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REVIEW

Xenobiotic-induced apoptosis: significance and potential application as a general biomarker of response

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The process of apoptosis, often coined programmed cell death, involves cell injury induced by a variety of stimuli including xenobiotics and is morphologically, biochemically, and physiologically distinct from necrosis. Apoptotic death is characterized by cellular changes such as cytoplasm shrinkage, chromatin condensation, and plasma membrane asymmetry. This form of cell suicide is appealing as a general biomarker of response in that it is expressed in multiple cell systems (e.g. immune, neuronal, hepatal, intestinal, dermal, reproductive), is conserved phylogenetically (e.g. fish, rodents, birds, sheep, amphibians, roundworms, plants, humans), is modulated by environmentally relevant levels of chemical contaminants, and indicates a state of stress of the organism. Further, apoptosis is useful as a biomarker as it serves as a molecular control point and hence may provide mechanistic information on xenobiotic stress. Studies reviewed here suggest that apoptosis is a sensitive and early indicator of acute and chronic chemical stress, loss of cellular function and structure, and organismal health. Examples are provided of the application of this methodology in studies of health of lake trout (*Salvelinus namaycush*) in the Laurentian Great Lakes.

Keywords: apoptosis, xenobiotic, biomarker.

Introduction

Biomarkers can be defined as environmental chemical-induced alterations at the molecular, biochemical, cellular, or organismal level: that depart from homeostasis; whose techniques of detection may be applied in experimental effect studies or field monitoring; and whose assays may be destructive or nondestructive to the test organism (Molven and Goksøyr 1993, Walker 1998a,b). Assays that detect apoptotic events are appropriate as biomarkers of response in that they qualitatively and quantitatively monitor the sequence of changes associated with xenobiotic-induced cell stress and toxicity in multiple tissues. The qualitative and quantitative aspects of apoptosis as a biomarker involve comparative toxicology, and estimation of level of toxic exposure to target cells, both of which complement *in vivo* understanding and risk assessment.

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Table 1. Molecular, cellular, and morphological hallmarks of apoptosis.

Stages	Events
Early	Protease activation (e.g. caspases) in the nucleus and cytoplasm; disruption of mitochondrial transmembrane potential
Intermediate	Calcium flux; actin cleavage; loss of intercellular junctions and surface extensions; membrane asymmetry (e.g. phosphatidylserine externalization); loss of cellular potassium and water; intracellular acidification
Late	Nuclear chromatin coalescence; endonuclease activation; DNA fragmentation; membrane bound apoptotic body formation

Concepts of apoptosis

Programmed cell death occurs for a multitude of reasons: during the sculpting of tissues (e.g. embryonic development and metamorphosis), as a counterbalance to cell proliferation in the immune system without evoking an inflammatory response, and as a response to chemical damage or infection. The concept of programmed cell death (i.e. gene-directed cellular self destruction), with the term apoptosis, were introduced based on their biological significance and morphology and emerged between the 1960s and 1980s (Klion and Schaffner 1966, Lockshin 1969, Kerr *et al.* 1972, Wyllie *et al.* 1980). Whereas the term 'programmed cell death' has generally been used to imply the execution of a genetic programme for cell death, the term 'apoptosis' refers to the constitutively expressed elements of signalling pathways that control the execution of cell death, which are present in essentially all cells. A complete understanding of these signalling pathways has not yet been realized; however, several of the core components of these pathways have been identified (Leist and Nicotera 1997). Although the apoptotic process is heterogeneous depending upon cell type and cycle status, stimuli, and intracellular ATP levels, there are generally recognized cellular and morphological hallmarks (table 1).

The primary effectors of apoptosis appear to be a family of protein-cleaving enzymes called caspases (cysteine-containing aspartic acid proteases), although some caspase-independent pathways have been demonstrated (Miller *et al.* 1997, Lavoie *et al.* 1998). The activation of these proteases is a central biochemical feature of apoptotic induction. Phosphatidylserine externalization in the apoptotic process occurs downstream to caspase activation, post disruption of mitochondrial transmembrane potential, concurrent with cytoplasmic acidification, and prior to DNA strand breaks (Meisenholder *et al.* 1996, van Engeland *et al.* 1998, Hirata *et al.* 1998). For instance, Fas ligand is expressed in several tissue types and cells (e.g. immune, reproductive) and induces apoptosis in target cells by binding to the Fas surface receptor, thereby activating a cascade of proteases, downstream of which occur extranuclear (e.g. mitochondrial permeability transition, cytoplasmic shrinkage, phosphatidylserine externalization, apoptotic body formation) and nuclear changes (e.g. DNA fragmentation, chromatin condensation, nuclear shrinkage and fragmentation) (Hirata *et al.* 1998). The Fas ligand and surface receptor complex is one of the best defined apoptosis pathways (Enari *et al.* 1998). A model indicating the early signalling events that regulate apoptosis is summarized in figure 1.

The induction of active cell death results in the cleavage of critical cellular substrates including cytoskeletal components gelsolin and actin, the DNA repair

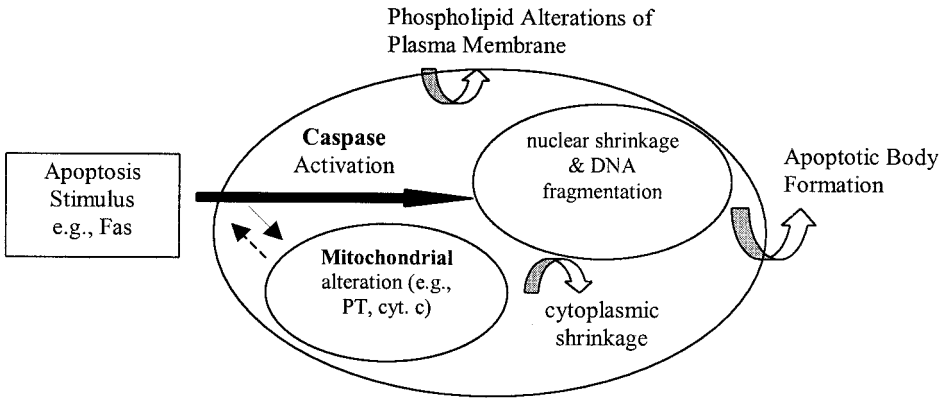

Figure 1. Schematic representation of signalling pathways in apoptosis. The central events in apoptosis induction include the expression of death genes, activation of the caspases, and perturbation of mitochondria such as permeability transition (PT) and release of cytochrome c (cyt. c).

enzyme polyADP-ribose polymerase (PARP), protein kinase C, whose role is in cell cycle progression, and lamins that aid in maintaining nuclear envelope integrity (Leist and Nicotera 1997). DNA cleavage is believed to be essential for the irreversible completion of apoptosis.

Modulators of apoptosis

The rate of apoptosis is dictated by a balance between intracellular factors that either facilitate or delay cell death. Cell proteins involved in apoptosis include the DNA repair associated p53, Bcl-2 and its homologs (Bax, Bad, Bcl-X_L Bcl-X_s), the transcription factors Myc and NFκB, and numerous others (Osborne 1996, Nagata 1997). The following table indicates some of the key molecules that modulate the signalling pathways leading to apoptosis (table 2).

However, apoptosis is not merely a physiological mode of cell death as it can be induced in a pathological manner by environmental chemical toxicants, depending upon chemical species, exposure level and duration, receptor sites, and energy supply of the cell. Induction of apoptosis in lake trout thymocytes exposed *in vitro* to the pesticide lindane is shown in figure 2 (see Sweet *et al.* 1998a).

Although the chemical concentrations eliciting xenobiotic-induced apoptosis remain relatively uncharacterized, published evidence suggests that lower levels preferentially trigger apoptotic cell death, and higher levels are routed to necrotic pathways (Kass 1997, Mangipudy *et al.* 1998). The evidence that supports this hypothesis include 'damage response coupling' (Bayly *et al.* 1997) and 'ATP

Table 2. Mechanisms of induction or resistance to apoptosis[a].

Apoptotic pathway	Regulator (gene or gene product)
Pro-death	*c-myc* (oncogene); p53 (transcriptional); Bax, Bad, Bcl-X_s; cytochrome c
Anti-death	Bcl-2 and Bcl-X_L; Ras (role in cell proliferation); survivin (role in tumours); NFκB; thiols

[a]NB: this table is not exhaustive, but rather emphasizes the well established participants.

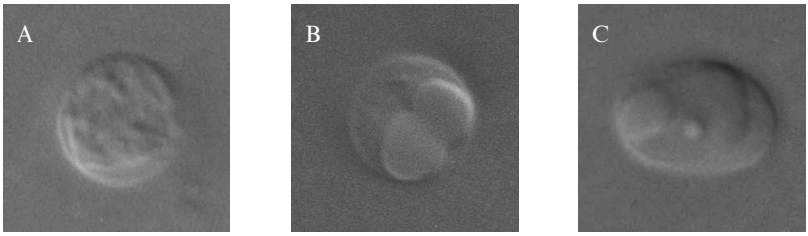

Figure 2. Micrographs of lake trout thymocytes (~7 μm diameter) showing normal condition (A) and 50 μM lindane-induced apoptosis (B and C), in an *in vitro* study. Note cellular membrane blebbing and budding, and nuclear condensation in lindane-exposed cells.

switching' (Kass *et al.* 1996, Leist *et al.* 1997a, Mangipudy *et al.* 1998). The first concept, 'damage response coupling', suggests that toxicant-induced responses are sensed by the cell and the injury is signalled in order to execute apoptosis. This implies that chemically stressed cells can actively arbitrate their own death. The second concept, 'ATP switching', posits that cellular ATP levels can act as a biochemical checkpoint governing the switch between apoptotic and necrotic patterns of cell death. This hypothesis considers cellular energy both as a metabolic parameter and as a regulator of toxicant-induced apoptotic (lower xenobiotic dose, lower toxic stress, energy intact) and necrotic (higher xenobiotic dose, higher toxic stress, energy low) cell death. More recent evidence suggests that the most notable biochemical determinants of apoptosis and necrosis include the following: dissipation of mitochondrial transmembrane potential, production of reactive oxygen species, loss of ATP, levels of calcuim and thiol antioxidants (McConkey 1998); and sensitivity of caspases to oxidative inactivation (Hampton and Orrenius 1997; Lemaire *et al.* 1998). Taken together, these lines of evidence suggest that apoptosis serves as a biomarker for certain low level exposures to xenobiotics.

Indeed, environmental chemical contaminant exposure has been shown to modulate and often accelerate physiological cell death. Hence, the ability of chemical contaminants to induce apoptosis and initiate the aforementioned cleavage events may lead to curtailed cell cycle progression, altered homeostatic and repair mechanisms, disassembled structural components and detachment, malignant transformation, and targeted death.

Evidence of xenobiotic-induced apoptosis

A multitude of toxic agents induce apoptosis (e.g. ethanol, ionizing radiation, reactive oxygen and nitrogen species, chemotherapeutic drugs). Xenobiotics such as polychlorinated dioxins, polychlorinated biphenyls, and tributyltin are reported to trigger cellular apoptosis and provide an explanatory mechanism for tissue atrophy, dysfunction, and other stress responses (Schwartzman and Cidlowski 1993, Corcoran *et al.* 1994, Lai *et al.* 1994, McConkey *et al.* 1996, Kamath *et al.* 1998). For instance, the immunotoxicity of PCBs in murine splenocytes was attributed to enhanced rates of apoptosis upon exposure (Yoo *et al.* 1997). In addition, a number of hepatotoxins (e.g. dichloroethylene, thioacetamide), and

teratogens (e.g. thalidomide) have also been shown to induce apoptosis in mammalian models (Aseffa *et al.* 1997, Mangipudy *et al.* 1998). Apoptotic cell death has served also as a marker of DNA damage and the genotoxic effects of metals such as hexavalent chromium (Blankenship *et al.* 1997) and cadmium (Hamada *et al.* 1997, Bagchi *et al.* 1998, Habeebu *et al.* 1998).

Freshly isolated human alveolar macrophages underwent apoptosis upon exposure to residual oil fly ash and urban particles, suggesting that *in vivo* this exposure could lead to pulmonary inflammation and disease (Holian *et al.* 1998). Chemical contaminants in airborne dust particles were implicated in inducing apoptotic cell death and immunomodulation in mice (Kozlowska *et al.* 1996). Carbon monoxide exposure was shown to induce oxidative stress and subsequently apoptotic cell death in murine thymocytes (Turcanu *et al.* 1998). Apoptosis has also been shown to occur in sulphur-mustard treated endothelial cells, suggesting it may be used as a marker of chemical warfare agent induced dermatoxicology (Dabrowska *et al.* 1996).

Although the majority of investigations into xenobiotic-induced apoptosis have involved mammalian models, lower vertebrate models with fish provide ecological and comparative vertebrate toxicological information (LeBlanc and Bain 1997). Trout epithelial cells exposed to micromolar concentrations of cadmium showed morphological changes, and induced expression of heat shock proteins, indicative of apoptosis (Lyons-Alcantara 1998). Studies in fish sampled from inshore contaminated sites and those from offshore reference sites revealed enhanced apoptosis in liver cells from the more contaminated sites (Moore *et al.* 1994). Also, results from studies investigating the alteration in DNA content of fish blood cells from contaminated and relatively uncontaminated waters indicate that DNA content tends to be more variable in fish populations residing in more contaminated aquatic areas (Lingenfelser *et al.* 1997). This work suggests that xenobiotic exposure and stress may induce DNA instability; and if unrepaired or improperly processed these alterations may be associated with apoptotic cell death.

Other studies investigated the ability of industrial effluents to stimulate apoptosis *in vivo* in fish (Witters *et al.* 1996, Janz *et al.* 1997). The aquatic toxicants tributyltin and mercury were demonstrated to induce heat shock protein-70 and apoptosis in a marine sponge (Batel *et al.* 1993). Heat shock protein induction (e.g. *hsp60* for heavy metal exposure) is indicative of cellular stress and apoptosis, and was used as a biomarker in salmon hepatocytes exposed to environmental pollutants (Grosvik and Goksøyr 1996, Martin *et al.* 1996). Further, modulation of the levels of the stress hormone cortisol has served as an indicator of environmental metal (Daoust *et al.* 1984, Bury *et al.* 1998) and hepatotoxicant (Murchelano and Bodammer 1990) exposures, which has been shown to induce apoptosis in a variety of fish cells.

Janz and Van Der Kraak (1997) have discussed the possibility of using apoptosis in teleost ovarian development as a marker of endocrine disruption by industrial effluent chemical constituents. Apoptosis has been proposed as a mechanism for endocrine disruption by chemical carcinogens (Telang *et al.* 1997). Increased apoptosis of gonad and skin cells in fish exposed to crude oil have been utilized as indicators of reproductive impairment (Marty *et al.* 1997). Julliard *et al.* (1996) suggest that low level exposure to waterborne contaminants can specifically induce cell death in the olfactory epithelium of trout. Sweet *et al.* (1998a) tested the hypothesis that piscine immunocytes can undergo enhanced apoptosis upon

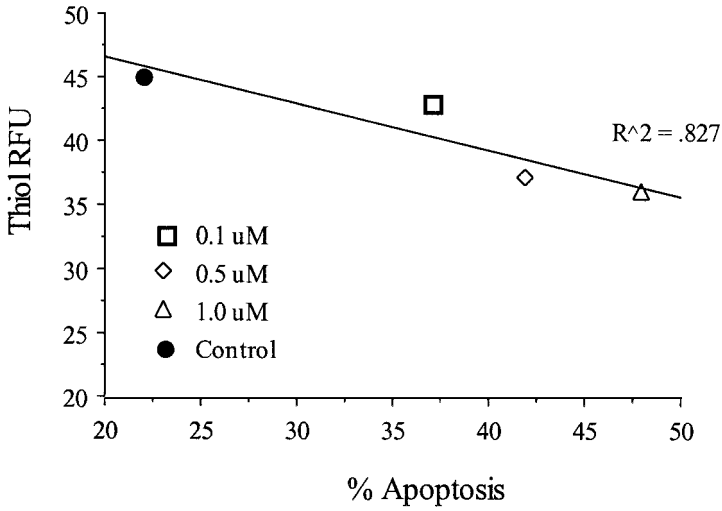

Figure 3. Scattergram split by treatment of lake trout thymocytes treated with mercuric chloride for 5 h at 17°C, in an *in vitro* study. Apoptosis detection was determined by cytofluorometric binding of fluorescein conjugated annexin-V and exclusion of propidium iodide (Apoptosis Detection Kit, R & D Systems, Inc., MN), and thiol relative fluorescence units (RFU) determined by ThioGlo (Covalent Associates Inc., MA) intensity on a microplate spectrofluorometer (Perkin-Elmer HTS 7000).

exposure to accumulated xenobiotics in blood and tissue. Results from this work demonstrated increased apoptosis in Aroclor 1254 and hexachlorocyclohexane treated thymus cells.

Other published evidence suggests that oxidative stress in vertebrate cells is associated with chemical-induced apoptotic cell death (Forrest *et al.* 1994, Jacobson 1996, Simonian and Coyle 1996, Oishi *et al.* 1997), as well as altered mitochondria and thiol reserve (Insug *et al.* 1997, Shenker *et al.* 1998). Xenobiotic-mediated oxidant responses play an important role in cellular adaptive responses (i.e. antioxidant induction) and toxic effects (i.e. lipid peroxidation, DNA oxidation), and has been proposed as a biomarker for environmental contamination (Di Giulio 1991). The oxidant-induced stress response may also serve as a model for how damage-response coupling occurs. For instance, the toxicant mercury depletes cellular glutathione and other sulphydryl compounds (figure 3, L.Sweet *et al.*, unpublished data), induces apoptosis, indirectly acts as a prooxidant (Richter 1997), and has other multi-modal cytotoxic effects. It remains unclear whether there is a causal relationship between oxidative stress, glutathione levels, gene expression, and changes in protease activity (Tan *et al.* 1998).

The pattern that emerges from the aforementioned literature is that relevant xenobiotics may be contributing to irreversible cell damage, tissue dysfunction, and stressed organisms.

Methods of apoptosis detection

This section highlights the techniques employed to assess apoptosis. This paper will not detail all techniques, but rather present some of the most validated assays with utility in detection of the general biomarker apoptosis. A more thorough description of the methods and procedures in detection of apoptosis, and technical advantages of each, can be found in a variety of other references (Cotter and Martin 1996, Spector *et al.* 1997, Harmon *et al.* 1998, Juan and Darzynkiewicz 1998, Ormerod 1998,

Pharmingen 1998). Apoptosis as a biomarker of xenobiotic cellular modulation has utility in a variety of media including blood (e.g. immunocytes), urine (e.g. polymeric DNA detection from dying cells), and tissue (e.g. liver).

Morphological assays

On a cellular level, the characteristic morphological features of apoptotic cells (i.e. chromatin condensation, loss of organelle structure, DNA fragmentation, budding and blebbing, and vacuolization) may be visualized with microscopy with or without fluorochromes. Morphological techniques by which apoptosis can be identified include light, transmission and scanning electron, fluorescence, and confocal microscopy. For instance, transmission electron microscopy is useful in revealing apoptotic bodies and vacuolization, while confocal microscopy has the advantage of working with optimum resolution in fluorescence mode. Potential artifacts associated with microscopy (e.g. fixation) are an important consideration for investigators utilizing these techniques.

Assays of membrane permeability

Fluorescence light microscopy has utilized differential uptake of fluorescent dyes that stain DNA, or cytoplasmic binding dyes, including the following: acridine orange, ethidium bromide, Hoechst 33342/33258, 7-amino-actinomycin, and propidium iodide (Duke and Cohen 1992, Negri *et al.* 1997, Shenker *et al.* 1998). These methods rely on the differences in plasma membrane permeability of viable, apoptotic, and necrotic cells. For example, propidium iodide and Hoechst dyes are excluded from viable and early apoptotic cells, and thus their staining is used as an indicator of loss of membrane integrity and necrosis. Viable cells are able to exclude or pump both dyes out, whereas apoptotic cells exclude propidium iodide but not Hoechst dyes.

Phosphatidylserine translocation

In plasma membranes, aminophospholipids (e.g. phosphatidylserine and phosphatidylethanolamine) that normally reside in the inner leaflets, are translocated during apoptosis to the outer leaflets of the intact plasma membrane (Emoto *et al.* 1997, Fadok *et al.* 1998). In particular, annexin-V, a calcium-dependent phospholipid binding protein, staining of the outer membrane has found utility both *in vitro* and *in vivo* as a marker of apoptosis due to its selective affinity for phosphatidylserine (Martin *et al.* 1995, van Engeland *et al.* 1996, 1998, Gerke and Moss 1997, van den Eijnde *et al.* 1998).

This apoptosis assay may be especially useful as a biomarker of xenobiotic stress in that phosphatidylserine is a common cellular component of animal, plant, and bacterial cells (Prasad 1996). For instance, with biotin-labeled annexin-V, investigators were able to visualize apoptosis during embryogenesis in mice (van den Eijnde *et al.* 1997). Although the majority of investigations have utilized annexin-V in mammalian models, we believe annexin-V is one of the most promising

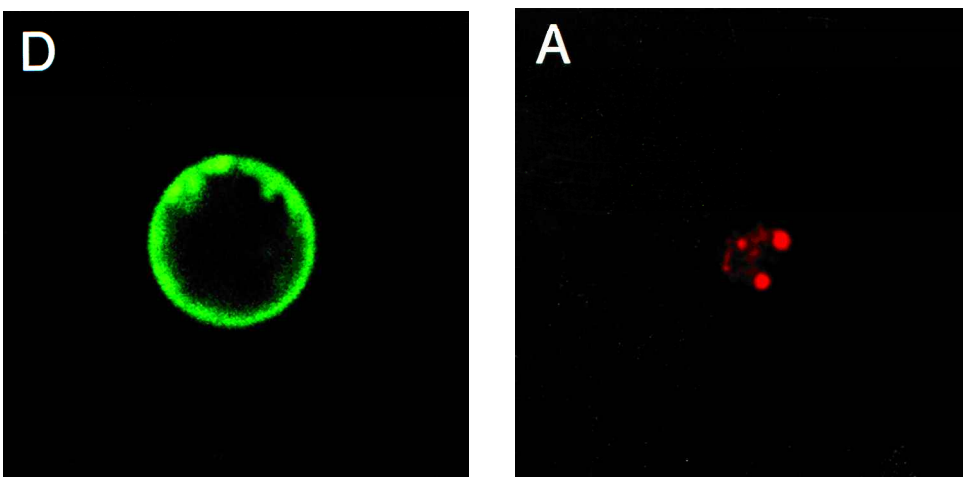

Figure 4. Fluorescent images of lake trout thymocytes undergoing apoptotic cell death, as detected by phosphatidylserine externalization with annexin-V (R & D Systems Inc) (left) and caspase-3 expression (PhiPhiLux, OncoImmunin, Inc.) (right) (Sweet *et al.*, unpublished data). Viable cells do not stain and are not shown.

fluorescent probes for apoptosis detection in lower vertebrates (figure 4, L. Sweet *et al.*, unpublished data). The ability to observe contaminant effects on key cellular and molecular events in organismal development may prove toxicologically important, especially as a biomarker for teratogenesis.

Caspase assays

Fluorogenic probes for one of the major executioners of apoptosis, caspase-3, are increasingly employed as early markers of cell death (e.g. Pharmingen, CA; OncoImmunin Inc., MD). Caspase-3 resides as an inactive proenzyme in the cytosol of non-apoptotic cells, yet is activated during early apoptosis and acts as an effector protease that cleaves cellular substrates (e.g. PARP in the nucleus) and precipitates morphological alterations characteristic to apoptosis (Hirata *et al.* 1998). Activated caspases have been detected in both the nucleus and cytoplasm of apoptotic cells (Duriez and Shah 1997). These fluorogenic substrates for caspases emit increased fluorescence when proteolysed at specific subunits during early and mid apoptosis, yet decline during the later stages. Fluorogenic caspase substrates are also very promising fluorescent probes for apoptosis detection in environmental and human toxicology models (figure 4, L. Sweet *et al.*, unpublished data), although one drawback to measuring apoptosis using fluorescent stains is the variability of active dye uptake in different cell types. Overall, the application of fluorescent probes to detect membrane and protein alterations associated with apoptosis allows the observation of early levels and effects associated with xenobiotic exposure. The detection of early events and responses associated with chemical-induced apoptosis is important in determining alterations in cellular homeostasis and stress at lower concentrations, as well as mechanisms of effect.

Detection of PARP cleavage by caspases has been utilized as a sensitive marker for identification of apoptotic cell death, since it is one of the earliest proteins targeted for specific cleavage during the apoptotic process (Duriez and Shah 1997). PARP is involved in DNA repair and functions to limit chromosomal aberrations, and is activated by single and double strand breaks. However the significance and specificity of PARP cleavage and caspase activation for apoptosis, as compared with necrosis or other cellular events, in some experimental systems remains unclear (Leist *et al.* 1997b, Sallmann *et al.* 1997, Wilhelm *et al.* 1998).

Assays of DNA damage

Those studies that include examination of histological sections may employ the TUNEL (i.e. terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) (Negoescu *et al.* 1997, Labat-Moleur *et al.* 1998) technique that measures DNA strand breaks. During apoptosis, the higher order chromatin structure is degraded by endonuclease action into smaller DNA fragments (i.e. double and single stranded DNA) that are detected by a reaction with exogenous 'terminal deoxynucleotidyl transferase' (TdT) (Pharmingen 1998). The end labelling documents nicking of DNA. The potential limitation to the identification of apoptosis in tissue sections is that levels of active cell death do not always reflect the extent of actual cell death, due to the short time period (i.e. hours) over which apoptosis occurs and the rapid rate of clearance of apoptotic cells. Further, end labelling techniques should not be considered a specific marker of apoptosis because they are not always able to discriminate between apoptotic, necrotic, and autolytic cell death, especially *in vivo* (Grasl-Kraupp *et al.* 1995).

In situ end labelling of DNA has also been applied to detect apoptotic cell death simultaneously with cell proliferation in tissue sections (Mundle *et al.* 1994). To clarify, when DNA polymerase is used the technique is called '*in situ* end labelling', yet when TdT is used the usual term is TUNEL (Renvoize *et al.* 1998). This method uses DNA polymerases to catalyse a reaction that labels ends of DNA strand breaks. Hence, the intensity of cell labelling is expected to demonstrate the extent of DNA damage; however, it may not be the best to clearly differentiate between DNA fragmentation due to apoptosis versus necrosis. Further, the presence of DNA strand breaks is not unique to apoptotic cells (Darzynkiewicz *et al.* 1992, Eastman and Barry 1992). Nonetheless, the advantages to end labelling assays of apoptosis include direct detection of lesions at the molecular level, potential identification of cell-cycle position, and applications for clinical materials (e.g. tumours).

Enzyme-linked immunosorbent assays (ELISAs) have also been employed to measure and quantitate nucleosomal particles (e.g. DNA fragments or histone complexes) characteristic of apoptosis in cytoplasmic fractions or cell supernatants (Iyer *et al.* 1996, Kirichenko *et al.* 1996, Jones *et al.* 1997, Yan *et al.* 1997).

On the molecular level, gel electrophoresis reveals DNA pieces (i.e. oligonucleosome-length) characteristic of apoptosis. During apoptotic cell death, endonuclease activation results in the formation of high molecular weight DNA fragments and internucleosomal cleavage, sufficient to allow chromatin to collapse (Walker and Sikorska 1997). The smaller DNA fragments are separated by size using agarose gel electrophoresis in the presence of a constant electrical field, leading to ladder formation. The higher molecular weight fragments associated with early DNA cleavage may be separated by pulse field electrophoresis (Spector *et al.* 1997). The toxicant-induced (e.g. mercury, DDT) laddering pattern has been extensively described in a variety of mammalian cell types (Rossi *et al.* 1997, Renvoize *et al.* 1998, Tebourbi *et al.* 1998), however, not in piscine immunocytes (figure 5). It is also important to note that

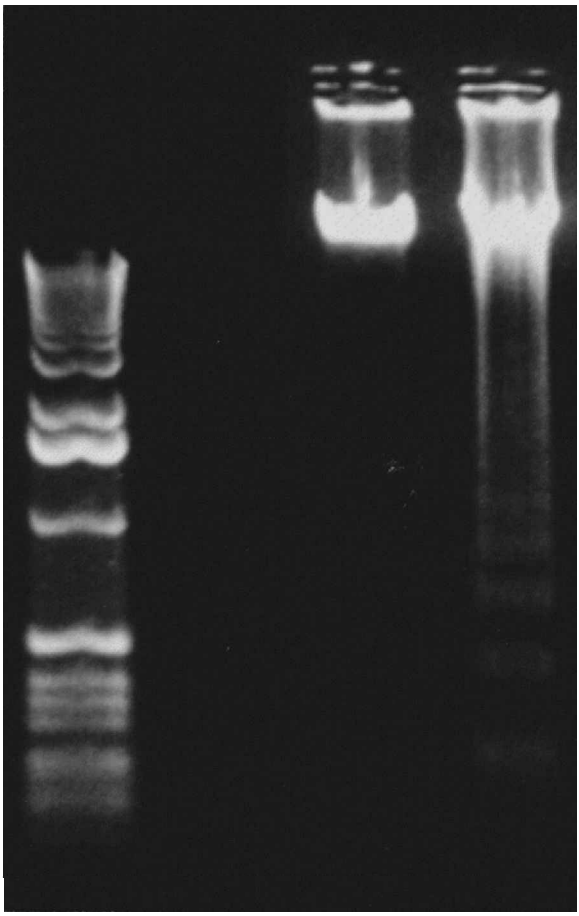

Figure 5. Observation of DNA ladder bands by agarose gel electrophoresis. Lake trout thymocytes were treated with the pesticide lindane for 7 h at 5°C. Lane 1, DNA ladder standard as size marker; lane 2, solvent (0.1% dmso) control lane; lane 3, treatment of 100 μM lindane, *in vitro* (Sweet *et al*. unpublished data).

not all cell systems undergoing apoptosis produce endonucleocytic cleavage and DNA laddering (Leist and Nicotera 1997).

Mitochondrial transmembrane potential

The mitochondrial transmembrane potential disruption feature of apoptosis in different cell types has been assayed via fluorochromes such as rhodamine 123 (Lizard *et al*. 1995, Hirsch *et al*. 1998). Disruption of the inner mitochondrial transmembrane potential is reported to occur prior to other morphological features of apoptosis (e.g. plasma membrane perturbations, chromatin condensation) (Marchetti *et al*. 1996). This type of assay may be especially appropriate as a marker for DNA damaging chemicals (e.g. PAHs, benzo(*a*)pyrene) that preferentially target the mitochondrial genome (Sweet *et al*. 1998b).

Flow cytometric assays

Flow cytometric assays for the detection of apoptosis include the following: DNA content analysis and *in situ* labelling of DNA fragments with tracer dUTP (Hanon *et al*. 1996); merocyanin 540, a fluorescent lipophilic probe that binds preferentially to apoptotic cell membranes with loosely packed lipids (Frey 1995, 1997); DAPI (diamidino-2-phenylindole) (Hotz *et al*. 1994); ethidium bromide (Ferlini *et al*. 1996); as well as the aforementioned dyes propidium iodide (Darzynkiewicz *et al*. 1992),

annexin-V, Hoechst (Dive *et al.* 1992), and caspase-3 substrates. For instance, Sweet *et al.* (1997) employed cytofluorometric techniques utilizing differential binding of propidium iodide and annexin-V, as confirmed with acridine orange/ethidium bromide fluorescence, to assess levels of apoptotic cell death in untreated and organochlorine contaminant-treated fish thymocytes. The use of annexin-V and propidium iodide fluorochromes in the flow cytometric detection of apoptosis and necrosis has been documented in numerous other cell systems (Koopman *et al.* 1994, van Engeland *et al.* 1996, 1998, Gerke and Moss 1997).

Apoptosis is detected widely in cells exposed to low levels of xenobiotics and may prove to be an effective cellular marker of xenobiotic stress, in addition to providing mechanistic insight into chemical action and bioavailability (Cantrell *et al.* 1998). However, because not all of the aforementioned measurable components of apoptosis are expressed in each cell type, it will be important to utilize assays of several different events simultaneously to assure detection of the process. For example, we suggest that multiparameter flow cytometry using three fluorescent probes (e.g. annexin-V, caspase-3 substrates, and propidium iodide) for apoptotic events, as well as forward and side light-scattering properties characteristic of cell morphology, will provide sensitivity and confidence in apoptosis detection. The utility of apoptosis as a biological marker will continue to the degree that the aforementioned assays give evidence for xenobiotic stress.

Use and interpretation

Of particular importance to the evaluation of a biomarker in environmental toxicology are specificity and sensitivity (Decaprio 1997). Chemical alteration of apoptosis has been characterized as a non-specific response biomarker, inasmuch as it can be associated with a multitude of chemical stressors (Andersen and Barton 1998). It can be argued that nonspecific biochemical markers, such as apoptosis, offer unique advantages in assessing the effects of complex chemical mixtures and metabolites on organismal health (Mineau 1998) and in environmental monitoring, (Peakall 1992) including: more sensitive, less variable, highly conserved between phyla, and more adaptable to various environmental compartments (e.g. air, water, sediment pollution).

In general, apoptosis has mostly served as a marker in cells, tissues, organs, and blood; however, there is emerging evidence of measuring the products of apoptosis in urine (Umansky 1997). With regard to environmental toxicology issues, apoptosis as a biomarker of cytotoxicity may be used in animal models, such as fish, in order to evaluate the potential for human exposure. Apoptosis may also prove useful as part of a suite of biomarkers of different categories and sensitivities or when a site is subject to uncharacterized chemical mixtures. In instances when a series of biomarkers is employed to assess chemical risk, it is important to have a species-specific database on the homeostatic or normal range for each cellular marker (Fossi 1998).

It is also important to consider the validity of apoptosis as a biomarker of response. It is likely to be most valid for the purposes of providing insight into early biochemical events and effects of chemical exposure to reproductive, genetic, and immunological toxicants. For instance, cells exposed to chemical agents may induce genetic instability, as manifested by altered rates of apoptosis, altered gene expression, inactivated tumour suppressor genes, and increased probability of neoplastic transformation. However, it is important to recognize that certain confounding or modifying factors, operating at the time of assessment, may exist that modulate apoptosis (i.e. organismal age and diet, reproductive, and seasonal variation). For example, confinement-stress, as well as immunological response events, have been shown to induce apoptosis in piscine cells (Greenlee *et al.* 1991, Alford *et al.* 1994, Weyts *et al.* 1997). Also, trace elements (e.g. zinc) and metals (e.g. cadmium, chromium) were responsible for both induction and inhibition of

the apoptotic process as a function of exposure duration and concentration (Fraker and Telford 1997, Shimada *et al.* 1998b).

Other important attributes in considering apoptosis as a biomarker include the stability and reversibility of the response (Walker 1998b). Apoptotic events are typically short-lived, occurring on the order of hours, and may only affect limited numbers of cells. Hence, the selection criteria for apoptosis assays, and the interpretation of the results, must consider that this process involves a continuum of early (e.g. protease activation) and late (e.g. DNA fragmentation) characteristic events that are asynchronous and of differing duration. Also, some events such as calcium elevation are not invariably associated with the apoptotic process in all systems. Furthermore, when considering the stress response of a cell to xenobiotic stimulation, it is important to understand the molecular controls and events that couple cell stress to the irreversible commitment to apoptosis. It remains to be discovered what exclusive event characteristic of apoptosis has to be manifest before a cell is fully committed to suicide, although extensive degradation of DNA is considered to be an irreversible event (Muller *et al.* 1994).

Apoptosis induction by chemicals, and alteration in homeostasis, if subtle, may not in every instance result in a relevant clinical effect; however, it may alter the probability of adverse health effects such as neoplasia. Tissues and cells have a natural rate of cellular proliferation that is balanced by a rate of cell death. Essential to this balance is apoptotic cell death and cell division. Hence when apoptosis is reduced or stimulated, or when cellular proliferation is excessive (e.g. damaged DNA deregulated cell cycle), pathological conditions may result.

Chemicals that promote apoptosis may be expected to inhibit tumour development, if one accepts the hypothesis that the apoptotic process preferentially eliminates preneoplastic cells (James *et al.* 1998). In this regard, apoptosis ensures that mutated, non-functional, misplaced, and harmful cells do not proliferate. Conversely, chemicals that induce a resistance to apoptosis may tend to accelerate genetically damaged cells (Bayly *et al.* 1997, James *et al.* 1998) and act in the mechanism of tumour promotion (Wright *et al.* 1994). It has been argued that apoptosis is a biomarker of cytotoxic events, and that diminished apoptosis may be a predictor of disease processes (Trosko 1995).

In turn, xenobiotic-induced apoptosis above homeostatic levels may have deleterious effects on an organism including: loss of tissue function, tissue atrophy, and altered functionality. For example, chemical-induced oxidant production may play a key role in mediating tumour promotion via DNA damage or inhibiting apoptosis (Zelikoff *et al.* 1996, Evans *et al.* 1997).

In the context of a biomarker, there are potential therapeutic and toxicologic roles in characterizing the inhibition of apoptosis. For example, it has been shown that pretreatment of cells with such compounds as protease inhibitors, glutathione extrusion inhibitors (Ghibelli *et al.* 1998), *N*-acetyl-L-cysteine (Dabrowska *et al.* 1996), and antioxidant vitamins (Blankenship *et al.* 1997) can rescue cells from xenobiotic-induced stress and apoptosis. Also, inhibition of the normal apoptotic process during animal development (e.g. inhibiting tissue sculpting or deletion) may alter the resulting phenotype and functioning of the organism (Jacobson *et al.* 1997). The characterization of chemicals that inhibit apoptosis during normal developmental stages may prove useful in organismal protection.

Nonetheless, whether xenobiotics induce or inhibit apoptosis it is important that assays of xenobiotic effects and responses in target tissues be obtained for

physiologically and environmentally relevant exposure concentrations and duration. In addition, it is important to identify target cells or tissues, because apoptotic thresholds are known to differ between cell types and *in vitro* or *in vivo* studies. It is also important to note the various physico-chemical modulators of xenobiotic bioavailability including temperature, pH, protein binding (e.g., albumin), metabolic activation or inhibition of potency, receptor sites (e.g. aryl hydrocarbon), and concurrent exposures (e.g. bacterial endotoxin).

Finally, there is the criterion of practicality in considering how useful apoptosis is as a biomarker. Indeed, there are other biomarkers of cellular stress responses (e.g. stress protein induction) that are appropriate for detecting the modulatory effects of chemical exposure. However, the measurement of apoptosis may be easier, quicker, and cheaper than organismic level stress indices, insofar as it is readily quantifiable and modulated by a variety of compounds. For example, flow cytometric detection of apoptosis is highly cost effective and sensitive in initial screening for xenobiotic hazard, and in the evaluation of chemical mixtures. Morphological and fluorescence-activated cellular parameters detected by flow cytometry may be obtained at the single-cell level and may be used to assess cytotoxic action of xenobiotics. *In vitro* apoptosis assays provide dose–response data and replicates with reduced numbers of test organisms. Furthermore, apoptosis assays of xenobiotic effects using blood cells do not require animal euthanasia, and may also provide early evidence for linking a toxicant to diminished immune and physiological function, and survival.

Thus, characterizing xenobiotic-induced or inhibited apoptosis provides the opportunity to detect subtle, and reversible, changes in the normal activity of cells, and aid in explaining organismal stress and time from exposure to toxicity. Its utility as a biomarker of response remains an important yet relatively uncharacterized component of human and environmental toxicology.

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