Apoptosis and cytotoxins

John A Hickman* and Catherine C Boyle†

*School of Biological Sciences, University of Manchester, Manchester, UK; †Department of Clinical Biochemistry, University of Edinburgh, Edinburgh, UK

Most xenobiotics ultimately become lethally cytotoxic, according to concentration. Toxins with completely disparate mechanisms of action induce apoptotic cell death. This suggests that the threshold for the onset of cell death can be determined by the relative expression levels of genes which promote or suppress apoptosis. The selectivity of a toxin may thus be determined not only by the selective imposition of perturbation or the amount of damage inflicted, but also by how readily that cell engages apoptosis. Measuring damage to cells, therefore, does not necessarily predict outcome. The threshold for apoptosis is determined not only by the static phenotype of the cell, which may confer a high or low survival potential, but also by its capability when stressed to modulate the expression of genes which control survival. The trophic environment of a cell can also influence the threshold for death. These findings have a profound impact on concepts defining toxin selectivity and on attempts to use mechanistic information to predict toxicity to organisms.

Toxins induce apoptosis in vivo

Many natural products and chemical compounds are cytotoxic. For some agents this represents a hazard and is an unwelcome effect. Such compounds define for themselves a toxicology as noxious substances. Other cytotoxins have been harnessed as the mainstream of present day antitumour therapy. Unfortunately, as drugs, they possess many of the properties of the noxious toxins. Almost without exception toxins induce death by apoptosis. The test of whether a compound induces apoptosis has naturally rested largely on observation of the classical morphological features of the apoptotic cell, discussed elsewhere in this issue. Perhaps the most critical feature of the concept of a programmed, apoptotic cell death is not that it has a conserved morphology but that it is regulated by the expression of certain genes1. This provides a mechanistic understanding of why some cells die readily and others do not. Morphological analysis of toxin-induced cell death may be confounded in vitro and in vivo by kinetic considerations: if cells die rapidly in vitro they may not be able to retain their membrane integrity.
and rapidly move to express the appearance of a necrotic cell. In vivo, similar kinetic considerations may influence whether one observes necrosis or apoptosis: if membrane integrity is lost rapidly, or if cells are not rapidly cleared by phagocytosis (see this issue), it is possible to interpret the death as being by necrosis. Under some circumstances, if the degree of injury to the cell is overwhelming, and/or the integrity of membrane gradients are compromised, necrosis without apoptosis will ensue. In the absence of secure morphological evidence, particularly in vivo, a reasonable test of whether a toxin induces apoptosis, as defined as a programmed cell death, is whether the amount and/or kinetics of cell death is genetically modulatable without changing the amount of toxin-induced damage accumulated.

In experiments performed in vivo, a wide variety of agents induce apoptosis in different organs. Irradiation of a mouse induces significant amounts of apoptosis in the thymus and in splenocytes, as well as in the epithelial crypts of the small intestine and colon. The intestinal tract has been the subject of intensive studies of the induction of apoptosis by cytotoxins in vivo. Interestingly, even when high doses of whole body radiation are administered to mice (10 Gy) apoptosis remains the mode of cell death in the crypt epithelia, with no indications of necrosis. In recent experiments of ours, we have attempted to ascertain what the relationship is between the amount of apoptosis observed acutely in the intestinal tract after radiation or a cytotoxin and ‘toxicity’ as defined more broadly by a toxicologist. Whilst the symptoms of toxicity present themselves as a continuing morbidity (with weight loss, diarrhoea) apoptosis is strictly a pathological snapshot, usually of early events which may then translate to those later pathological changes observed in histological sections. We measured morbidity, changes in animal body weight and changes in histology (crypt integrity/cellularity, crypt height) and compared these with acute changes in mitotic indices and apoptosis to establish if there was any relationship. Using the gut toxin 5-fluorouracil (5-FU), patterns of acute apoptosis indeed translated to standard aspects of toxicity. As is discussed elsewhere in this issue, toxins that induce damage to DNA, either directly or indirectly, such as 5-FU, induce apoptosis in a p53-dependent manner. The changed kinetic patterns of apoptosis observed in mice in which are p53 null (—/—) provides a critical test of the relationship between acute induction of apoptosis and the onset of pathological change and animal morbidity. In these p53 null (—/—) mice, our preliminary data strongly support the view that reduced patterns of apoptosis relate to reductions in pathological changes typical of 5-FU toxicity. Similarly, important recent experiments have sought to answer the question of whether intervention in the apoptotic process itself, by either inhibition of the proteases involved in cellular execution, or by inhibition of the initiation
of apoptosis via overexpression of \textit{bcl-2}, can influence the onset of neuronal toxicity induced by either neuronal toxins or hypoxia\textsuperscript{11,12}. Again, the evidence supports the view that toxicity does indeed reflect the engagement of death by apoptosis\textsuperscript{11,12} and that inhibition of apoptosis by expression of \textit{bcl-2} (a suppressor of apoptosis) permits the maintenance of functional aspects of neurones\textsuperscript{13}.

\textbf{Toxicity is modulated by genes which control apoptosis}

The observations that disparate toxins induce apoptosis changes a traditional framework defining the determinants of a toxin-induced cell death. It is generally considered that both the quality and quantity of damage delivered are the sole determinants of the final fate of the cell. For example, a leading text on toxicology\textsuperscript{14} states:

The magnitude of the effect is related to dose... this assumption is often a source of misunderstanding. It is really a composite of three assumptions that occur frequently: (i) there is a molecular receptor site (or sites) with which the chemical reacts to produce the response; (ii) the production of a response and the degree of response are related to the concentration of the agent at the receptor site; and (iii) the concentration at the site is, in turn, related to the dose administered.

This description of how a toxin induces a biological response assumes that the damaged or perturbed cell is a passive recipient. This is not always the case. The induction of a cellular response, made in an attempt to restore homeostasis to the whole organism, may involve a number of endpoints, only one of which is death (Fig. 1). The ‘choice’ between the different end-points and the relative ease of their engagement, even at the same level of toxin–‘receptor’ interaction, differs according to cell type.

There are essentially two phases to the ‘response’: the first is directly related to events at the so-called ‘receptor site’, for example how much inhibition of an enzyme may have occurred or the quantity and quality of DNA damage. The second phase might be considered as a homeostatic adjustment to that perturbation. At some point damaged cells ‘commit’ to a particular pathway and the molecular events which allow this ‘decision’ to be made are now becoming clear. It is possible that a reiterative programme is initiated, perhaps involving stoichiometric balances of opposing signals—for a cell cycle check point \textit{versus} cell death in proliferating cells, for example. Thus, although two different cell types might have received an equal quantity of enzyme...
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Fig. 1 Possible stages in the processing of responses to cellular damage or perturbation. After initiation, DNA damage for example, there may be a reiterative series of responses made (circular arrows). This can be influenced by epigenetic factors, such as cell-cell, cell-matrix contacts or the trophic environment. The stoichiometry or balance of these possibly opposing responses (e.g., survival versus death) ultimately leads to a point where an individual cell becomes committed to a 'response', such as a cell cycle checkpoint (cytostasis) or to apoptosis (death). Individual cells in a population may commit differently, giving a mixed population of response (some death, some cytostasis) according to the amount of damage imposed. This type of scheme clearly implies that the amount of damage imposed to initiate the response is not the only determinant of outcome.

inhibition or of DNA damage, the individual cellular response to that perturbation may be very different. Consequently, the 'selectivity' of a toxin and its dose response curve is not determined by estimations of the concentration of the agent at the receptor site alone. Instead, selectivity and the threshold dose for the expression of toxicity will also be determined by differences in, for example, genomic and signalling responses to the products of the receptor and the toxin and the perturbation that they impose.

Whilst it is clear that the cellular response induced by a toxic insult is concentration and context dependent, there is also evidence to suggest that cells from the same population may respond in different ways to a toxic insult. A recent study showed that exposure to the non-genotoxic carcinogens CPA or nafenopin stimulated DNA synthesis and that their withdrawal induced apoptosis. Interestingly, the responses were observed in different hepatocyte populations: those that remained quiescent during exposure were preferentially deleted by apoptosis following withdrawal of the toxins. This suggests that even within the hepatocyte phenotype, toxic insult can establish and maintain a
hierarchy for cell death which can respond to changes in the environment (here the withdrawal of toxic insult)\textsuperscript{15}.

**‘Sensing’ toxin-induced damage**

Clearly there are ways in which cells ‘sense’ damage. Perhaps the most well defined of these sensors are with respect to protein damage, which then initiates a heat shock response\textsuperscript{16}, and DNA damage which initiates repair\textsuperscript{17}. How the ‘sensing’ of DNA damage in turn sets in train responses such as cell cycle inhibition (a ‘checkpoint’) or cell death is dealt with elsewhere in this issue, particularly with respect to the activation of p53. In ways which are not entirely clear, individual cells may either ‘sense’ damage differently or after ‘sensing’ they may engage responses which differ qualitatively and/or quantitatively. A good example of this differential is illustrated by a study of the expression of the tumour suppressor protein p53 after whole body radiation: whereas accumulation of the protein was observed in murine thymocytes, splenocytes and osteocytes, expression was not observed in hepatocytes. Furthermore, whilst apoptosis was observed in the thymocytes and splenocytes it was not observed in the osteocytes\textsuperscript{3}. Analysis of toxin-induced DNA damage to two types of tumour cell lines, derived from testicular and bladder cancers, showed that at equidamaging concentrations of the toxin etoposide (equal numbers of DNA strand breaks), survival was very different, even when cell lines were shown to both have functional p53. p53 protein was elevated after damage in both cell types\textsuperscript{18}. The testicular cancer cell lines readily underwent apoptosis and failed to initiate a proficient G1 cell cycle checkpoint, with only a modest expression of the checkpoint protein waf-1/p21, whereas a bladder tumour cell line also with wild-type p53 showed a strong G1 checkpoint after the synthesis of waf-1/p21, the cyclin-dependent kinase inhibitor. Comparison of the clonogenic survival of these cell lines showed that at equidamaging concentrations, the bladder cell line, which underwent a checkpoint permissive for DNA repair, was less sensitive than the testicular lines\textsuperscript{18}. Quantitation of damage did not therefore predict outcome.

**Sensors and signals**

There are other ‘sensors’ of the damage induced by highly reactive toxins which, directly or indirectly, damage not only the genome but other cellular targets. Damage induced by irradiation, UV light and certain
alkylating agents appears to be 'sensed' by components of the plasma membrane and activates signalling pathways which are normally associated with ligation by cytokines and growth factors. One such pathway is the JNKs (Jun kinases, also known as the stress-activated kinases, SAPKs) which mediate the transcriptional activation of the immediate-early genes c-jun and c-fos. In murine fibroblasts it has been suggested that JNK activation induced by the alkylating agent methylmethanesulphonate may signal for survival rather than cell death whereas, in other studies, with other toxins, JNK activation by stress was associated with apoptosis. Toxin-induced synthesis of the signalling molecule ceramide, either by stimulation of ceramide synthase or activation of a sphingomyelinase, directly stimulates an apoptotic signalling cascade and again suggests that membranes can be 'sensors' of a variety of disparate toxins. In addition, a cytotoxins have been shown to activate signals for apoptosis via the APO-1/FAS pathway (see elsewhere in this issue).

Genotoxic damage of cells in vitro initiates the transcription of a variety of genes leading to growth arrest with associated stimulation of repair, as is the case with GADD45. However, in some contexts damage can also lead to the up-regulation of the receptors for growth factors, such as EGF and PDGF, and for transcription factors such as NFMB, a somewhat paradoxical response at first sight. NFMB activation has recently been shown to inhibit apoptosis when activated through the tumour necrosis factor receptor, so that in addition to a signalling cascade which may promote cell death another cascade may be activated to attempt to promote survival. In recent studies of ours, we have found that treatment of Rat-1 rat fibroblasts in vitro with a non-genotoxic toxin (dimethylformamide) increased the expression of IGF-1 receptors at concentrations of toxin below that causing a 50% fall in viability. Interestingly, the toxin-induced up-regulation of IGF-1 receptors was associated with an increase in survival, so that the cells effectively shifted their dose response curve to the right in response to the toxin (Fig. 2). Transfection and over expression of IGF-1 receptors or pretreatment with PDGF, which up-regulates IF-1 receptors, also shifted the dose response curve, making the cells less sensitive to the toxin. Insulin-like growth factor is a poor mitogen for fibroblasts, but provides a strong survival signal, increasing the threshold at which apoptosis can be engaged. Insulin-like growth factor is thought to play a key role in carcinogenesis. Because it inhibits apoptosis in certain cell types, inappropriate expression or up-regulation of expression under conditions of stress may promote the survival of cells with a damaged genome. The up-regulation of growth/survival factors in response to toxins illustrates the way in which a genomic response to toxicity can consequently modulate the dose-response curve. There are analogies
Fig. 2 Cartoon to illustrate how an adaptive response to toxic insult may change the dose-response curve upon chronic exposure to a toxin. Up-regulation of survival-factor receptors, and subsequent intracellular signalling after ligation of trophic factor, may change the threshold at which cell death is initiated (right) either by shifting the dose-response-curve to the right (broken line) or changing the threshold at which toxicity is engaged (full line). Other examples of this type of adaptive response are given in the text.

here with the heat shock response, which induces cellular thermotolerance: an otherwise lethal heat shock is ineffective once a moderate heat shock triggering the synthesis of protective heat shock proteins, effectively shifting the dose response curve to the right.16

Endogenous modulators of survival and protection against toxin-induced death

The stoichiometry of expression of members of the bcl-2 family of genes is thought to be a major determinant of the survival potential of a cell (see Brown, this issue). The balance between pro- and anti-apoptotic proteins, such as bax and bcl-2, respectively, has been suggested to set the cellular threshold for death.35 Although this suggests a 'set-point' for survival, the expression of members of the family is regulated by toxins in some cell types. For example, genotoxic agents which activate p53 have been shown to increase the synthesis of the pro-apoptotic bax in some cells, possibly because of a p53 transcriptional activation site on the bax gene promoter.16 Recent studies of ours have shown that ligation of CD40 on B-cells provides cells with a clonogenic survival advantage.
after genotoxic insult, probably because CD40 rapidly up-regulated the expression of bcl-xL, a suppressor of apoptosis. This result is interesting because it again suggests that the trophic environment of a cell may play a critical role in determining fate after toxic insult, by modulating the endogenous survival threshold. It also raises important questions about the relevance of performing and extrapolating from toxicity experiments in vitro where the experimenter defines a fixed trophic environment, which is likely to be heterogenous in vivo. We consider this to be of critical importance in the resistance of some tumours to cytotoxic therapy, where small numbers of cells may occupy a survival ‘niche’.

A wide variety of studies have shown that over-expression of bcl-2 or bcl-xL changes the kinetics of the onset of toxin induced apoptosis. Nearly all of these reports concern the toxicity of cytotoxic antitumour drugs with widely different mechanisms of action. They show that overexpression of bcl-2 or bcl-xL promotes pleotropic drug resistance. Formally, a study of ours showed that overexpression of bcl-2 in a B-cell lymphoma line had no effect on the damage imposed by 5-fluorodeoxyuridine (inhibition of thymidylate synthase, a fall in thymidine pools, the induction of breaks in nascent DNA) but very significantly delayed the onset of apoptosis. This again challenges the notion that the toxicity of an agent is defined by the amount of toxin-induced damage, as discussed above. However, many of the in vitro studies of toxins in cells overexpressing bcl-2 and bcl-xL have only measured acute changes in survival. Usually, this has been over a 48–72 h period using vital dyes as an indicator of viability. The long-term fate of the bcl-2 protected cells has rarely been established, for example by a clonogenic assay. This is critical if it is to be suggested that ‘downstream’ events from toxin-induced damage are the final arbiters of survival, translating into long-term tolerance of the toxin. In fact, there are conflicting in vitro data, showing on the one hand that expression of bcl-2 provides a true long term survival to lymphoid cells treated with radiation or cytotoxins and on the other that fibroblasts are provided with no long-term survival after treatment with aphidicolin. However, in vivo studies of neural toxicity imposed by hypoxia arising from ischemia have shown convincingly that over-expression of bcl-2 driven by neuron-specific enolase or phosphoglycerate kinase promoters protects against brain infarction by 50%. Moreover, axotomized neonatal neurons from transgenic animals overexpressing bcl-2 not only survived but retained functional electrophysiological properties. These data show that ectopic bcl-2 expression is critical to long-term neuronal survival after toxin challenge. Incidentally, bcl-2 knockout mice display normal morphology and it is considered that the homologue bcl-xL may be more critical in developed neurones, so that ectopic bcl-2 expression can supplement its suppression of apoptosis.
The effects of bcl-2 expression may be to delay the onset of apoptosis rather than to promote long-term survival per se in some cells. In a clever study, Schimke and colleagues showed that the kinetic advantage of limited but extended survival in vitro provided by bcl-2 expression may allow sufficient time for the toxin-treated cell to up-regulate mechanisms of defence against particular classes of toxins which can initiate gene amplification. Here, resistance to the toxin per se does not arise from the expression of bcl-2 alone but as a consequence of its activity to delay death for long enough to permit repair/resistance mechanisms to emerge against a background of chronic toxin challenge. One might speculate that this effect of bcl-2 expression to promote mechanisms of accumulating survival in the presence of chronic toxin exposure might be quite extensive. In that respect, although an accumulating survival threshold may allow the cell to become more and more tolerant, it may also promote tolerance to genotoxic damage, thus allowing the accumulation of mutations with potential for the emergence of malignancy.

**Perspectives**

In this review, we have suggested that toxin induced apoptosis is important because it implies that the toxin-induced death of the cell is, in part, determined by the genes modulating this process. Some evidence in favour of this hypothesis has been reviewed. Further experiments need to be performed to establish that the end-points recognised by toxicologists can be related to changes in the patterns of apoptosis brought about by manipulation of these genes. The studies of bcl-2 overexpression in the brain on the effects of hypoxia are an excellent example. Further studies of gene knockout animals will also be important. In the context of multicellular organisms the concept that death by apoptosis is part of an adaptive response, additionally suggests that adaptive responses to toxins which are initiated in the 'downstream' aspects of toxicity (Fig. 1) are important arbiters of toxicity. Responses to toxic insult which modulate the amount of damage are well documented (drug metabolising enzymes, DNA repair, etc.) but genomic responses to damage, made before, during or after limited repair, are critical in determining the selectivity of a toxin. Measuring damage is, therefore, insufficient to allow prediction of outcome. Furthermore, some of these responses, even if conserved across different phenotypes, may be engaged differently depending upon cell–cell, cell stroma and trophic environments. These paradigms, invoking context, and adaptability according to context, present problems when attempting to analyse causative mechanisms of...
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It is not surprising that, recently, attempts to model in vivo toxicological tests in vitro have foundered. Despite the excitement of a growing definition of the molecular controls of toxin-induced cell death, based on the reductionist approach of the molecular biologist, toxicology demands a strategy of holistic, integrative biology in order to be able to predict and modulate toxicity. Only in a whole organism can the effect of contextual elements be determined with any certainty.

Acknowledgements

Both authors wish to thank the Wellcome Trust for support of their work, through their Molecular and Cellular Toxicology initiative. JAH also thanks The Cancer Research Campaign and Zeneca Pharmaceuticals for long term funding.

References

5. Response of intestinal cells of differing topographical and hierarchical status to ten cytotoxic drugs and five sources of radiation Br J Cancer 1983; 47: 175–85
10. Pritchard DM. Unpublished observations


36 Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 1995; 80: 293–9


41 Yin DX, Schimke RT. Inhibition of apoptosis by overexpressing Bcl-2 enhances gene amplification by a mechanism independent of aphidocolin pretreatment. *Proc Natl Acad Sci USA* 1996, 93: 3394–8