally inactivates p53 and TAp73. DNA damage results in cullin-mediated ubiquitination of the antiapoptotic ΔNp73 protein. Thus, DNA damage leads to preferential proteosomal degradation of ΔNp73 and increased levels of TAp73. TAp73 induces expression of proapoptotic genes

Both TAp73 and ΔNp73 levels are regulated by proteosomal degradation, but in different ways. In response to DNA damage, ΔNp73, the antiapoptotic isoform, is ubiquitinated and degraded by the proteosome.[9] Levels of p73 isoforms are independently regulated by cullins, ubiquitin ligase members of the NEDD8 family.[10] In response to DNA damage, levels of ΔNp73 decrease and TAp73 can go on to efficiently induce apoptosis (Figure 1).

TAp73 synergises with chemotherapeutic drugs to induce apoptosis of cancer cells and direct evidence for the involvement of p73 proteins in cancer comes from a negative correlation of ΔNp73 with overall survival of neuroblastoma patients. These results led Melino to propose that the ratio of TAp73 to ΔNp73 may dictate the response to chemotherapy and suggest that TAp73 is the oncologist's friend, whereas ΔNp73 may prove to be the foe in tumorigenesis.[11]

## Orchestration of cell death: a tale of death receptors and mitochondria

Apoptosis can be induced either by activation of death receptors or by perturbation of mitochondria[12–14] (Figure 2). G Cohen (Leicester, UK) explained how the mitochondrial pathway can be engaged in response to death receptor signalling, facilitating apoptosis by release from the mitochondria of cytochrome *c* or the inhibitor of apoptosis (IAP) antagonists Smac/Diablo and Omi/HtrA2. Part of the function of IAP antagonists is to release partially processed caspase-3 from XIAP, allowing it to undergo autocatalytic processing to its mature form.[15] Cohen then focussed on the function of the

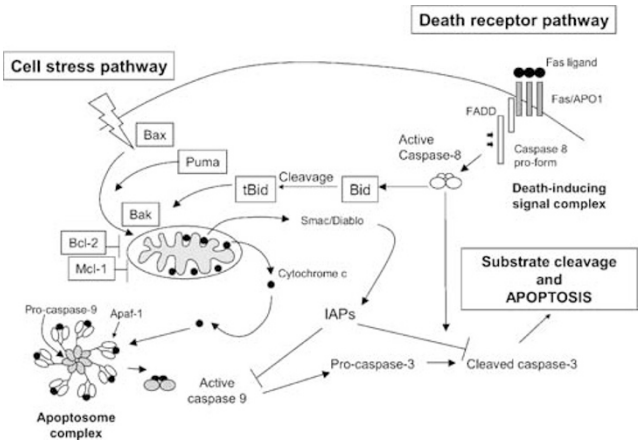


**Figure 2** Death receptor and cell stress pathways to apoptosis. In the death receptor, or 'extrinsic pathway', to apoptosis, ligation of death receptors of the tumour necrosis factor (TNF) receptor family, such as Fas/APO-1, by their natural ligands results in trimerisation of the receptors. Adapter molecules such as FADD associate with the intracellular death domain of the receptor and recruit procaspase-8 to form the death-inducing signal complex (DISC). Procaspase-8 is rapidly activated by autocleavage. Subsequently, in Type I cells, caspase-8 can cleave caspase-3 leading to further caspase activation and apoptosis. Type II cells rely on amplification of the caspase signal by the mitochondrial pathway and release of cytochrome *c* and other apoptotic molecules such as Smac/Diablo from the intermembrane space of the mitochondria. The Bcl-2 family of proteins controls this release: antiapoptotic members of the Bcl-2 family (e.g. Bcl-2, Bcl-xL, Mcl-1) inhibit the release, whereas proapoptotic members of the family, Bax and Bak promote the release. In Type II cells caspase-8 cleavage of Bid facilitates cytochrome *c* release by Bax and Bak. The released cytochrome *c* catalyses the formation of the apoptosome, a heptameric complex of Apaf-1 and procaspase-9. Caspase-9 is autoactivated and can then cleave and activate caspase-3. In the cellular stress or 'intrinsic pathway' to apoptosis, Bax is translocated to the mitochondria, sometimes facilitated by other proapoptotic Bcl-2 family members such as Puma, where it can induce cytochrome *c* release in conjunction with Bak

death-inducing signal complex (DISC) that is formed as a result of receptor aggregation and recruitment of the adapter proteins and procaspase-8. The death ligand, TRAIL (TNF-related apoptosis-inducing ligand), holds promise for cancer treatment since many tumour cells are more sensitive to TRAIL-induced apoptosis than normal cells.[16] However, primary chronic lymphocytic leukaemia (CLL) cells are resistant to TRAIL-induced death due to low-level expression of death receptors. Caspase-8 was recruited to the DISC, but was not efficiently activated. However, addition of exogenous receptors to CLL cell lysates allowed caspase-8 to be processed to its active form.[17] Knowledge of how tumour cells become resistant to TRAIL will help to determine mechanisms to sensitise such cells to TRAIL-induced apoptosis by combination with other agents.

Fas-associated death domain protein (FADD) is reported to be the universal adaptor used by death receptors to recruit and activate the initiator caspase-8. The CD95/Fas receptor and the TRAIL-R1 and R2 receptors bind FADD directly, whereas recruitment to tumour necrosis factor receptor, TNF-R1, is indirect through another adaptor TRADD (TNF-Receptor-Associated Death Domain protein). It is generally thought that TRADD recruits FADD leading to apoptosis. Although caspase-8 and FADD were obligatory for TNF-mediated apoptosis in Jurkat, HeLa and U937 cells, they were

not recruited to a TNF signalling complex.[18] This means that in response to TNF signalling, caspase-8 must be activated elsewhere within the cell on another activation scaffold. Cohen pointed out that these results are in agreement with the finding of Micheau and Tschopp[19] that a second complex involving TRADD, RIP, FADD and caspase-8 can form in the cytoplasm, and he referred us to the work of Gomez-Angelats and Cidlowski[20] who reported that FADD can be localised to the nucleus as well as the cytoplasm. Hence, Cohen challenged our view of how death ligands signal apoptosis, and it will be exciting to watch the development of this field over the coming months.

Andrew Halestrap (Bristol, UK) described how the Mitochondrial Permeability Transition Pore (MPTP) plays a central role in cell death associated with reperfusion injury during cardiac surgery. Necrotic cell death is the primary cause of the damage, but around the damaged area, or infarct, there exists a ring of apoptotic cells. The brain can also suffer apoptosis due to ischaemia–reperfusion in stroke. Apoptosis in response to cell stress or damage is mediated through the release of proapoptotic proteins contained in the mitochondrial intermembrane space. These include cytochrome *c*, which activates the caspase cascade; AIF, which facilitates DNA fragmentation; and Smac/Diablo, which releases caspases from inhibition by IAP (inhibitor of apoptosis) proteins. One mechanism for intermembrane protein release involves selective permeation of the outer mitochondrial membrane through recruitment of protein such as Bax, Bad and Bid. These proteins, either in their own right, or through interaction with outer membrane proteins such as the voltage-dependent anion channel (VDAC), induce a channel in the outer membrane. An alternative mechanism by which these proteins are released may be through MPTP opening. This causes mitochondrial swelling, outer membrane rupture and nonspecific release of intermembrane proteins, resulting in activation of caspases. Subsequent to this, one of two things can happen: if the pore closes again, apoptosis results; if the pore remains open, failure of ATP synthesis and the breakdown of ATP leads to necrosis. According to the severity of the insult to the cell, mitochondria determine not only whether a cell should die, but also the nature of that death. Therefore, it is important to understand how the MPTP can be controlled to inhibit cell death. Halestrap's group have shown that two free radical scavengers, pyruvate and the anaesthetic propofol, have cardioprotective effects associated with diminished opening of the MPTP. A major goal is therefore to develop novel, specific, and potent inhibitors of the MPTP that can enter the heart and rapidly reach the ischaemic area.[21]

## Destruction from within

Understanding the mechanisms of apoptosis execution will assist in the treatment of diseases in which apoptosis occurs inappropriately, for example by development of pharmacological agents to block caspase activation or apoptosome assembly. Apoptosis has largely been attributed to the activation of caspases, which cleave many substrates to produce the characteristics of an apoptotic cell,[22,23] however it is worth noting that developmental apoptosis is often unaffected by many caspase knockouts, and so other changes might be important that are caspase-independent.[24] Caspases are initially synthesized as inactive zymogens that require cleavage and reassembly of the subunits to be active. Apical caspases are recruited to activation scaffolds (for example, the apoptosome or the DISC) to facilitate activation. Alternatively, caspase activation may occur as a result of direct proteolytic processing by other caspases or noncaspase proteases, such as granzyme B, an enzyme produced by natural killer cells and cytotoxic lymphocytes.

Seamus Martin (Dublin, Ireland) investigated the components of the apoptosome using reconstitution experiments, which permit investigation of the timing of recruitment of individual components. In the absence of cytochrome *c*, caspase-9 was monomeric and did not coimmunoprecipitate with Apaf-1. Apoptosome assembly was triggered by cytochrome *c* addition and Apaf-1 immediately oligomerised. Interestingly, cytochrome *c* seems to perform a 'hit and run' function, since it was not present in the assembled apoptosome complex. The caspase inhibitory protein XIAP was also rapidly recruited to the apoptosome as a major constituent and was found to act as a tether for the stable recruitment of mature caspase-3.[25] By contrast, no recruitment of cIAP-1, cIAP-2, Hsp70, Hsp90, Bcl-2 or Bcl-xL was detected.

Martin's group is seeking caspase and granzyme B targets in a systematic way using two-dimensional gel electrophoresis. Granzyme B can activate caspases 3, 7 and 8, and through caspase-3 activation, indirectly activate caspases 2, 6 and 9. These studies have identified numerous new caspase substrates, as well as several novel granzyme B substrates. Future work is aimed towards identifying substrates that play important functional roles in apoptosis.

## Disposing of the evidence

*In vivo*, apoptotic cells are normally efficiently disposed of by phagocytosis without eliciting an inflammatory response. C Gregory (Edinburgh, UK) drew our attention to the recent review of Savill *et al*.[26] on apoptotic cell clearance, which involves a complex array of receptors, soluble factors ('bridging molecules') and apoptotic cell-associated ligands. Aberrant clearance of apoptotic cells may be a contributory factor in autoimmune diseases and persistent inflammatory disorders. *In vivo*, apoptotic cells appear rare, even in tissues such as the thymus in which extensive apoptosis is a normal feature during regression. Gene knockout strategies in mice are starting to assist in the elucidation of the relative importance of individual component molecules of the clearance pathways *in vivo*. Animals deficient in these molecules can lead to diverse phenotypes, ranging from total absence of demonstrable clearance defects to apoptotic cell persistence and autoimmunity. Gregory has shown that apoptotic clearance is dependent, in part, on CD14. Membrane-anchored CD14 supports the function of other receptors such as the phosphatidyl serine receptor (PSR) on macrophages by acting as a tethering molecule allowing the firm binding of apoptotic cells to macrophage surfaces.[27] CD14−/− mice show inefficient phagocytosis of apoptotic cells, which accumulate in multiple tissues of unchallenged animals, and

are particularly evident in the thymus and spleen. To test the capacity of CD14−/− mice to clear cells, labelled cells were injected into the peritoneal cavity of mice. Wild-type animals had much better clearance of apoptotic cells than the CD14−/− mice. Interestingly, despite the defective clearance of the apoptotic cells in these mice, aged CD14−/− mice fail to show increased titres of autoantibodies. Therefore, defects in tethering can leave the anti-inflammatory mechanisms intact and the mice suffered no detrimental effects. The results suggest that the role of CD14 in apoptotic-cell clearance *in vivo* can be uncoupled from the anti-inflammatory mechanisms that accompany apoptosis. So, the different phases of recognition and anti-inflammatory signalling can be separated from each other. The importance of this work relates to our understanding of, not only autoimmune diseases and inflammatory disorders, but also the complex inter-relationships between apoptotic cells and macrophages recruited to a growing tumour. For example, factors produced by macrophages, such as interleukin 10, are important in promoting survival of Burkitt lymphoma cells *in vitro*. Gregory is using animal models of human Burkitt lymphoma to ask whether the process of engulfment is important in the pathogenesis of the tumour.

## Cell survival: the ups and downs

J Downward (Cancer Research UK, London) focussed on the use of RNA interference to dissect out regulators of apoptosis. Ultimately, RNA interference or similar strategies might be used to induce apoptosis in diseases linked to upregulation of specific genes, for example, viral diseases, inflammatory diseases and cancers.[28] One such target might be the survival gene *Akt*. Downward hypothesised that the kinase Akt (protein kinase B) may protect against apoptosis not only by phosphorylation of Bad, but by some other mechanism preventing the conformational change in Bax and resultant translocation of Bax to the mitochondria. To identify Akt targets, he first used an inducible system for Akt expression, taking advantage of the fact that some Akt substrates are 14-3-3 binding proteins. One Akt target was Yes-associated protein (Yap), a transcriptional coactivator that, in the absence of Akt phosphorylation, enhanced p73-induced transcription of *Puma*, as well as *Bax*.[29] Yap RNAi-attenuated cisplatin-induced killing of MCF-7 breast carcinoma cells, and when combined with p53 RNAi resulted in almost complete resistance. Other 14-3-3 binding proteins that arose from the initial screen are being identified as Akt targets from the use of Akt RNAi. Downward is also using RNAi screening approach to find out which losses of protein expression lead to apoptosis resistance. For example, in the MCF-10A spontaneously immortalised human breast epithelial cell line, genes identified by this technique as sensitising cells to anoikis included an Akt inhibitory protein, a small GTPase-activated protein kinase, p53 and another tumour suppressor gene. In a similar screen for genes involved in sensitising cells to Fas-induced apoptosis, p53, GSK3$\alpha$, a different Akt inhibitory protein and an NF-$\kappa$B inhibitor were identified. Although not all of these changes may be directly in the death pathways, they may reflect components of parallel pathways that impinge on them to regulate apoptosis sensitivity. Dissection of these pathways will surely lead to new therapeutic targets.

One possible therapeutic target that is overexpressed in human leukaemias and lymphomas is Mcl-1, an antiapoptotic member of the Bcl-2 family. G Packham (Southampton, UK) showed that antisense-mediated ablation of Mcl-1 induces apoptosis in Akata6 B cell lymphoma cells.[30] During normal B-cell maturation, B cells move from the mantle zone of the lymphoid follicle to the germinal centre, during which process Bcl-2 is switched off and Mcl-1 is switched on. All human B-cell lymphomas express either Bcl-2 or Mcl-1.

Mcl-1 protein has a short half-life. It is unusual among the Bcl-2 family members in that it contains PEST sequences, which are characteristic of proteins with a rapid turnover. However, the PEST sequences may not be entirely responsible for the short half-life for Mcl-1. Packham showed that Mcl-1 is cleaved during cisplatin-mediated apoptosis by caspase-3 at two cleavage sites, asp127 and asp157, that lie in the PEST sequence. The cleavage of Mcl-1 is a major mechanism by which the full-length protein is lost from apoptotic cells. The cleavage generates a fragment containing the BH1-3 and transmembrane domains of Mcl-1 which has proapoptotic function in NIH3T3 cells. Therefore, Mcl-1 is an essential survival molecule for B-lymphoma cells and is downregulated and cleaved by caspases to a death-promoting molecule during apoptosis. In contrast to Mcl-1, Bcl-2 and Bcl-$X_L$ were not substantially downregulated and were relatively resistant to caspase cleavage. Interfering with Mcl-1 expression or function appears to be an effective means of inducing apoptosis in Mcl-1-positive B-cell lymphoma. Alternatively, it may be possible to harness the overexpression of Mcl-1 that has now been shown to be a feature of some solid tumours as well as leukaemias and lymphomas. Exploitation of the unique sensitivity of Mcl-1 to caspase-mediated cleavage that converts it to a proapoptotic molecule forms an attractive therapeutic strategy.

As well as reducing the expression of antiapoptotic genes, tissue-targeted gene expression may be an appropriate strategy for treatment of degenerative diseases. J Uney (Bristol, UK) is using adenoviral viral systems to express antiapoptotic heat-shock proteins, Hsp70, Hsp40 and Hsp27 in neurones and is investigating the role these Hsps play in regulating apoptosis. The protective effects of Hsp70 have generally been attributed to its ability to stabilize/refold damaged or nascent proteins; however, Hsp70 can also inhibit the activation of C-Jun-N-terminal kinase (JNK) and p38 kinases, as well as caspase-3. Loss of cholinergic neurones is associated with memory loss and possibly Alzheimer's disease. Uney emphasised the importance of JNK in apoptosis of cholinergic neurones in response to nerve growth factor depletion. Inhibition of JNK activity protected these cells from apoptosis in response to the NGF withdrawal and also protects dopaminergic neurones in the mitochondrial permeability transition pore model of Parkinson's disease. Uney has shown that nonintegrating adenoviral vectors can be used to mediate powerful and sustained gene expression in neurones *in vivo*;[31] hence gene therapy to treat chronic neurological illness could soon be translated into a practical reality.

## Cell survival: the ins and outs

Although the retinoblastoma susceptibility gene *RB1* is inactivated in a wide range of human tumours in association with escape from replicative senescence, in colonic carcinomas Rb is frequently overexpressed, sometimes as a result of an increase in allele copy number. This overexpression possibly reflects antiapoptotic functions of the protein. A Williams (Bristol, UK) has recently shown that the Rb protein interacts with Bag-1, an apoptotic regulator, in human colonic adenoma and carcinoma cells.[32] The specificity of the interaction was demonstrated by expression of human Papillomavirus E7 oncoprotein. In colonic cells, Bag-1 adopts both a nuclear and cytoplasmic localisation, but immunocytochemistry showed a predominance of nuclear staining. When infected with HPV E7, the Rb/Bag-1 complex was disrupted and there was an increase in spontaneous apoptosis. In addition, the cytoplasmic localisation of the Bag-1 protein increased. To verify that Rb was able to regulate the subcellular localisation of Bag-1, SAOS-2 cells, which are Rb-null, were investigated. In these cells Bag-1 was chiefly cytoplasmic, but transfection of the ABC pocket domain of Rb led to redistribution of Bag-1 to the nucleus, whereas introduction of the C domain only did not. There are three Bag-1 proteins that are generated from a single mRNA by alternate translational initiation (Bag-1L, Bag-1 M and Bag-1S) and these are indistinguishable by immunocytochemistry. Bag-1L contains a nuclear localisation sequence. Williams reported that Bag-1L protects colonic tumour cells against apoptosis induced by either $\gamma$-irradiation or the vitamin D analogue, EB1089. Similarly, Bag-1S, when genetically modified by the addition of a nuclear localisation sequence, was able to confer resistance to apoptosis. In response to $\gamma$-irradiation, Bag-1 appeared to exit the nucleus, much in the same way as when the Rb-Bag-1 interaction was disrupted. Using EGFP-tagged Bag-1 designed for isoform-specific expression, Williams showed that there was a pronounced exit of Bag-1 M in all cells 24 h after irradiation that could not be blocked by leptomycin B. Clearly, not all of the cells underwent apoptosis in response to radiation and the question remains as to how the life or death decision is then made, but the findings suggest that Rb overexpression in colon carcinomas may result in retention of Bag-1 within the nucleus and this may favour cell survival.

## Dying in a social situation

J Holly (Bristol, UK) highlighted the concept that social signals between cells must override any intrinsic mechanisms that may promote the inappropriate survival of individual damaged cells. The insulin-like growth factor binding proteins (IGFBPs) have intrinsic actions that are independent of IGF in modulating cell stress responses and consequently cell survival. The endogenous production of IGFBP-3 from epithelial cells is under the transcriptional regulation of p53 and there is feedback control such that IGFBP-3 enhances the p53 response to DNA-damage and the consequent apoptosis. IGFBP3 is secreted into the extracellular space, such that it could sensitise other damaged cells in the area to apoptosis.[33] In addition, IGFBP-3 can enhance apoptosis completely independently of p53. Holly compared the ability of IGFBP3 and IGFBP5 to sensitise cells to stress-induced apoptosis. In general, IGFBP3 promoted apoptosis, whereas IGFBP5 suppressed it.

To address if this could potentially be utilised for treatment of breast cancer, Holly questioned whether cancer cells might respond differently to their nonmalignant counterparts. The breast cancer cell line Hs578T and a spontaneously immortalised, but anchorage-dependent breast epithelial cell line, MCF-10A, were used as a model system. In the cancer cell line, IGFBP3 potentiated apoptosis induced by ceramide, whereas in the anchorage-dependent cell line IGFBP3 inhibited apoptosis. The effects of both IGFBP-3 and IGFBP-5 on anchorage-independent epithelial cells proved opposite to their effects on anchorage-dependent cells. However, the actions of IGFBP3 and IGFBP5 could be switched by changes in activation of specific integrin receptors. For example, although IGFBP3 is antiapoptotic in MCF-10A cells, in the presence of anti-integrin $\beta 1$ antibody it became a survival factor. Similarly, changing the extracellular matrix altered the response of the cells. Whereas on plastic, collagen IV or laminin, IGFBP3 potentiated apoptosis and IGFBP5 was antiapoptotic, the situation was completely the opposite when cells were cultured on fibronectin: IGFBP3 acted as a survival factor – whereas IGFBP5 became proapoptotic! Therefore, although the IGFBPs exert differential effects on malignant cells *versus* nonmalignant cells, whether apoptosis is inhibited or promoted in response to DNA damage can be totally reversed depending on the cell's interaction with extracellular matrix components.[34] This work sends us a message of caution in our eagerness to develop new apoptosis-based treatments – the environment in which the cell resides is a powerful modulator of apoptosis sensitivity.

## Tissue and mouse models of apoptosis

T Cotter (Cork, Ireland) described *in vitro* and *in vivo* models of photoreceptor cell death in the retina as a model of disease and development. Retinitis pigmentosa is a disease in which the retina degenerates, leading to total blindness by middle age. The photoreceptor cell line 661W has been a useful *in vitro* model in addition to two *in vivo* models of photoreceptor apoptosis: the light-induced retinal degeneration model and the rd mouse, that has a mutation in the phosphodiesterase gene. The 661W cell line undergoes caspase-dependent apoptosis in response to staurosporine or to serum starvation. Both inducers of apoptosis led to activation of caspases 3 and 9, but serum deprivation also induced caspase-12 and calpain activity, suggesting involvement of the endoplasmic reticulum stress pathway. The overall finding was that growth factor withdrawal led to cell death that was predominantly caspase-dependent, but with a smaller calpain involvement.

By contrast, photoreceptor apoptosis in both *in vivo* models appeared to be independent of caspase-9, -8, -7, -3 and -2 activation. In the light-induced model of apoptosis, TUNEL positivity and DNA laddering were obtained 6–24 h after light exposure. Whereas retinas of mice only 10 days old

expressed Apaf-1, no Apaf-1 could be detected in retinal cells of 60-day-old mice. Cytochrome *c* release and caspase-9 activation could not be detected. Cytochrome *c*-dependent proteolysis and activation of caspase-9 could be restored in a neonatal cell-free system, but not in an adult cell-free system, due to an age-related decrease in expression of Apaf-1 in the normal developing mouse retina. In the rd mouse however, this age-related downregulation of Apaf-1 was not observed, but cytochrome *c* release and caspase-9 activation could not be detected in adult mice. Cotter suggested that prevention of cytochrome *c* release might be a mechanism by which mature, terminally differentiated photoreceptors protect against apoptotic stimuli once caspase-dependent developmental apoptosis is complete. Since photoreceptor death in the degenerating retina is caspase-independent, targets other than caspases must be identified for therapeutic intervention.[35]

R Grafström explained tissue homeostasis in the oral epithelium, how *in vitro* models of oral cancer development show defects in the regulation of differentiation and apoptosis. In normal oral epithelium, apoptosis associates with proliferation, implying regulatory coupling of these processes, and that apoptosis also serves to eliminate cells with proliferation-inflicted errors potentially prone to transformation. During transition from the basal to the outermost supra-basal layer, keratinocytes gradually lose proliferative ability and undergo terminal differentiation. Although oral epithelium is similar in structure and differentiation patterning to the skin, the buccal mucosa, for example, is keratinised differently with a parakeratotic terminal differentiation pattern in which the nuclei are retained.[36] The final phase of terminal differentiation in normal undamaged epithelium involves an apoptosis-like event, serving to eliminate cells already committed to death.

Organotypic culture generates stratified layers that recapitulate the three-dimensional tissue structure *in vivo*. In organotypic culture generated from normal oral epithelium, the percentage of cells undergoing apoptosis is low. Normal oral epithelial cells need survival factors, which are usually present in keratinocyte growth medium in the form of bovine pituitary extract (BPE). Removal of BPE induces, not apoptosis, but terminal differentiation. Similarly, high calcium levels induce terminal differentiation. Achievement of an immortal state seems to occur early during oral cancer development, and is coupled with loss of terminal differentiation. Owing to defects in terminal differentiation, immortalised cells can become more susceptible to apoptosis. This was illustrated in organotypic cultures by a high percentage of apoptotic cells in an SV40 large T antigen-immortalised buccal epithelial cell line. Interestingly, the immortalised cell line, which showed defective differentiation, exhibited a higher apoptotic index in organotypic culture than a malignant well-differentiated buccal carcinoma cell line. Grafström suggested that immortalised oral epithelial cells, having lost their primary mechanism of cell death, terminal differentiation, might be at higher risk than other cells for cancer progression.

A Clarke (Cardiff, UK) has modelled colorectal cancer in mice to investigate how several genetic changes drive colorectal carcinogenesis with the ultimate aim of developing sufficient understanding of the disease to be able to tailor chemotherapy to the genetic profile of the tumour.[37] Microsatellite instability is an early event in some forms of colorectal carcinogenesis. The mismatch repair (MMR) complex recognises $O^6$-methylguanine lesions, and may either give rise to direct apoptosis, or may lead to cycles of futile repair and apoptosis through loss of energy. Clarke's group have recently analysed mice mutant for the methyl binding domain (MBD) protein, Mbd4. This protein interacts with Mlh1 and functions as a thymine glycosylase. *Mlh1* and *Mbd4* are intestinal tumour suppressor genes that are mutated at high frequency in mismatch repair-deficient colorectal tumours that exhibit microsatellite instability. *Mbd4* deficiency accelerates intestinal tumour development and alters the mutation spectrum in mice heterozygous for the $Apc^{Min}$ allele.[38] Mbd4 was essential for the normal apoptotic programme following DNA damage and *Mbd4* deficiency can confer increased long-term clonogenic survival *in vivo*.[39] Clarke suggested that this might be a result of interaction with the death molecule FADD. A possible mechanism might be that Mbd4 retains FADD within the nucleus until DNA damage is recognised, whereupon FADD is released. This idea is supported by enhanced sensitivity to Fas ligand-mediated death in the absence of Mbd4. The nuclear localization of FADD and its interaction with a genome surveillance/DNA repair protein that can regulate apoptosis could suggest a novel function of FADD distinct from direct participation in death receptor signalling complexes.[40] In summary, Mbd4 may suppress tumorigenesis not only by suppressing 5-methyl CpG deamination, but also by mediating apoptosis in response to DNA damage.

C Watson (Cambridge, UK) studies regression (involution) of the mammary glands of mice after weaning as a model system of apoptosis in the breast. Mammary epithelial cells are induced to undergo extensive apoptosis during the first 3 days of post-lactational regression. Apoptosis is associated with a sequential activation of the executioner caspases 3, 6 and 7, suggesting distinct mechanisms of cell death during the early and late phases of involution. Using conditional gene targeting and mammary epithelial cell culture models, Watson's group have identified a number of transcription factors that mediate the apoptotic process during involution; these include Stat3 and NF-$\kappa$B. The cytokine, LIF, is increased during the involution switch and activates Stat3 *in vivo*, which then increases the rate of apoptosis in the mammary gland.[41] Comparing transcriptional profiles at different stages of early mammary gland involution by Affymetrix microarray analysis revealed an unexpected association of the apoptotic response with an inflammatory response.[42] Many of the inflammatory mediators/acute phase response proteins are targets of Stat3 and/or NF-$\kappa$B. Furthermore, a number of apoptosis regulators and components of various signalling pathways are dramatically regulated between 12 and 72 h involution. By defining the apoptotic pathways that control epithelial cell death during involution and remodelling of the mouse mammary gland, Watson will undoubtedly discover some of the fundamental biology that underlies evasion of apoptosis during breast cancer, thus helping to identify targets to facilitate breast cancer treatment.

## Concluding remarks

The meeting brought together scientists using different model systems and technologies to dissect the molecular pathways of apoptosis and cell survival as we try to understand how the life of our cells lies in the balance. The tissue specificity and environmental controls on apoptosis sensitivity are clearly critical to translating this knowledge into disease treatment. Communicating our experiences of different model systems was not only inspirational to each of us present at the conference, but also fundamental to avoiding the many pitfalls that could await us as we push towards clinical use of our findings.

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