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Meeting Report

DATELINE Edinburgh

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Abbreviations: IUBMB, International Union of Biochemistry and Molecular Biology; ICE, Interleukin-1β converting enzyme; PAR, proline- and acid-rich transcription factor; GST, glutathione transferase; IL-1, Interleukin 1; BUdR, bromodeoxyuridine; TUNEL, terminal dUTP nick end-labeling; aSMase, acidic sphingomyelinase; PT, permeability transition; GFP, green fluorescent protein

This past summer, the International Union of Biochemistry and Molecular Biology (IUBMB) dedicated their 4th annual conference to the topic of 'The Life & Death of the Cell.' The conference was held in the picturesque city of Edinburgh on a week when Scotland enjoyed what probably represented its sunniest weather of the entire year. Despite the fair weather, however, the participants were eager to spend the entire day in the Conference Center, thanks to the exciting programme and outstanding cadre of speakers. The topics covered at the annual conference were widely diverse, and included parallel sessions on: cell cycle control in yeast and xenopus; mechanisms of cell differentiation and senescence; compartmentalization of signal transduction in animal and plant cells; and mechanisms of protein targeting and turnover. Of greatest interest to Apoptosis and Cell Death researchers were sessions on oxidative stress, effectors of cell death; and apoptosis dysfunction in disease. Some highlights of the meeting, particularly as they relate to cell death regulation, are summarized below.

In an elegant plenary lecture, Andrew Wyllie (University of Edinburgh) presented an overview of the current status of apoptosis research, discussing the morphological and biochemical characteristics of this type of cell death as well as some general findings concerning its genetic regulation. He then proceeded to describe his own more recent studies of the role of p53 in apoptosis induced by DNA damage. The main conclusion from this work is that p53 involvement in this situation differs between cell types, with lymphocyte apoptosis having a strong p53-dependence, enterocytes containing both p53-dependent and independent mechanisms, and hepatocytes exhibiting p53-independent cell death after DNA damage.

The broad theme of oxidative stress was the subject of six lectures on the opening day. After Barry Halliwell's (King's College; London) introductory keynote lecture, Gisela Storz (NIH; Bethesda) discussed the redox regulation of bacterial transcription factors. Her group has identified the hydrogen peroxide-induced conversion of protein-SH to protein-SO_3^- as one modification that alters the transcriptional activity of bacterial oxyR. Similar studies of some of the redox-sensitive mammalian transcription factors are clearly needed.

Michael Jacobson (University College London) followed this with a dissection of some ideas concerning the molecular mechanisms of apoptosis. Research from his group has emphasized that while many agents induce apoptosis (and conversely, many agents can also inhibit apoptosis in certain cases), this issue is distinct from identification of the conserved set of proteins that actually undertakes apoptotic cell death. For example, he has previously shown that Fas/APO-1 transduced apoptosis can occur independent of ambient oxygen tension, indicating that reactive oxygen species are unlikely to be essential mediators of this form of cell death. His experiments with cells depleted of functional mitochondrial electron transport have similarly been used to support the idea that oxidative phosphorylation is unimportant in apoptosis, although other functions attributed to this organelle potentially may be directly or indirectly involved. In contrast, all forms of apoptosis investigated by Jacobson's group to date are sensitive to inhibition by peptide inhibitors of proteases related to interleukin-1β converting enzyme (ICE-like proteases). This finding is very much in accord with recent published studies concerning apoptosis in the nematode *Caenorhabditis elegans* and various human lymphocyte cell lines, and emphasizes the central importance of ICE-like cysteine proteases in the execution phase of apoptosis.

Roy Burdon (U. Strathclyde; Glasgow) followed with a discussion into the control of cell proliferation by either external hydrogen peroxide or reduced thiols such as glutathione and N-acetyl cysteine, after which Sten Orrenius (Karolinska Institute; Stockholm) presented recent findings concerning the redox modulation of apoptosis. Using dithiocarbamates, Dr Orrenius showed that while chronic oxidative stress induces cell death (apoptosis at low concentration and necrosis when higher amounts of the reagent are present), in the short term these reagents can also inhibit apoptosis. This inhibitory effect is also displayed when the dithiocarbamates are oxidised, and the results of various biochemical experiments presented suggest that it reflects an inhibition of ICE-like protease maturation. As these enzymes contain a cysteine nucleophile and require autocatalysis for processing into a mature form, mixed disulphide formation between a thiol toxicant and protein-SH is proposed to

be responsible. The session on oxidative stress was ended by Lawrence Loeb (U. Washington; Seattle), who presented results from a biochemical investigation into some of the DNA base pair modifications that occur after exposure to reactive oxygen species. Cytidine to thymidine substitutions (both C to T and C-C to T-T) were the most common base changes detected, and Loeb suggested that this could represent a primitive sensor by which a cell could respond to oxidative stress by changing protein function.

The second morning began with several sessions about basic cell biology (differentiation, lipid signaling and protein targeting respectively), followed by four afternoon lectures focusing on some of the molecular mechanisms underlying apoptotic cell death mechanisms. Michael Hengartner (Cold Spring Harbor Labs) introduced the *C. elegans* model system, which has been so instructive in revealing the genetically conserved mechanisms underlying apoptosis. More recently he has been involved in the characterization of a novel gene product called ces-2 that regulates the specific ability of serotoninergic neurosecretory motor neurons to undergo a programmed apoptosis during nematode development. *Ces-2* appears to negatively regulate the activity of a second gene, *ces-1*, which in turn suppresses expression of the cell death machinery. Expression of *ces-2* thus indirectly promotes apoptosis. A mammalian homolog of *ces-2*, a type of proline- and acid-rich transcription factor called PAR, is already known, and Hengartner predicts that these PAR proteins will be found to control apoptosis in certain types of mammalian cells.

Results from work concerning the control of cell number in the developing mammalian nervous system was the subject of a presentation by Yves Barde. His research emphasises the importance of appropriate developmental apoptosis in controlling the number of neurons finally present, and also the role of cytokines in determining which cells are to live and which to die. Some fascinating findings from a cell free-approach to the study of apoptosis were then presented by William Earnshaw (U. Edinburgh), a pioneer in this area. Extracts prepared from chicken hepatoma cells have previously been shown to induce an apoptotic-like degradation of exogenously added nuclei, and the essential role of ICE-like proteases in this activity has been demonstrated. More recently, Earnshaw and colleagues have shown that the different ICE-like proteases present in the hepatoma cell extracts have distinct substrate specificities. Thus, Mch2α preferentially cleaves nuclear lamin A, while mature CPP32/apopain favors poly(ADP-ribose) polymerase. This is important, as it has previously been unclear whether the multiple ICE-like protease activities present in apoptotic cells are redundant or each specific for a distinct subset of protein substrates.

The apoptosis session was then completed by John Savill (U. Nottingham) who provided a survey of the mechanisms employed by healthy cells to phagocytose their apoptotic neighbors. Several different recognition mechanisms are known (for example involving thrombospondin or a phosphatidyl serine receptor), with each probably being important in different conditions. Recent work identifying a phagocyte ABC-like transmembrane protein with homology to the *C. elegans ced-7* gene (already implicated in the control of phagocytosis) was discussed, as was evidence that thymocyte macrophages use the class A scavenger receptor to recognise and ingest the many apoptotic cells that naturally occur in the thymus.

The final morning of the meeting began with sessions on cell signaling and protein folding, as well as series of lectures concerning apoptosis and pathology. Arturo Zychlinsky (New York University) presented the results of investigations into the mechanisms by which the bacterium Shigella induces apoptosis in macrophages. Upon entering the bowel, Shigella crosses the epithelium and infects macrophages located in the submucosa lymphoid aggregates. Once infected, these bacteria induce apoptosis in host macrophages. Genetic analysis of Shigella mutants resulted in the identification of a bacteria-encoded gene ipa-B that is required for the induction of apoptosis. Dr Zychlinsky showed that injection of a GST-ipa-B fusion protein into peritoneal macrophages is sufficient to induce apoptosis, thus demonstrating that other bacteria-encoded proteins are not required for this response. Using GST-ipa-B fusion protein, cellular proteins were then pulled-out from the cytosolic extracts of macrophages, revealing proteins having apparent molecular masses of $\sim$45, 32, 20, and 10 kDa upon analysis by SDS–PAGE. The size of these proteins immediately suggested to Dr Zychlinsky the possibility of ipa-B association with ICE, which is known to be highly expressed in peritoneal macrophages and which is present as either a $\sim$45 kDa pro-protein zymogen or as an active protease with $\sim$20 kDa and $\sim$10 kDa subunits. Immunoblotting confirmed the presence of the p20 and p10 subunits of processed ICE. What does ipa-B do to ICE, upon binding? That remains undetermined. Dr Zychlinsky showed that the peptidyl ICE inhibitor YVAD-fmk can partially block Shigella-induced apoptosis of macrophages and also prevents release of IL-1β from these cells. Thus, ICE or other ICE-related proteases that are inhibitable with YVAD-fmk appear to be involved in the mechanism of apoptosis, raising the possibility that ipa-B somehow facilitates processing and activation of pro-ICE or enhances the pro-apoptotic functions of ICE once activated by other means. Though IL-1β is released from Shigella-infected macrophages, IL-1 Receptor Antagonist (IL-1RA) fails to prevent the induction of apoptosis indicating that IL-1β is not directly involved. Future studies employing macrophages derived from ICE knock-out mice and investigations of the effects of ipa-B on ICE processing *in vitro* and in cell extracts should yield insights into the question of the functional significance of the ipa-B interaction with processed ICE. So far, no cellular homologs of ipa-B are known.

Dr Marie-Lise Gougeon (Pasteur Institute; Paris) continued the theme of apoptosis induction by infectious agents, reviewing the role of HIV in the dysregulation of programmed cell death in the immune system. At least four mechanisms may contribute to the excessive apoptotic loss of T-cells seen in individuals with HIV infection, including: (1) direct killing of infected CD4+ helper cells by the virus; (2) loss of Th functions (such as cytokine secretion) that are required for lymphocyte survival; (3) secreted tat

protein-induced increases in Fas-ligand on T-cells; and (4) indirect triggering of cell suicide by other mechanism, affecting both CD4+ helper and CD8+ cytolytic T-cells. Dr Gougeon presented some of the evidence that HIV gp120 binding to CD4 on Th cells primes them for induction of apoptosis when subsequently stimulated through the antigen receptor by specific antigen or superantigens. However, other mechanisms also appear to render lymphocytes more vulnerable to activation-induced death, because not only CD4+ T-cells, but also CD8+ T-cells and B-cells experience higher percentages of activation-induced apoptosis compared to lymphocytes derived from normal, non-infected persons. There is a statistically significant correlation between the percent of lymphocytes that can be induced to undergo activation-induced apoptosis *in vitro* and absolute CD4 counts in HIV-infected patients, consistent with the idea that activation-induced apoptosis may represent a major mechanism for the loss of CD4+ T-cells that occurs during progression of HIV infection to AIDS. In general, higher percentages of circulating activated T-cells (CD45-RO+) also correlate with activation-induced apoptosis. Interestingly, Chimpanzees can become chronically infected with HIV and replicate the virus, yet do not develop AIDS. Analysis of Chimp lymphocytes showed that HIV-infected animals lack the accumulation of activated T-cells seen in humans and also do not become more vulnerable to activation-induced apoptosis, strengthening the connection between an 'activated' phenotype and susceptibility to antigen receptor-mediated apoptosis (which is in large part a Fas/Fas-ligand dependent process). Dr Gougeon also reported evidence of other defects in T-cells derived from HIV-positive humans, including an increased rate of spontaneous *in vitro* apoptosis in CD8+ T-cells in association with reduced levels of Bcl-2, Fas-positivity, and reduced levels of the co-stimulatory protein CD28. In this regard, CD8+ T-cells from HIV-infected persons appear to become progressively more susceptible to anti-Fas antibody induced apoptosis during disease progression, based on comparisons of non-infected persons with asymptomatic HIV-positive individuals and persons with AIDS. The central hypothesis proposed by Dr Gougeon was that chronic HIV infection perpetuates a T-cell activation mechanism that renders lymphocytes more vulnerable to apoptosis induction by several types of stimuli.

On the theme of cell death dysregulation and cancer, Tim McDonnell (MD Anderson Cancer Center; Houston) reviewed the importance of Bcl-2 in multistep carcinogenesis. Previous studies by Dr McDonnell using transgenic mice where Bcl-2 was over-expressed in B-lineage lymphocytes (Bcl-2/IgH) have demonstrated that progression to monoclonal lymphomas requires additional secondary genetic events, with activation of the *c-myc* gene representing a common one. Thus, by blocking apoptosis, Bcl-2 over-expression may create a permissive environment for development of other genetic alternations, such as activation of *c-myc* which is known to induce both proliferation and apoptosis. Myc induced apoptosis is readily blocked by Bcl-2, as well as certain survival factors. Not unexpectedly then mating Bcl-2/IgH and Myc/IgH transgenic mice has been shown to result in rapid development of fatal tumors. In contrast, Dr McDonnell showed that crossing these Bcl-2 transgenic mice with p53 knock-out mice resulted in no faster progression of tumors compared to p53 loss alone, suggesting that p53 and Bcl-2 belong to the same oncogene/tumor suppressor gene complementation group, whereas Myc falls into a different complementation group. Consistent with this idea, Myc has been shown to cooperate with p53-loss in similar types of mouse experiments. One caveat however is that p53 deficient mice tend to develop thymomas and T-cell malignancies, whereas the Bcl-2 transgene was expressed specifically in B-cells. In the keratinocytes of the epidermis, Bcl-2 is normally expressed in a gradient with higher levels in the long-lived stem cells lining the basement membrane compared to the more differentiated cells that advance towards the body surface. To explore the *in vivo* effects of Bcl-2 here, Dr McDonnell produced transgenic mice with Bcl-2 expression driven by a keratin gene promoter which resulted in high levels of Bcl-2 protein production throughout the epidermis, thus disrupting the normal gradient of Bcl-2 expression. Despite deregulated Bcl-2 expression, the cytoarchitecture of the epidermis was normal and no obvious thickening developed, though occasional hyperplastic areas could be identified in these animals. Interestingly, K6 keratin, which is normally expressed only around hair roots was expressed throughout the epidemis in these Bcl-2 transgenics, suggesting that Bcl-2 may have altered differentiation pathways or promoted the survival of cells with an abnormal differentiated phenotype. BUdR-positive cells could also be identified higher in the epithelium beyond the basal cell layer to which they are normally confinded. Importantly, Bcl-2 over-expressing keratinocytes were shown to be more resistant to UV-irradiation-induced apoptosis. Moreover, in a tumor promotion model where the phorbol ester TPA and the carcinogen DMBA were applied to the skin of Bcl-2 and normal littermate control mice, Bcl-2 over-expression was associated with faster progression of benign papillomas to malignant carcinomas but interestingly had no effect on the frequency with which papillomas formed or the latency period required to form papillomas. Thus, in the epidermis, Bcl-2 appears to contribute to tumorigenesis at a relatively late step in the multi-hit process, probably acting as a progression factor, whereas in B-cell lymphomas it represents a very early event which functions primarily as an initiator of malignant transformation.

Dr McDonnell also presented data about Bcl-2 and Bax expression and function in prostate and prostate cancer cell lines. Previous investigation from McDonnell's and other groups have documented an association between Bcl-2 and progression of prostate cancers to androgen-independence. Gene transfer-mediated over-expression of Bcl-2 in the androgen-responsive human prostate cancer line resulted in hormone-independent tumor cell growth in castrated male SCID mice. Surprisingly, not only did Bcl-2 reduce the amounts of apoptosis (TUNEL-positivity) that normally occurs in these tumors upon castration, but it also prevented the decline in cell cycling (BUdR labeling) suggesting that Bcl-2 can create a more permissive

intracellular environment for cell proliferation. This observation stands in stark contrast to recent reports that have suggested that Bcl-2 over-expression can have an anti-mitogenic effect in some types of cells, and implies that the effect of Bcl-2 on cell cycle is likely to be highly dependent on cell context. Castration was also shown by Dr McDonnell to induce increases in p53 and Bax expression in the rat ventral prostate, suggesting that androgen-deprivation in the prostate may promote apoptosis at least in part by inducing the expression of these proapoptotic proteins. It remains to be determined whether apoptosis proceeds normally in Bax knock-out mice; mixed results have been reported for p53 deficient mice, with some groups finding at least a partial inhibition of prostate involution after castration and others not.

The Bcl-2 protein resides in both the nuclear envelope and outer mitochondrial membrane. Presentations by Dr McDonnell and by John Reed (Burnham Institute; La Jolla, CA) contrasted the roles of Bcl-2 at these two subcellular compartments. Dr McDonnell presented evidence that Bcl-2 can prevent the entry of Ca^{2+} into the nuclei of the prostate cancer line LNCaP when induced to undergo apoptosis by the Ca^{2+} ATPase inhibitor thapsigargin or by chemotherapeutic drugs. Nuclei isolated from Bcl-2 over-expressing cells were also shown to take-up far less Ca^{2+} in response to ATP, compared to control nuclei. Bcl-2 was also demonstrated to prevent p53 entry into the nuclei of LNCaP cells after irradiation, and also blocked the transactivation of p53-reporter gene plasmids in this model. NF-kB translocation into the nucleus was not blocked by Bcl-2, however, implying specificity in terms of which particular proteins are excluded from the nucleus. Given that Bcl-2 does not prevent p53 translocation into the nucleus in several other types of cells, the combined data available to date suggest that the ability of Bcl-2 to prevent p53 nuclear entry is cell context dependent. In this regard, a protein (p53BP2) has been described that can bind to both Bcl-2 and p53, raising the possibility that the presence or absence of this protein or other as yet unidentified co-factors may determine whether Bcl-2 can act as a barrier to the entry of p53 or other transcription factors into the nucleus.

In contrast to these effects of Bcl-2 on the nucleus, Dr Reed emphasized the role that Bcl-2 has been shown to play in mitochondrial physiology, where it can prevent mitochondrial permeability transition (PT) (megachannel opening) and reduce the release of Ca^{2+} from mitochondria treated with PT-inducing uncouplers of oxidative phosphorylation. Dr Reed showed that when expressed in yeast, the Bax protein localizes to mitochondria and confers a lethal phenotype that can be specifically suppressed by Bcl-2. Deletion of the C-terminal membrane anchore from Bax ablates its targeting to mitochondria and abrogates its cytotoxic activity in yeast. Appending heterologous sequences onto Bax from a yeast mitochondrial outer membrane protein (Mas70p) restored targeting to mitochondria and cytotoxic function. Though the mechanism by which Bax kills yeast remains to be clarified, Dr Reed speculated that Bax may induce mitochondrial PT in yeast. Further evidence for an important role for Bcl-2 in the mitochondrial membrane was provided by studies of the interaction of Bcl-2 with the protein kinase Raf-1 in intact cells. Using a fusion Raf-1 protein tagged with the Green Fluorescent Protein (GFP), Dr Reed showed that over-expression of Bcl-2 results in targeting of Raf-1 to mitochondria. The BH4 domain of Bcl-2 was shown to be required for this effect. Moreover, when active Raf-1 was artificially targeted to the outer mitochondrial membrane by fusion with appropriate sequences from Mas70p, Raf-1 suppressed apoptosis in IL-3-dependent 32D cells almost as effectively as Bcl-2, whereas mitochondria targeted inactive Raf-1 (kinase-dead) accelerated the rate of cell death upon removal of IL-3. Active Raf-1 targeted to the plasma membrane with the K-Ras C-terminal CAAX box containing domain had no effect on apoptosis. Thus, the recruitment of Raf-1 to the surface of mitochondria by interactions with the BH4 domain of Bcl-2 appears to promote cell survival through unknown mechanism, which does not evidently involve the MAPK pathway. A kinase-dead Raf-1 (dominant negative) also abrogated the cell survival protective effect of Bcl-2 in 32D cells, suggesting that the Raf-1/Bcl-2 interaction could be critical for Bcl-2 function under some circumstances. Finally, Dr Reed presented evidence that another Bcl-2 binding protein, BAG-1, which also requires the BH4 domain for its interactions with Bcl-2, can bind to and activate Raf-1 probably through a Ras-independent protein-protein interaction mechanism.

Zvi Fuchs (Sloan Kettering; New York) presented his group's findings concerning radiation-induced apoptosis in acidic sphingomyelinase (aSMase) knock-out mice, making side-by-side comparisons with p53 deficient animals. Radiation induced cell death in some tissues is dependent on aSMase (e.g. lung), whereas in other tissues p53 is the dominant regulator (e.g. thymus), but in most tissues p53 and aSMase seem to split the duty. The findings represent perhaps the strongest evidence to date that ceramide is critically important for induction of cell death *in vivo*.

Continuing with the p53 theme, David Lane (University of Dundee; Scotland) reviewed the multiple functions of p53, but then focused on the various controversies concerning the question of how does p53 induce apoptosis? In some scenarios, the ability of p53 to transactivate the expression of target genes is critical, but not in others. Thus, the take-home message seemed to be that p53 has several potential mechanisms at its disposal for inducing apoptosis, and the most dominant one varies depending upon the type of cell and the stimulus.

One of the more intriguing things about p53's role in apoptosis induction is the exquisite tissue-specificity with which it functions. For example, in mice subjected to radiation, increases in p53 protein levels and elevated expression of p53-response genes such as Gadd45 can be seen in many tissues, whereas apoptosis occurs only in selected types of cells (e.g. hematopoietic precursors; thymocytes and pre-B-cells; epithelial stem cells in the small intestine). Dr Lane showed the results of experiments where a p53-responsive lacZ reporter gene was employed in transgenic mice to monitor radiation responses in various tissues *in utero*, during develop-

ment. During early development, essentially types of cells upregulated the p53 reporter gene after radiation but as differentiation progressed, the responses became more limited. Moreover, the ability of radiation to trigger apoptosis also became highly selective during development and did not necessarily correlate with activation of the p53 reporter gene. A variety of explanations were offered for the tissue-specificity of p53 induced apoptosis *in vivo*, including (a) quantitative and functionally important differences in the levels of p53 protein accumulation; (b) differential promoter selectivity, possibly as a result of p53 protein phosphorylation; and (c) the influence of other transacting activators and repressors that modulate the effects of p53 on its target genes.

Several other lectures during the conference also had apoptosis as the main theme. Pierre Goldstein's presentation described some recently initiated studies with the primitive eukaryotic organism *Dictyostelium discoidium* which demonstrate how the ability to undergo apoptotic cell death appears to be an ancestral eukaryotic trait. Upon starvation this unicellular protist transforms into a multicellular fungus-like structure. In some cells, this transition is accompanied by morphological changes resembling apoptosis. Goldstein predicts that a search for the gene products responsible might uncover homologies in the mechanisms of cell death shared between *Dictyostelium* and higher eukaryotes.

Taken together, the data presented at the meeting, both in the main session as well as in the numerous excellent and thought-provoking posters not discussed here, provided substantial new insights into the fundamental molecular mechanisms responsible for cell death regulation in normalcy and disease. Congratulations are due to the IUBMB, the organizing committee, Scientific Advisory Panel, and all the meeting participants for a job well done!