1 Background to Molecular and Cellular Toxicology

1.1 What do we mean by molecular and cellular toxicology?

The Society of Toxicology\(^1\) defines Toxicology as ‘the study of the adverse effects of chemical, physical or biological agents on people, animals and the environment’ and toxicologists as ‘scientists trained to investigate, interpret and communicate the nature of those effects’. In the disciplines of molecular and cellular toxicology, toxicologists make use of the many new techniques which are becoming available in the molecular life sciences to understand the underlying mechanisms by which these agents damage cells, tissues and entire organisms. The main aims of toxicity testing, whether during pre-clinical drug development, in the course of safety assessment of cosmetic ingredients and consumer products or while evaluating the potential consequences of exposure to industrial and environmental chemicals, are to construct a toxicological profile of the chemical and to identify a threshold dose (if any).

The topic of this book is how molecular and cellular techniques can be used to study the toxicity of exogenous chemicals, referred to in the trade as xenobiotics. The primary target organs for xenobiotic toxicity are usually those which are exposed to xenobiotics and their metabolites because of the roles they play as portals of entry, sites of metabolism and/or organs of excretion. The molecular and cellular consequences of exposure are summarised in Figure 1.1.

Despite the many scientific advances made in the life sciences over the last couple of decades, which include spectacular advances in the fields of molecular biology, biotechnology and bioinformatics, the basic concepts of regulatory

\(^1\) http://www.toxicology.org/
Figure 1.1  Consequences of exposure to a toxic insult (source: Dr Cliff Elcombe, CXR Biosciences Ltd. Reproduced with permission of Dr Cliff Elcombe)

Toxicology have hardly changed over the same period. For example, although the classical LD$_{50}$ (dose giving 50% lethality) test for oral toxicity and the Draize tests for eye or skin irritancy are widely considered to cause unacceptable suffering to laboratory animals, they are still widely used and the development of non-animal alternatives has been slow, to say the least. However, the implementation of both the 7th Amendment to the European Union (EU) Cosmetic Ingredient Directive and the Registration, Evaluation, Authorisation and restriction of Chemicals (REACH) regulations during the early 2000s has provided a strong stimulus for further developments.

There is an acute need for this to be reflected in a paradigm shift in the field of toxicology to take advantage of the new opportunities offered by modern developments in the life sciences, including new in vitro models, alternative whole organism (non-mammalian) models and the exploitation of ‘omics methods, high throughput screening (HTS) technologies and molecular imaging technologies.$^2$

1.2 Tissues and their maintenance

Tissues are made up of cells of various types plus the extracellular space which surrounds them. The extracellular space is filled with extracellular matrix, the proportion and structure of which depends on the tissue type. Epithelia, for example, consist mainly of sheets of epithelial cells with very little extracellular matrix whereas connective tissue contains few cells and a lot of extracellular matrix.

$^2$ Reviewed from a 3Rs perspective by Van Vliet (2011).
The proteins of the extracellular matrix are linked to cytoskeletal proteins through the plasma membrane and are able to influence cell development, migration, proliferation, shape and function. All tissues have certain basic requirements including mechanical strength, access to nutrients and removal of waste, connection to the nervous system, removal of debris and protection against infection. Specialised (differentiated) cells provide these and other functions.

During the process of embryonic development, the fertilised ovum proliferates and the resulting daughter cells differentiate to form three germ layers:

- The endoderm gives rise to the epithelia of the gut and its associated organs (lung, liver and pancreas).
- The ectoderm gives rise to the outer surface epithelia (epidermis, buccal epithelium and outer cervical epithelium) and neuroectodermal tissues.
- The mesoderm gives rise to the embryonic mesenchyme and thence to the connective tissue and supporting tissues including bone, cartilage, muscle, vascular tissue and haematopoietic system.

The products of this process are the various differentiated tissues of the body. Even when removed from their normal environment, differentiated cells retain their specialised characteristics; for example, glandular cells still secrete mucin, fibroblasts still make extracellular matrix and macrophages still carry out phagocytosis. Differentiated cells can still respond to the environment and some cell types can adapt quite dramatically: for example, fibroblasts can convert into cartilage cells, liver cells can express different enzymes and mammary cells can switch milk proteins on or off. Some cells, however, are terminally differentiated, having become so specialised that they have lost the ability to divide.

1.2.1 Stem cells

Terminally differentiated tissues are maintained by stem cells, precursors which are not themselves differentiated but are committed to produce a particular type of terminally differentiated cell. A stem cell can be defined as ‘a cell which can proliferate either symmetrically or asymmetrically in response to an appropriate external signal’; in other words, under one set of circumstances it will divide to produce two stem cells and under other circumstances it will divide to generate one stem cell and one progenitor cell which can give rise to a differentiated cell lineage. The signals to which stem cells can respond include growth factors, levels of oxygen and antioxidants and growth substrates (e.g. feeder layers, extracellular matrix).

Stem cells can divide without limit and on division the daughter cells have a choice either to remain as a stem cell or embark on terminal differentiation. The final differentiated state of the majority of stem cells is pre-determined (e.g. muscle satellite cell, spermatogonium), although some stem cells are pluripotent (can differentiate into many cell types). Organ-specific stem cells have two defining properties, the ability to self-renew and the potential to differentiate into

\footnote{For a review, see Kang and Trosko (2011).}
organ-specific cell types. The various types of stem cells have different potencies (i.e. abilities to generate different classes of progeny):

- **A totipotent stem cell** can generate an entire new organism. The definitive totipotent stem cell is the fertilised egg; following implantation, the totipotent fertilised egg becomes committed to form an embryonic pluripotent stem cell.
- **A pluripotent stem cell** can give rise to any other type of cell but not to an entire new organism. Pluripotent cells give rise to committed progenitor cells which can only mature into one type of cell (i.e. each one is unipotent) and this maturation process involves differentiation, which is controlled by growth factors and the surrounding environment.
- **Multi-potent stem cells** can produce a limited number of cell types and are committed to become part of a particular organ. They give rise to lineages of progenitor cells.
- **Progenitor cells** are committed to a particular lineage (e.g. the haematopoietic system) and give rise to terminally differentiated cells, which do not divide further.

1.3 Tissue damage

Living tissues are constantly exposed to environmental changes to which they respond with modifications of metabolism and growth.

- **Primary (direct) injury** involves an interaction between the chemicals and the components of the cell. Toxic cell injury requires high concentrations of toxic compounds and, in some cases, metabolic activation. It may involve membrane damage (e.g. lipid peroxidation induced by carbon tetrachloride in the liver).
- **Secondary (indirect) injury** involves changes in the cellular environment (e.g. oxygen tension, nutrient supply, hormone levels).

1.3.1 Consequences of tissue injury

The primary responses following tissue damage due to an injury are cell death and acute inflammation. The pathological stimuli responsible may be endogenous (e.g. hormones, autoimmunity, anoxia) or exogenous (e.g. radiation, drugs/chemicals, infections, mechanical trauma, heat or nutritional imbalances). The pathological changes observed following a toxic insult give an indication of the vulnerability of certain organ systems, and their nature and severity may give an insight into the toxicity of the compound. However, pathological changes, as revealed by microscopy, do not necessarily provide information about the sub-cellular and molecular processes involved.

The long-term consequences of injury depend on the ability of the tissue to regenerate and on whether the damaging agent persists. They include regeneration, healing by repair and chronic inflammation. The final outcome
may be restoration (complete healing with full functionality) or fibrosis/scar formation. Following an episode of tissue damage, the following may occur:

- Full regeneration: an optimal response, but only occurs in the liver in higher organisms.
- Removal/repair of necrotic tissue leading to restitution or fibrosis (scar formation).
- Alterations to necrotic tissue (e.g. calcification).

**Acute and chronic inflammation** Acute inflammation is the commonest early response to tissue damage and destruction. The classical clinical indications of acute inflammation are *rubor* (redness), *calor* (heat), *dolor* (pain), *tumor* (swelling) and loss of function. If the injury is not too severe and the damaging agent has been removed, this will rapidly subside and the tissue will start to heal itself, either by restoration or by scar formation. Restoration occurs when there is minimal damage to the tissue architecture and comprises restoration of the normal structure and function of the tissue without forming a scar. It requires the supporting stroma to be intact and the damaged cells must be able to regenerate as in, for example, liver regeneration following acute liver damage. Regeneration depends on the ability of cells to divide, which means that it usually involves stem cells such as those in the gastrointestinal (GI) tract, urinary tract, skin, lymphoid tissue and the haemopoietic system. Cells such as hepatocytes which can come out of quiescence are also able to regenerate, but terminally differentiated cells such as cardiac myocytes and neurons cannot regenerate.

If, however, the damaging agent persists, the tissue will become chronically inflamed. In chronic inflammation, the processes of necrosis, organisation and repair all occur simultaneously. Chronic inflammation occurs in situations such as long-term alcohol abuse, where the ability of the liver to restore itself is overwhelmed by continual exposure to alcohol and progresses through chronic inflammation to fibrosis, cirrhosis and ultimately liver failure. The macrophage, which arises as a result of monocyte differentiation in response to interferon γ, is the main effector cell in chronic inflammation. Activated macrophages are also called epithelioid cells and can fuse to form multi-nucleate histiocyte giant cells, which have both phagocytic and secretory roles in chronic inflammation.

**Restoration** Restoration occurs when there has been minimal damage to the tissue architecture and cells can re-grow. The end result of this process is restoration of normal tissue structure and function without scarring. In order for this to occur the acute inflammatory response must be terminated appropriately. The support stroma must remain intact and the damaged cells must be able to regenerate. This process is most clearly seen in response to acute liver damage (e.g. two-thirds partial hepatectomy), when the full mass and function of the liver is restored within a few days. The ability of a tissue to regenerate depends on the ability of its cells

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4 For a beautifully illustrated review of cellular processes involved in healing, see Abreu-Blanco et al. (2012).
to divide, so this process is dependent upon the presence of stem cells (e.g. in the 
GI tract, urinary tract, skin, lymphoid tissue and haematopoietic system) or cells 
which can come out of quiescence (e.g. liver, kidney). Terminally differentiated 
cells (e.g. cardiac myocytes, neurones) cannot regenerate, so tissues made up of 
these cell types are particularly vulnerable to injury.

**Scar formation** If a tissue is too severely damaged for restoration to be possible, 
healing can occur by means of organisation and repair, leading to the formation 
of a scar. Macrophages phagocytose dead tissue and inflammatory exudate and 
existing capillaries bud into the damaged area leading to the formation of vascu-
lar granulation tissue (organisation). Proliferation of fibroblasts within this tissue 
causes it to develop into fibrovascular granulation tissue, which gradually fills with 
collagen secreted by the fibroblasts to form a collagenous scar (repair). This pro-
cess can be impaired by inadequate nutrition, ischaemia, infection, disease (e.g. 
diabetes) and the presence of foreign material.

### 1.3.2 Reversible changes in cells and tissues

Each tissue is an intricate mixture of different cell types and this organisation is 
maintained even though individual cells are constantly dying and being replaced. 
An appropriate balance between cell growth and cell death is therefore essential 
for the maintenance of homeostasis. An excess of cell growth over cell death leads 
to disorders of cell accumulation (e.g. cancer) and insufficient cell growth comb-
ined with excessive cell death leads to disorders of cell loss (toxicity/atrophy). 
Changes in cellular growth patterns may involve changes in the size of cells or in 
their number. Alterations in the differentiation state of cells (dedifferentiation or 
metaplasia) may also occur.

Xenobiotics may induce the following reversible changes in the pattern of cel-
lular growth:

- **Hypertrophy** is an increase in the size of the individual cells within a tissue. 
  It may be a physiological adaptation (e.g. hypertrophy of skeletal and cardiac 
  muscle in athletes), an adaptive response to stress (e.g. hepatocyte hypertro-
phy due to enzyme induction and proliferation of the smooth endoplasmic 
  reticulum) or a pathological effect (e.g. heart muscle in hypertension). The 
  opposite of hypertrophy (cell shrinkage) is called *atrophy*.

- **Hyperplasia** is an increase in cell number leading to an increase in the vol-
  ume of an organ. By definition, it can only occur in cell types which have 
  retained the ability to divide, and is therefore not seen in terminally differ-
  entiated tissues (brain, skeletal muscle). Hyperplasia may be a physiological 
  process (e.g. in the lactating breast) or a repair process (e.g. wound healing). 
  It can also have pathological consequences because it is necessary for fixing 
  (i.e. making irreversible) DNA damage, which increases the risk of neopla-
  sia. Hyperplasia can occur in response to toxic stimuli in epithelial cells (e.g. 
  renal tubule, pulmonary alveolar epithelium, intestinal epithelium and epi-
  dermis), blood cells, thyroid cells and bone tissue. The liver, despite having a 
  very low rate of cell proliferation under normal circumstances, can come out
of quiescence and respond with a spectacular proliferative response following chemical or physical damage. The opposite of hyperplasia (reduced cell proliferation) is called hypoplasia.

- **Metaplasia** refers to the reversible replacement of one type of adult cell by a simpler mature cell type due to abnormal differentiation of a stem cell. This is commonly an adaptation to stress (e.g. as a result of chronic inflammation). A classical example of metaplasia is the replacement of the columnar epithelium of respiratory tract (the bronchial lining) with squamous epithelium in smokers (squamous metaplasia). This is a reversible event and therefore not classified as part of the neoplastic process, but it can lead to dysplasia, which is irreversible.

### 1.3.3 Irreversible changes in cells and tissues

When a tissue is exposed to a sub-lethal dose of a toxin it may undergo reversible adaptive changes in order to cope with the insult. Such changes may be accompanied by morphological alterations, but these will regress if the insult is removed. However, if adaptive changes are insufficient to overcome the insult, the cell will progress to irreversible damage. The likelihood of this happening depends upon the cell type and its metabolic state at the time of injury; for example, ischaemia will cause irreversible damage after a few minutes in neurons, after 1–20 min in cardiac myocytes and after 1–2 h in epithelial cells of the renal proximal tubule. Severe or chronic toxic insults can therefore lead to irreversible changes in cell growth and differentiation.

- **Dysplasia** is an abnormal change in the arrangement and size of cells in a tissue. It can sometimes be reversed, but is generally considered to represent a point of commitment to the carcinogenic process.
- **Neoplasia** literally means new growth: a group of cells which are growing in an uncontrolled manner.
- **Anaplasia** refers to the regression of the physical characteristics of a cell towards a more primitive or undifferentiated type and is a common feature of malignant tumours.

### 1.4 Tissue responses to injury

The main targets for damage within the cell are the cell membrane, mitochondria, cytoskeleton and DNA. The biochemical mechanisms involved include ATP loss, release of calcium into the cytoplasm, reactive oxygen metabolites, structural damage to membranes and cytoskeleton and DNA damage which can be lethal or lead to mutations.

#### 1.4.1 Oxidative stress

Oxidative stress underlies a vast number of human diseases as well as mechanisms of toxicity of drugs and chemicals; in addition, exposure to environmental
chemicals can cause a variety of human diseases by mechanisms which involve oxidative stress.

Oxidative stress has been implicated in the toxicity of a plethora of drugs and environmental chemicals. One of the key intracellular molecules involved in this process is the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine (GSH)) (Figure 1.2). Glutathione is the most abundant low-molecular-weight thiol found within cells; by cycling between its reduced state (GSH) and its oxidised state (glutathione disulfide) it helps to maintain the appropriate redox status within the cell. Many inducers of oxidative stress exert their toxic effects by causing GSH depletion as a consequence of generation of reactive oxygen species, which arise from a variety of sources, being either endogenously generated or produced by environmental agents such as xenobiotics, UV irradiation and infectious organisms. In the event that a cell produces more reactive oxygen species than can be detoxified the result is DNA damage, lipid peroxidation and cell death. The consequence of this is an acute or a chronic disease.

The three major types of reactive oxygen species are as follows:

- **Superoxide anion radical** ($O_2^-$), which is present constitutively in cells because of leakage from the mitochondrial respiratory chain.
- **Hydrogen peroxide** ($H_2O_2$), resulting from the dismutation of $O_2^-$ or directly from the action of oxidase enzymes.
- **Hydroxyl radical** ($^\cdot$OH), a highly reactive species that can modify purine and pyrimidine bases and cause DNA strand breaks resulting in DNA damage.

Reactive oxygen species are natural by-products of cellular metabolism, and oxidative stress is tightly regulated by the balance between their production and removal. Stress response pathways have evolved to protect cells against oxidative stress and environmental challenge, as well as to repair damage. Indeed, all organisms have enzymes which can scavenge superoxide and $H_2O_2$ (Imlay, 2008). Oxidative stress is a very complex problem because of the number of different pathways which exist in order to protect against different oxidants. In mammalian cells, for example, the enzymes involved include superoxide dismutases (SODs), catalase, glutathione S-transferases (GSTs), glutathione peroxidase, reduced nicotinamide adenine dinucleotide phosphate (NADPH)-quinone oxidoreductase, haem oxygenase-1 (HO-1), dual-specificity phosphatases, thioredoxin and peroxiredoxins.

The main intracellular site where reactive oxygen species are generated is the mitochondrion, and mitochondrial energy metabolism is quantitatively the most important source of reactive oxygen species in the majority of eukaryotic cell types (Kowaltowski et al., 2009; Khansari et al., 2009; Murphy, 2009; Kagan et al., 2009).
The production of reactive oxygen species in mitochondria is a normal consequence of respiration, but the different species generated during this process can differ markedly in their reactivity and lifetime; for example, hydroxyl radical reacts almost instantaneously with adjacent molecules whereas semiquinones may be stable for days, weeks or months (Pryor et al., 2006). In fact, mitochondria can produce reactive oxygen species even under conditions of hypoxia and this may have implications for mitochondrial redox signalling (Murphy, 2009).

The primary reactive oxygen species generated by mitochondria is superoxide, which is produced by one-electron reduction of O$_2$ and metabolised by SOD in the inter-membrane space. The resulting H$_2$O$_2$ is relatively unreactive; however, if it is not metabolised, it may go on to form hydroxyl radical via a Fenton reaction (Kowaltowski et al., 2009; Pryor et al., 2006). If there is a lack of balance between reactive oxygen species generation and antioxidant defence mechanisms, reactive oxygen species can leak from the mitochondrion causing damage to cellular targets, including cell membrane fatty acids (forming lipid peroxides), cellular proteins (damaged proteins may accumulate up to toxic levels causing cell death) and DNA (causing DNA strand breaks and deletions). The consequent damage has a number of sequelae including modulation of survival signalling molecules, triggering of cell death pathways and production of proinflammatory cytokines and chemokines (Khansari et al., 2009; Roberts et al., 2009; Pan et al., 2009).

Oxidative stress and chronic inflammation are characteristic of a wide variety of human diseases (Brenneisen et al., 2005), but it is often difficult to determine whether oxidative stress is a primary cause of cell death or a physiological consequence of the induction of cell death pathways. This has led to the identification of a ‘growing need for simple, convenient, and reliable markers for the assessment both in vitro and in vivo of the metabolic/oxidative distress and of its modulation … [by] … pharmaceutical products’ (D’Alessandro et al., 2011).

Oxidative stress also plays a role in cellular senescence and cancer. Unbalanced regulation of the production of reactive oxygen species appears to initiate cellular senescence programmes via multi-faceted mechanisms including the direct induction of mutations (Pan et al., 2009), and the altered metabolic state of cancer cells (associated with aerobic glycolysis) makes them particularly susceptible to reactive oxygen species damage linked to the accumulation of mutations (D’Alessandro et al., 2011). Indeed, the interaction between reactive oxygen species and cellular senescence has been suggested as a target for cancer therapy (Pan et al., 2009).

The transcription factor Nrf2 plays a key role in cellular responses to oxidative stress. Under normal conditions, Nrf2 is located in the cytoplasm and is bound to the accessory protein Keap1, which targets Nrf2 for proteasome-mediated degradation. Under conditions of oxidative stress, however, Keap1 loses its ability to bind Nrf2, which is then able to translocate to the nucleus and bind to the antioxidant response elements in the 5′ regulatory regions of target genes.

Example: Oxidative stress and cardiovascular disease

Oxidative stress is known to play a role in the aetiology of cardiovascular diseases (atherosclerosis, coronary artery disease and myocardial infarction) as

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6 For a review, see Limon-Pacheco and Gonsebatt (2009).
well as stroke, dementia, Parkinson’s disease and cancer. Excessive oxidative stress and chronic inflammation are characteristic features of cardiovascular disease, which features increased production of reactive oxygen species, compromised antioxidant defences (e.g. GSH depletion) and increased circulating levels of proinflammatory cytokines (Lee et al., 2011). Oxidative stress may be involved in the pathogenesis of cardiovascular disease, and the Nrf2 pathway has been implicated in this process, especially in the sedentary elderly. Proposed strategies for prevention range from lifestyle changes including dietary supplementation (increased consumption of broccoli, curcumin) and increased exercise to pharmaceutical interventions such as prophylactic treatment with the ubiquitin-proteasome inhibitor MG132, which may protect cardiomyocytes against oxidative stress via Nrf2-mediated up-regulation of antioxidant genes.

1.4.2 Necrosis and apoptosis

The two main mechanisms by which cells can die are necrosis and apoptosis (also known as programmed cell death). If repair mechanisms and changes in gene expression are insufficient to allow the cell to cope with a toxic insult, the consequence is cell death by one of these two mechanisms. Many of the key players in apoptosis have now been identified and it turns out that many of the genes which control cell growth are also involved in apoptosis, allowing the processes of cell division and death to be tightly coregulated. This is crucial for the maintenance of homeostasis; indeed, evasion of apoptosis signals is a hallmark of human cancer, and the genes involved in regulation of the cell cycle and apoptosis represent potential molecular targets for the control of human diseases where inappropriate apoptosis is prominent (e.g. cancer and degenerative disorders).

The occurrence of necrosis is not determined by factors that are intrinsic to the cell but by changes in the cellular environment. In contrast, apoptosis is a physiological process and plays a role in the maintenance of tissue homeostasis. Apoptosis is an active process leading to cell death via an ordered sequence of events. Its morphologic appearance is very different from that of necrosis (Figure 1.3). Necrosis involves the swelling and rupture of the injured cells, whereas apoptosis involves a specific series of events that leads to the dismantling of the internal contents of the cell. Apoptosis is a normal part of development, being involved in processes including

- resorption of the tail during metamorphosis of a tadpole;
- removal of the webbing between the digits of hands and feet during mammalian embryonic development.

Necrosis is an unregulated mechanism involving dilation of the endoplasmic reticulum, dissolution of lysosomes and ribosomes, mitochondrial swelling and an increase in cell volume. Necrosis does not, however, involve gross changes in chromatin structure. In contrast, during apoptosis, the cytoplasmic organelles are well preserved and there is actual shrinkage of the cell and nucleus. The biochemical changes which occur during apoptosis include a moderate increase in

7 For a general review, see Han, Kim and Kim (2008).
intracellular [Ca$^{2+}$] and total shutdown of protein and RNA synthesis. Following condensation of the nuclear chromatin, activation of Ca$^{2+}$/Mg$^{2+}$ endonuclease produces distinctive chromatin fragments which may be viewed as a ladder on an agarose gel (Hooker et al., 2012).

Apoptosis is characterised by distinctive morphological changes including decreased cell volume, increased cell density, compaction of cytoplasmic organelles (except for the mitochondria, which remain morphologically normal) and dilation of the endoplasmic reticulum. Nucleolar disintegration, budding and separation of nucleus and cytoplasm into multiple small membrane-bound apoptotic bodies occurs, followed by progressive degeneration of residual nuclear and cytoplasmic structures and condensation of chromatin into crescent-shaped caps at the cell periphery.

A key process in apoptosis is the activation of a series of highly conserved cysteine proteases called caspases which act as common effector molecules in various forms of cell death. Caspases are produced as inactive precursors called procaspases which can be activated either by oligomerisation (initiator caspases e.g. caspases 8 and 9) or by proteolytic cleavage to create active enzymes in a proteolytic
cascade (effector caspases e.g. caspase 3). Once they are activated, caspases cleave other proteins within cells resulting in efficient and precise killing of the cell in which they are activated.

Apoptosis can be initiated via two different pathways, the extrinsic and intrinsic pathways, each of which involves the activation of specific caspases:

- **The extrinsic pathway** is triggered by the binding of so-called death signals such as tumour necrosis factor (TNF), Fas ligand or TNF-related apoptosis-inducing ligand to the corresponding receptors (TNFR, Fas receptor, DR4/5). This triggers the recruitment of adaptor proteins and activation of the initiator caspases 8 and 9. Interestingly, caspase 8 mutations have been detected in some cancers and can act as dominant negative mutations, blocking apoptotic cell death and having a profound impact on the cancer cell’s ability to undergo apoptosis (Fulda, 2009).

- **The intrinsic pathway** is mediated by mitochondrial damage, which leads to cytochrome c release. Cytochrome c interacts with an adaptor molecule (Apaf-1) and procaspase 9 to form a complex called an *apoptosome* leading to the activation of caspase 9. Inhibitor of Apoptosis Proteins (IAPs) inhibit caspases and procaspases and are themselves controlled by other mitochondrial proteins (Smac/DIABLO and Omi/HtrA2). This process is regulated by various factors including members of the Bcl-2 family, Bax and Bak.

Both pathways lead to the activation of so-called executioner caspases (caspase 3 and caspase 7) by caspases 8 and 9.

Apoptosis appears to be the major pathway of cell death triggered by DNA damage. It is thought to eliminate genetically damaged cells and, therefore, counteracts carcinogenesis. The genes involved in regulating this process represent potential molecular targets for the control of human diseases which feature inappropriate apoptosis (e.g. cancer and degenerative disorders).

**Role of calcium in cell death** Calcium signals are responsible for the regulation of many vital cell functions; cellular Ca\(^ {2+}\) overload or perturbation of intracellular Ca\(^ {2+}\) compartmentalisation can cause cytotoxicity. The point of no return is thought to be when the sarcolemma can no longer bind Ca\(^ {2+}\) and the mitochondria start to take up the excess calcium (Rasola and Bernardi, 2011). Cell death can be brought about by a loss of Ca\(^ {2+}\) homeostatic control, but can also be triggered by more subtle changes in Ca\(^ {2+}\) distribution within intracellular compartments.

Normal intracellular calcium levels range between 10\(^ {−7}\) and 10\(^ {−6}\) M, approximately four orders of magnitude lower than in the extracellular fluid, thanks to the activity of plasma membrane Ca\(^ {2+}\) ATPases and the Na\(^ +\)/Ca\(^ {2+}\) exchanger which remove Ca\(^ {2+}\) from the cell. This concentration gradient allows rapid influx of Ca\(^ {2+}\) into the cell if the plasma membrane channels open, for example in response to a toxic insult. An unphysiological increase in cytosolic calcium concentration (to >10\(^ {−5}\) M) can either cause necrosis or contribute to apoptosis by activating

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8 For a review from the perspective of renal toxicity, see Servais *et al.* (2008).
9 For a detailed review of this topic, see Zhivotovsky and Orrenius (2011).
physiological calcium-dependent processes and cellular responses that are not normally affected by calcium. These include changes in cellular shape, blebbing (a bleb is an irregular bulge in the plasma membrane of a cell caused by localised decoupling of the cytoskeleton from the plasma membrane), changes in ionic conduction, excessive contraction/transmitted release, enzyme activation (proteases, nucleases, phospholipases, transglutaminases) and inhibition (adenylate cyclase). This is associated with a cycle of decreased ATP levels due to activation of the Ca$^{2+}$ pump, subsequently causing reduced functioning of the pump itself due to insufficiency of ATP.

1.4.3 Neoplasia

The term *neoplasia* refers to the development of a collection of cells which grow in an uncontrolled manner, usually resulting in the formation of a tumour. Neoplasia may be benign or malignant and its causes include foreign chemicals (xenobiotics), hormones, viruses, inherited genes, radiation and physical agents (e.g. inert implanted plastic or metal film).

The cellular changes observed during neoplasia include hypertrophy, hyperplasia and alterations in differentiation which may be associated with changes in the histological appearance of cells. It is important to note that the terms cancer and tumour are not synonymous: the word tumour applies to any readily defined mass of tissue distinct from normal tissue but not necessarily made up of abnormal cells; in other words, the term tumour just means a lump. The definition of a benign tumour is that it is restricted to its site of origin; a malignant tumour is one which is metastatic.

Cancer is defined as a heritably altered, relatively autonomous growth of tissue which occurs when a cell or group of cells begins to multiply more rapidly than normal leading to the development of a malignant tumour: it is, by definition, a disease of aberrant cells and is a consequence of both uncontrolled cell division and the loss of normal patterns of differentiation leading to the autonomous growth of these abnormal cells. A true cancer is a malignant neoplasm made up of morphologically transformed, autonomously replicating (malignant) cells. The characteristics of morphologically transformed cells include uncontrolled cell division, loss of contact inhibition, defective cell cycle control, lack of balance between cell division and cell death, breakdowns in cell–cell communication and dedifferentiation.

Metastasis occurs when individual cells or small groups of cells break away from the primary tumour and migrate to other sites within the body, where they begin to grow into secondary tumours (metastases). Malignant tumours tend to be locally invasive, fast-growing and anaplastic whereas benign tumours are usually encapsulated and are generally slow growing.

1.4.4 The initiation–promotion paradigm

Carcinogenesis is defined as the process by which normal cells are transformed into cancer cells. A carcinogen, therefore, is any substance that causes cancer.

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10 For an overview of the history of ideas about carcinogenesis, see Mitrus *et al.* (2012).
The link between cancer and exposure to specific exogenous substances was first identified in the 18th century, when in 1761 John Hill noticed an increase in nasal cancer associated with long-term use of snuff and in 1775 Percival Pott observed that chimney sweeps often suffered from scrotal cancer. The role of industrial exposure to aromatic amines in the induction of bladder cancer was demonstrated in 1895, when Rehn reported an increase in bladder cell tumours in workers in the dye and rubber industries.\textsuperscript{11}

Some cancers arise as a result of genetic susceptibility or alterations in homeostasis (e.g. hormonal changes) while others are caused by exposure to carcinogenic substances. Experimentally, a compound is considered to be carcinogenic if its administration to laboratory animals induces a statistically significant increase in the incidence of one or more histological types of neoplasia compared with animals in the control group, which are not exposed to the substance.

According to the European Chemicals Agency (ECHA)\textsuperscript{12}:

Chemicals are defined as carcinogenic if they induce tumors, increase tumor incidence and/or malignancy, or shorten the time to tumor occurrence. Benign tumors that are considered to have the potential to progress to malignant tumors are generally considered along with malignant tumors. Chemicals can induce cancer by any route of exposure (e.g. when inhaled, ingested, applied to the skin, or injected), but carcinogenic potential and potency may depend on the conditions of exposure (e.g., route, level, pattern, and duration of exposure).

The concept of cancer as a multi-step process was first proposed by Armitage and Doll in 1954 and extended by Moolgavkar and Knudsen in 1981 (Armitage and Doll, 1954, Moolgavkar and Knudson, 1981). The idea that the process of carcinogenesis could be divided into discrete stages arose when it was observed that tumours could be induced by painting mouse skin with polycyclic aromatic hydrocarbons (PAHs) then with croton oil, but not if these substances were applied in the reverse order. This is now known as the initiation–promotion paradigm (Figure 1.4) and reflects the fact that the carcinogenic process involves both genetic damage and changes in the growth of cells and tissues. Cell replication is necessary for fixing DNA damage (i.e. making it irreversible), therefore increasing the risk of neoplasia, and hyperplasia is central to the promotion phase during which tumour growth occurs.

**Initiation** A cancer originates from an initiated cell which multiplies clonally, escapes apoptosis and accumulates a collection of genetic and/or epigenetic alterations which allow it to escape from normal control mechanisms. Mutagenesis, the process by which mutations occur, is a key aspect of carcinogenesis. A mutation is a change in the sequence of bases in a DNA molecule, and any insult which causes a mutation is known as a *mutagen*. Carcinogenesis almost always involves mutagenesis, and many carcinogens are also mutagens, but it is a complex process involving defects in many different biological control mechanisms so mutagenesis is not, in and of itself, sufficient to generate cancer.

\textsuperscript{11} For interesting reviews on this topic, see Oliveira et al. (2007).
\textsuperscript{12} ECHA Guidance on information requirements and chemical safety assessment Chapter R.7a: Endpoint specific guidance, Version 2.0, November 2012, Section R.7.7.8.1.
Initiation involves the acquisition of irreversible genetic alterations as a consequence of mutation of one or more key genes. This may occur spontaneously or as a result of DNA damage caused by chemical or physical damage (e.g. due to ionising radiation). Initiation gives rise to a single initiated cell which is phenotypically indistinguishable from the surrounding normal cells but is predisposed to give rise to a neoplastic lesion. It is a rapid and irreversible process and the characteristics of initiation are transmitted to daughter cells when the initiated cell divides. The ability of chemicals which induce DNA damage (genotoxicity) to cause initiation and ultimately tumour development has led to an intense focus on the identification and regulation of genotoxic chemicals.

**Promotion** Promotion involves an increase in the number of initiated cells to produce a benign tumour. Increases in cell number can result from either increased cell proliferation or reduced cell death, so increased cell division and evasion of apoptosis are both considered to be critical in the promotion process. Promotion is believed not to require further DNA damage.

The importance of cell proliferation in the promotion process is twofold: (i) prior to DNA replication, DNA damage can be repaired, but proliferating cells have less time to repair DNA damage, and DNA replication during cell division can fix this damage, making it permanent and irreversible. This can be particularly important if the DNA damage occurs in a stem cell which can give rise to multiple progeny, and pluripotent stem cells are now believed to be the key target cells in carcinogenesis. Indeed, cells which are fully differentiated or committed to differentiation are unlikely to give rise to tumours as they are already programmed to die. (ii) Cell proliferation converts an individual initiated cell to a detectable lesion (a preneoplastic focus and subsequently a benign tumour).

By definition, the promotion process involves hyperplasia which may be augmentative (proliferation over and above that which is required for tissue maintenance) or regenerative (proliferation which is necessary for tissue repair) (Figure 1.5). Tumour promoters contribute towards the fixation of DNA damage in the form of mutations, enhance epigenetic processes which alter gene expression and cause changes in cellular growth control. Some promoters are tissue specific whereas others can act on more than one tissue type.

Long-term treatment with certain promoting agents can induce neoplasia, apparently without the need for initiation, the process known as *non-genotoxic carcinogenesis*. Some investigators believe that this indicates a genotoxic effect which has been missed by conventional genotoxicity assays, but it is equally
It is possible that non-genotoxic carcinogenesis occurs as the result of promotion of pre-existing, spontaneously initiated cells.

**The cell cycle** During the cell cycle, a cell must replicate its DNA and duplicate its contents, then divide in two. In the case of unicellular organisms each cell division cycle makes a new organism whereas in the case of multi-cellular organisms many rounds of cell division are required to make a new organism. Cell division is also needed in the adult body in order to replace cells which have been lost as a consequence of apoptosis or necrosis.

Eukaryotic cells divide and grow at different rates. The eukaryotic cell cycle can be resolved into four phases (Figure 1.6):

- **G1 phase** (growth of the cell)
- **S-phase** (DNA replication)
- **G2 phase** (growth and error checking)
- **M phase** (cell divides)

Cells can leave the cell cycle during and go into quiescence (G0 phase); indeed, some cell types (e.g. hepatocytes) spend most of their time in G0. During M phase the cell divides (cytokinesis) and its DNA is distributed evenly between the daughter cells.

The cell cycle must be controlled to allow time for synthesis of new proteins, replication of DNA and checking for DNA damage. Checkpoints are required in order to ensure that each process is complete before the next one starts. If this does not happen, delays and interruptions occur; this is what happens in cancer. At a cell cycle checkpoint the cell cycle pauses until the appropriate feedback signals have been received. The key checkpoints in the eukaryotic cell cycle are

- start (in G1, just before the beginning of S-phase), when the cell becomes committed to the cell cycle
- entry to M phase (at the end of G2)
- exit from M phase
1.4 TISSUE RESPONSES TO INJURY

Cell cycle checkpoints are tightly controlled and monitored by proteins which include the cyclins and cyclin-dependent kinases (CDKs). The mitotic cyclins bind to CDK molecules during G2, permitting entry to mitosis, while the G1 cyclins bind to CDK molecules during G1 and are required for entry into S-phase. The CDKs act by phosphorylating specific proteins on serine or threonine residues.

As a consequence of the importance of cell cycle control, increases in cell replication are indicative of tumour promotion. Changes in cell replication may be detected by a variety of methods including:

- Incorporation of $^3$H- or $^{14}$C-labelled thymidine into cellular DNA (detected by autoradiography and scintillation counting, respectively)
- Incorporation of bromodeoxyuridine (BrdU) into cellular DNA
- Up-regulation of proliferating cell nuclear antigen, an endogenous protein whose expression increases during S-phase

These techniques can, in theory, be applied to any tissue but it is easier to detect increased cell proliferation over background in tissues with a low background rate of replication, such as the liver.

Under normal circumstances adjacent cells communicate with each other to ensure that proliferation is properly controlled. Cell–cell contact is a key aspect of this process. When non-transformed adherent cells are grown in culture they become arrested in G0 when they are touching each other on all sides, thus forming a confluent monolayer. This process, which is known as contact inhibition, is controlled by cyclin-dependent kinases and mediated via cell membrane proteins such as N-cadherin.

Cell–cell adhesion in epithelial cells is mediated by integrins, transmembrane proteoglycans and calcium-dependent cell–cell adhesion molecules.
called cadherins. In particular, the calcium-dependent homophilic interactions of E-cadherin, which induce contact inhibition, maintain the epithelial cell phenotype and prevent migration. E-cadherin communicates with the cellular interior by catenin-mediated interactions with the actin cytoskeleton. One of the functions of E-cadherin is to sequester β-catenin, reducing the levels observed in the cytoplasm. Cytoplasmic levels of β-catenin are also regulated by proteolysis. Accumulation of β-catenin occurs physiologically during embryonic development and pathologically during tumorigenesis. In contrast, α-catenin, which associates with desmosomal cadherins as well as E-cadherin, is believed to have an inhibitory effect on processes associated with tumour development.

Disruption of cell–cell communication facilitates the dedifferentiation of cells to a more mesenchymal phenotype. This is a normal process during embryonic development but occurs pathologically as benign tumours progress towards a more invasive/metastatic phenotype. Loss of expression of E-cadherin and β-catenin is a key aspect of this process, which is known as the epithelial–mesenchymal transition and is associated with the induction of proliferative, mesenchymal and invasive genes leading towards a more malignant phenotype.

**Preneoplastic lesions** A preneoplastic lesion is a recognisable group of cells which has undergone the early stages of the carcinogenic process but is not yet fully committed to forming a tumour and can, under certain circumstances, regress to form apparently normal tissue. For example, a well-characterised series of phenotypic changes occurs during the early stages of rodent liver carcinogenesis *in vivo*. The methods used to detect these changes often involve the use of so-called initiation-promotion protocols (Pitot, 2007), in which animals are treated with a single dose of a potent genotoxic compound such as 2-acetylaminofluorene (2-AAF) (initiation) followed by regular treatment with a compound such as phenobarbital (PB) which induces cell proliferation (promotion). In some cases two-thirds partial hepatectomy is used to stimulate cell proliferation because the rodent liver can survive removal of two-thirds of the liver. Following this surgery it undergoes a period of rapid growth as a result of which the full weight of the liver is restored in less than a week.

Other early markers of tumour development include preneoplastic enzyme changes (e.g. up-regulation of γ-glutamyl transpeptidase (γGT) and glutathione-S-transferases (GSTs) in rodent liver), development of morphologically altered preneoplastic foci, early changes in ploidy and nuclearity, oncogene activation, activation of growth factors and altered cell–cell communication. All these can be seen to be logically related to the acquisition of cancerous properties by the cells: up-regulation of detoxifying enzymes may confer resistance to the cytotoxic effects of the carcinogen, morphological changes are diagnostic of the transformed phenotype, oncogene activation and growth factor up-regulation enhance proliferation and loss of cell–cell communication allows the transformed cell to ignore growth retarding signals from surrounding cells.

Examples of types of preneoplastic lesions which can arise in animals during chemical carcinogenesis include altered hepatic foci, prepapillomas of the skin and aberrant crypt foci in the colon. Among the best-characterised preneoplastic

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13 For a fascinating personal account of the background to these studies, see Pitot (2007).
lesions of toxicological relevance are hepatic preneoplastic foci (Figure 1.7), which develop in rodent liver in response to treatment with hepatic carcinogens. They are easy to identify, being characterised by an altered pattern of expression of various markers which can be detected by means of immunohistochemistry. The most commonly used markers are GST-P and gamma glutamyl transpeptidase (γGT); others include the diubiquitin-like molecule FAT10, α2-macroglobulin, fatty acid synthase and α-fetoprotein. One of the most useful of these markers is FAT10, which is also overexpressed in 70–90% of human hepatocellular carcinomas (HCCs). Single FAT10-positive cells appear early in the carcinogenic process, possibly as a consequence of epigenetic alterations. They have a growth advantage compared with normal hepatocytes, and are thought to represent a subpopulation of initiated cells which are resistant to cytotoxicity in the presence of a strong growth stimulus (French, 2010).

Studies on preneoplastic stem cells suggest that, at least in rats, susceptibility to the early stages of hepatocarcinogenesis is a consequence of the activation of several low penetrance genes and a single predominant susceptibility gene (French, 2010). Epigenetic phenomena also play a role; for example, hypomethylation is observed in the hepatocytes of susceptible F344 rats.

For an atlas of the difference types of liver lesions found in mice, see http://www.niehs.nih.gov/research/resources/liver.
Preneoplastic lesions in humans If clearly defined and easy to identify, preneoplastic lesions can be of great value both to the toxicologist and to the oncologist. Because they occur early in the carcinogenic process, the toxicologist can use them as an early indicator of potential future tumour development, while the oncologist can use them for early diagnosis and treatment. For example, dysplastic foci (<1 mm diameter) and nodules (1 mm – 1 cm diameter) have been identified as preneoplastic lesions in human liver. These may have either a large cell or small cell morphology; large cell dysplasia is not a precursor for HCC whereas small cell dysplasia is considered to be a preneoplastic lesion (French, 2010).

Identification of preneoplastic lesions can also play a key role in cancer screening. Cancer causes approximately 840 000 deaths annually in the EU and over 1 300 000 new cases are reported each year. In many cases, by the time a tumour causes symptoms, it is too advanced to treat, but if tumours can be detected when they are very small, or better still, people who have not yet developed a tumour but have a high risk of doing so can be identified; the chances of successful treatment are much improved. As well as saving lives, a well-organised screening programme can save the health services an immense amount of money and time. In order to establish a screening programme, two key requirements must be met: there must be a test or procedure which will detect the cancer before symptoms develop (preferably at the preneoplastic stage) and there must be evidence that treatment at this earlier stage of the disease will result in an improved outcome. The most successful screening programme to date is the one for cervical cancer.

Example: Early diagnosis and treatment: the cervical smear test

The cervical cancer screening programme depends upon the identification of preneoplastic cells in otherwise healthy women. In this programme, precancerous changes in the cervix are detected by looking for abnormal cells which arise in its lining.

Precancerous change in the cervix is called cervical intraepithelial neoplasia (CIN). In this condition, abnormal cells with large, oddly shaped nuclei are seen in the lining of the cervix. The process starts with CIN I and progresses through CIN II to CIN III. The majority of these changes will eventually revert to normal, but in a few cases they precede the development of a tumour.

When a woman goes for a smear test, cells are scraped from the cervix using a spatula, spread on a slide, stained using a method developed by Papanicolau in 1942 (the Pap test), and examined to identify any which are a strange colour or have an enlarged or bizarrely shaped nucleus. If a mild abnormality (dyskaryosis) is detected, the woman will be asked to return in 3–6 months. If the changes persist, she will be referred for colposcopy. In the colposcopy procedure a gynaecologist examines the cervix directly and removes any abnormal tissue. This procedure cures the problem at the same time as confirming the diagnosis.

The problem of cervical cancer screening is that the current screening programme depends upon the detection of dyskaryotic cells by trained cytology screeners. The process is labour-intensive, expensive, subjective and prone to a significant incidence of errors. Any method giving a simple yes/no answer would be a significant improvement, especially if it lent itself to automatic sample processing and analysis.
Could CYP1B1 be the answer? Immunohistochemical analysis of the expression of a protein called CYP1B1 in human tumours and corresponding histologically normal tissues (Murray et al., 1997), suggested that this cytochrome P450 (CYP) isozyme might have a future as a tumour marker since CYP1B1 expression was detected in 122/127 tumours and 0/130 normal tissue samples.

The question that arose from this was: At what stage of tumour development is CYP1B1 expression initiated? Cervical cancer was selected for further study for the reasons outlined earlier, and the results of this study indicated that expression of CYP1B1 was detectable in cervical lesions and individual exfoliated cervical epithelial cells from a subject with CIN. The location of the staining corresponded with the area occupied by abnormal cells. In contrast with the difficulty of picking out abnormal cells in a Pap smear, individual CYP1B1 positive cells could readily be detected in smear samples from patients with CIN (Figure 1.8). Thus, CYP1B1 appeared to be a good marker for precancerous changes in the cervix. The potential advantage of this test is that it gives an unequivocal result: normal cells are blue whereas abnormal cells are brown. This test would be much easier to interpret than the Pap test, and would lend itself to automation, making screening cheaper and less labour-intensive.

The result of initiation followed by promotion is a benign tumour. The definition of a benign tumour is that it does not metastasise. Benign tumours do undergo excessive growth and immortalisation, but are unable to spread metastatically and are generally slow growing. The promotion process is reversible and subject to physiological modification; if the promoting stimulus is removed regression can occur as a result of the induction of apoptosis.

Progression The final stage of carcinogenesis, leading to a malignant tumour, is progression. Progression involves the acquisition of metastatic capability by the tumour, converting it from a benign tumour to a malignant one. A malignant tumour is one which is metastatic and a true cancer is, by definition, a malignant neoplasm. Malignant tumours tend to be locally invasive, fast-growing and anaplastic. Metastasis occurs when individual cells or small groups of cells break away from the primary tumour and migrate to other sites within the body, where they begin to grow into secondary tumours (metastases). The processes involved in progression are less clearly defined than those involved in initiation and promotion, but are believed to include loss of cell–cell communication within the tumour and the acquisition by individual cells of the ability to detach from the main tumour, invade adjacent tissues, migrate via the blood stream and establish new colonies in distant tissues (metastasis).

Progression is the conversion of a preneoplastic/benign lesion to the so-called malignant phenotype. The characteristics of this phenotype include neoplastic differentiation, unlimited replicative potential, ability to evade apoptosis, sustained angiogenesis, telomerase overexpression, self-sufficiency in growth signals and insensitivity to anti-growth signals. Collectively, these changes confer on the tumour cells the capacity for tissue invasion and metastasis. Progression to malignancy involves both genetic and epigenetic mechanisms leading to the ability to proliferate indefinitely, independent of stimulation. This process is irreversible and involves changes in the biochemical, metabolic and morphological characteristics of the cells within the lesion. Malignant tumours
Malignant cells are able to separate from the parent tumour, migrate to distant locations and establish new tumours. The characteristics which make this possible include rapid growth, invasiveness and the ability to stimulate angiogenesis, with invasiveness being the earliest manifestation. Metastasis entails a strong component of tumour–host interactions.

Proteins which play key roles in the progression phase of carcinogenesis include the proteins CD44 and osteopontin, stress response genes that contribute to host defences. Osteopontin is an early mediator of host defences and provides protection against intracellular pathogens while CD44 is a transmembrane glycoprotein which acts as a homing receptor and is normally expressed in lymphocytes and macrophages. Both genes are dysregulated in autoimmune diseases.

One of the key aspects of the progression phase of carcinogenesis is acquisition of the so-called mutator phenotype (Loeb, 2011). This term was coined to convey the fact that cancer cells have an increased error rate during DNA synthesis, possibly arising as a consequence of mutations in genes which govern genetic stability (e.g. those responsible for DNA repair, DNA replication, chromosomal segregation and cell cycle checkpoint control).

For a review see Weber (2008).
Cells undergoing the processes of promotion and progression exhibit extremely diverse phenotypes, reflecting the different combinations of genetic and epigenetic alterations which occur during the neoplastic process. Selection of the cell population having the most favourable genotype/phenotype for survival occurs; indeed, only 1/100 individual GST-P+ hepatocytes goes on to form an altered hepatic focus, let alone a full-blown tumour. This phenotypic diversity and its molecular basis remain to be fully characterised.

**Epigenetic changes** Cancer cells have an altered epigenotype compared with the tissues from which they arise, and this epigenetic switch is characterised by changes in the level and placement of DNA methylation and histone modification, but much work remains to be done to resolve the role of epigenetic changes such as DNA methylation in the maintenance and reversibility of promotion and progression.\(^{16}\)

It is, for example, known that DNA methyltransferase 1 is overexpressed during tumour development. Hypomethylation of long interspersed nuclear elements (LINE-1 repeats), satellite DNA and moderately repeated DNA sequences is accompanied by hypermethylation of localised promoter-associated CpG islands which are usually unmethylated in normal cells, rendering them transcriptionally silent. In addition, deamination of methylated cytosine residues forms thymine, an error which is difficult to correct because DNA repair enzymes are unable to determine which base in the resulting mismatched pair is the correct one. Thus genome-wide hypomethylation can increase mutation rates leading to genomic instability in developing tumours, demonstrating the link between organisation of the genome and replication/repair. Changes in histone modifications are also observed in cancer; loss of lysine 16 acetylation and lysine 20 methylation in histone H4 is a hallmark of human cancer cells and is associated with hypermethylation at repetitive sequences.

Epigenetic alterations contribute to all phases of cancer development, including initiation, promotion, invasion, metastasis and resistance to chemotherapy; indeed, epigenetic biomarkers such as methylated DNA sequences, modified histones and microRNAs may be of value in diagnostic and prognostic monitoring. Furthermore, epigenetic processes are reversible: DNA methyltransferases and histone deacetylases therefore represent targets for chemotherapeutic inhibition. The antiretroviral drug azidothymidine is an example of a DNA methyltransferase inhibitor.

### 1.5 Key concepts in toxicology

#### 1.5.1 Risk and hazard\(^{17}\)

Evaluation of the harmful effects of substances in the environment may be based either upon hazard alone or upon an evaluation of risk. It always starts

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\(^{16}\) For an interesting, rather speculative discussion, see Szyf (2011).

\(^{17}\) This section has been extracted from the Hazardous Substances Advisory Committee *Approach on Hazard and Risk Assessment of Substances* (2013) with the kind permission of the Chair and Secretariat. For the full statement, see https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/209709/hsac-hazard-risk-assessment-substances.pdf.
by identifying potential targets and routes of exposure. This is followed by hazard identification/characterisation with the option of proceeding to exposure assessment and risk identification/characterisation. A substance’s potentially hazardous properties can include toxicity, persistence and ability to bio-accumulate. Risk is assessed by evaluating these properties against the predicted concentrations to which organisms are exposed. The hazard associated with a particular substance is its intrinsic ability to cause harm, while risk is the probability that such harm will occur in practice; it depends upon exposure, and the probability of risk from a particular hazard is almost always <100%.

The process involves three stages (i) hazard identification and characterization, (ii) exposure assessment and (iii) risk identification and characterisation, each of which is described in the following text:

- **Hazard identification and characterisation:** Screening tests must be amenable to testing many substances for
  - physicochemical properties (e.g. flammability, boiling point, water solubility and relative density); these determine how a substance behaves in different environmental compartments and influence its degradability and persistence;
  - toxicological properties for humans such as irritancy, sensitisation, acute and chronic effects including carcinogenicity, mutagenicity and reproductive effects; and
  - ecotoxicological properties including acute and chronic toxic effects on different species as well as processes such as bio-accumulation and bio-magnification.

  For ethical, time and cost-saving reasons hazards may initially be predicted from molecular structure (*in silico*), available information on structurally similar chemicals (read-across) and short-term tests. These are conducted *in vitro* wherever possible in order to avoid the unnecessary use of animals, and recent improvements in miniaturisation and robotics have allowed the development of a range of HTS approaches. As the objective is to identify potential hazards, these initial screening tests tend to be biased towards increasing sensitivity at the expense of specificity.

- **Exposure assessment:** It is exposure which determines whether a biological target, which may range from a single species to an entire ecosystem, is likely to experience adverse effects. Only if the target species is exposed to a harmful dose does a substance actually represent a risk.

- **Risk identification and characterisation:** The objective of risk identification and characterisation is to assess the probability and extent of occurrence of adverse effects of chemicals in the environment realistically, taking into account predicted exposure conditions over time. The potential for induction of adverse effects at the most sensitive stages of development (e.g. the embryo or foetus) is particularly important.

The validity and usefulness of any risk assessment are based on many factors. A major consideration is the quality and comprehensiveness of the underpinning scientific evidence. Good regulatory decisions are most likely to result from high quality evidence, taking into account all aspects of the risk assessment process.
1.5.2 Variability and uncertainty

If one measures a biological parameter, including toxicity, in a group of individuals the individual values obtained will differ because of two factors, variability and uncertainty. In the context of toxicology these may be defined as follows:

- **Variability** is defined as observable diversity in biological sensitivity or response, and in exposure parameters. It is caused by inherent biological differences between species, strains, sub-strains and individuals and cannot be reduced. Variability in response to toxic chemicals is determined by the fate of the chemical within the body (toxicokinetics) and the toxicity of the chemical and its metabolites (toxicodynamics). Variability in both toxicokinetics and toxicodynamics relates to a combination of factors that are inherent to the organism, and other factors relating to the physiology and environment of the individual, which change over time. The inherent characteristics include species, sex and genotype. The modulating factors include the physiology of the individual (e.g. age, stage of development, disease or nutritional deficiency, environment and lifestyle factors) and other chemical exposures originating from diet and lifestyle. In principle, variability is measurable, and lack of knowledge of variability is a source of uncertainty (i.e. uncertainty about the variability).

- **Uncertainty** is defined as imperfect knowledge concerning the present or future state of an organism, system or (sub) population under consideration. Uncertainty refers to lack of knowledge, which can often be reduced by undertaking appropriate studies or by increasing the sophistication or power of studies.

Variability is also sometimes called type A uncertainty, aleatory uncertainty, inherent uncertainty or irreducible uncertainty; however, the term variability is preferable because variability can be reproducibly quantified and therefore is not actually uncertain. The variability of a parameter represents true heterogeneity amongst individuals which cannot be reduced and has to be taken into consideration during risk assessment. Examples of this heterogeneity include physiological conditions and states, for example, pregnancy, the functional maturation of organs, body weight and composition, respiratory rate and food consumption.

The sources of variability in human susceptibility to toxic chemicals include both toxicokinetic and toxicodynamic factors. Toxicokinetics includes the processes of intestinal uptake and transfer (i.e. absorption) of substances by the body, the biotransformation these substances undergo, the distribution of the substances and their metabolites between tissues, and the elimination of the substances and metabolites from the body, while toxicodynamics is the process of interaction of chemical substances with target sites and the subsequent events which lead to adverse effects. Each of the stages in toxicokinetics and toxicodynamics is a potential source of variability and uncertainty.

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18 For a comprehensive discussion of this topic, see the Committee on Toxicity Report on Variability and Uncertainty in Toxicology of Chemicals in Food, Consumer Products and the Environment on http://cot.food.gov.uk/cotreports/cotwgreports/cotwgvut.
Other sources of variability in response are more generic; they include physiological factors (e.g. age, nutritional status, obesity and exercise) and disease states (e.g. diabetes mellitus). Age can be a particularly important factor, and risk assessments should always consider the potentially increased susceptibility to toxic chemicals of the developing child (embryo, foetus and child). Infants may be sensitive or resistant because of immaturity of enzymes and other processes involved in the elimination of chemicals (toxicokinetics), whilst infants and young children may show increased sensitivity at receptors or other macromolecules where toxins exert their effects (toxicodynamics). Growth, changing body composition and differentiation of tissues and organs in early life, childhood and adolescence may lead to increased susceptibility to environmental agents compared with adults; on the other hand, young animals, including humans, may be able to metabolise some agents better than do adults, and have a better ability to recuperate or compensate for adverse events than adults, in whom the capacity for architectural and functional regeneration of organs and tissues is reduced. At the other end of life, the elderly may have increased susceptibility to some types of toxic insult because of declining liver and kidney function, and this may be exacerbated by the fact that the majority of elderly people are being treated with multiple therapeutic agents (so-called polypharmacy).

### 1.5.3 Threshold and non-threshold dose responses

The conventional approach to risk assessment involves the identification of a threshold dose (i.e. one below which there is no toxic response) in laboratory animals and then the application of a so-called uncertainty factor or “safety factor” to allow for the possibility that humans are more sensitive to the observed effect than the animals in which the tests were conducted. The identification of threshold doses is particularly important in chemical risk assessment because it is considered to be possible to identify a safe level of exposure for a chemical which exhibits a threshold response, whereas for a chemical which exhibits no threshold it is assumed that no amount of exposure can be considered safe.\(^{19}\)

The parameters used in these calculations include:

- The no observed adverse effect level (NOAEL)
- Various benchmark doses (BMD)s

Under the REACH regulations, two new parameters, the Derived No Effect Level and Derived Minimal Effect Level, have been defined. The Derived No Effect Level is calculated from the NOAEL or an appropriate BMD and applies to effects which display a threshold, while the Derived Minimal Effect Level is used where no threshold can be defined and is considered to represent a low, possibly theoretical and hopefully tolerable risk.

\(^{19}\) For more on threshold effects, see http://www.popstoolkit.com/riskassessment/module/exposure+and+toxicity+analysis/toxicity/threshold+contaminants.aspx and http://www.toxicology.org/isot/RC/northland/blackburn.pdf.
Most chemically induced toxic effects exhibit a threshold, and it is therefore assumed to be possible to set a safe dose (e.g. an acceptable daily intake) based on a NOAEL with the application of appropriate safety and uncertainty factors. However, according to the initiation-promotion-progression theory of carcinogenesis, a single mutation could theoretically initiate a tumour; in other words, there may be no threshold for tumour induction. In the absence of a threshold, conventional threshold-based risk assessments cannot be applied; one solution to this problem is to adopt the margin of exposure (MoE) approach developed as a result of work sponsored by the European Food Safety Authority (EFSA) (Benford et al., 2010). The MoE is the ratio of a risk assessment parameter obtained from animal studies (e.g. a dose giving a particular incidence of tumours) to the anticipated exposure level. In the United States, an MoE of >100,000 is considered to indicate low risk, MoEs between 10000 and 100000 indicate moderate risk and a MoE <10,000 indicates high risk. The European approach is less rigid, and chemicals are considered on a case-by-case basis.

The vast number of compounds to which humans are exposed to and the cost and resources needed in order to assess all their hazards make it impossible to conduct a complete risk assessment for every chemical. A pragmatic approach to this problem is to attempt to identify a generic level of exposure which is considered acceptable for any chemical, the threshold of toxicological concern (TTC) (Munro et al., 2008). This approach is based on the concept that a reasonable assurance of safety can be given, even in the absence of chemical-specific toxicity data, provided that the exposure is sufficiently low: i.e. one can identify an exposure below which there is no significant risk to human health.

Proposed TTCs have been based on the knowledge gained from 50 to 60 years’ worth of risk assessments and balance the uncertainties inherent in extrapolation from existing toxicology data against a predicted or known low level of exposure. The approach was initially used for food packaging materials and subsequently extended to a wider range of chemicals. The original TTC for food packaging materials and additives was set at 0.5 ppb (equivalent to 1.5 μg/person/day) by the US Food and Drug Administration (FDA).

The development of TTCs for other compounds has attempted to take into account the molecular structures of the compounds under consideration. Compounds are categorised into three classes (Munro et al., 2008):

- **Class I**: Simple structures which are efficiently metabolised and have low potential toxicity.
- **Class II**: Less clearly innocuous than Class I, but no positive indications of toxicity or significant uncertainties.
- **Class III**: Have structural features which preclude the assumption of safety or suggest significant toxicity.

Statistical analysis of NOAELs indicated that TTCs could be set at 1800 μg/person/day for Class I, 540 μg/person/day for Class II and 90 μg/person/day for Class III (based on a body weight of 60 kg). Additional TTCs of 0.15 μg/person/day and 18 μg/person/day were defined for potential genotoxins and organophosphates, respectively. These values are now commonly used to evaluate food flavourings and are in the process of being extended to other categories of compounds,
although this can be problematic in some cases: for example, the application of the TTC of 0.15 μg/person/day to genotoxic impurities in medicinal products would result in the rejection of most drugs because of the use of highly reactive reagents in their synthesis. Overall, the TTC approach is considered to be a pragmatic solution to the problem of assessing the safety of large numbers of compounds for which limited data are available. It reduces the number of unnecessary animal tests and is conceptually simple and easy to apply. This approach is now accepted as part of the risk assessment of food flavourings and packaging materials and is in the process of being extended to other substances.

A concept which goes beyond thresholds is that of hormesis. This is based on the observation that the dose responses for some effects (including some genotoxic processes) can be J-shaped, that is, higher values observed at extremely low/zero doses than at low conventional doses. This may be explicable in terms of, for example, the induction of DNA repair enzymes by low doses of genotoxic agents leading to repair of endogenous lesions which would be left unrepaired in untreated animals/cells. It can be difficult to determine the shape of dose–response curves at very low doses in hazard assessment studies because the number of doses used is often inadequate, the dose spacing may be inappropriate and few doses below the NOAEL are considered, so it is difficult to prove or disprove the existence of hormesis. Some commentators believe that hormesis should be given much more credence during risk assessment, and it has, indeed, been suggested that hormesis should be the default assumption (Calabrese, 2005). However, this is still the subject of heated toxicological debate (Calabrese et al., 2011, 2012; Zeiger and Hoffmann, 2012).

1.5.4 The regulatory context

The role of the toxicologist is to ensure the safety of people who may be exposed to chemicals via the environment, in the workplace or voluntarily through using cosmetic ingredient, consumer products or pharmaceuticals. The data generated and reported by toxicologists must therefore be entirely reliable, and this requires total integrity on the part of everybody involved. While one would hope that all toxicologists work to the highest professional and ethical standards, this has not always been the case, and it has been necessary to implement regulations to ensure the maintenance of appropriate standards in the industry. Furthermore, in order to compare the potential toxic effects of different substances, data must be generated in a standardised manner. This is facilitated by the provision of test guidelines by a variety of international agencies.

The main regulations addressing the issue of data generation and reporting are the Good Laboratory Practice (GLP) regulations. These were first drafted in response to scandals such as the one relating to the initial safety assessment of the artificial sweetener aspartame in the early 1970s, when the manufacturer Searle was accused of bad practice in the conduct of toxicity tests and misrepresentation of the results obtained. The GLP regulations do not provide step-by-step instructions for toxicology testing; rather, they specify various generic aspects of good practice including the use of Standard Operating Procedures, the need for verification at all stages of a laboratory investigation, and criteria
for record keeping, reporting and archiving. Since the implementation of the GLP regulations, additional regulations have been drawn up: these include Good Manufacturing Practice for the manufacture of medicinal products, Good Distribution Practice regulations covering transportation of medicinal products, Good Clinical Practice regulations for clinical trials and Good Pharmacovigilance Practice relating to the monitoring of the safety of medicines. In the United Kingdom, the agency responsible for invigilating these regulations is the Medicines and Healthcare Products Regulatory Authority (MHRA).\textsuperscript{20}

International bodies including the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the Organisation for Economic Co-operation and Development (OECD) provide formal regulatory guidelines specifying procedures for various aspects of pharmaceutical manufacturing and \textit{in vivo/in vitro} testing. The ICH Guidelines are categorised under the headings of Quality Guidelines, Safety Guidelines, Efficacy Guidelines and Multi-disciplinary Guidelines,\textsuperscript{21} while OECD Guidelines cover tests for the physical–chemical properties of chemicals, environmental effects, degradation and accumulation in the environment, human health effects and other areas.\textsuperscript{22}

When conducting tests for regulatory purposes it is essential to ensure that they are conducted according to the appropriate guidelines and are, where appropriate, compliant with the GLP regulations; regulatory authorities such as the MHRA in the United Kingdom, the European Medicines Agency (EMA) in Europe and the US FDA specify the circumstances under which GLP-compliant data generated according to international guidelines are required.

### 1.5.5 Limitations of whole animal studies

\textit{In vivo} studies have a number of limitations which have led to ever-increasing efforts to develop better and more informative alternatives. Alternative approaches include endpoint assays, tissue slices, toxicokinetic modelling, structure–activity relationships, database interrogation and tissue culture (Kniewald \textit{et al}., 2005). The available \textit{in vitro} systems for studying target organ toxicity include perfused organ systems, precision-cut tissue slices, established cell lines, isolated cells in suspension and primary cell cultures. The most important factors in selecting an \textit{in vitro} system are retention of differentiated functions and demonstration of good \textit{in vivo–in vitro} correlations for the phenomena of interest. The greatest progress in the use of \textit{in vitro} test systems has been in the areas of target organ toxicity (particularly hepatic, renal and neural toxicity) and local ocular–dermal toxicity.

The drive to reduce animal usage in toxicity testing has led to the establishment of international bodies to oversee the move towards the use of alternatives. These include the European Centre for the Validation of Alternative Methods (ECVAM),\textsuperscript{23} which was established in 1991 in response to an EU Directive

\textsuperscript{20}http://www.mhra.gov.uk/Howweregulate/Medicines/Inspectionandstandards/index.htm
\textsuperscript{21}http://www.ich.org/products
\textsuperscript{22}http://www.oecd.org/env/ehs/testing/oecdguidelinesforthetestingofchemicals.htm
\textsuperscript{23}http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam
mandating active support for the development, validation and acceptance of methods to replace, reduce or refine the use of animals in laboratories and its US equivalent, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). Both these organisations support progress towards the reduced use of animals in toxicity testing by monitoring the evaluation of new methods and organising collaborative validation programmes.

3Rs and the place of in vitro tests in safety evaluation The 3Rs principle mandates the Replacement, Reduction and Refinement of the use of animals in scientific experimentation. The need for good in vitro toxicity tests has become even more urgent following the implementation of the REACH regulations in 2007. The need to test the toxicity of an estimated 30,000 existing chemicals that are currently marketed in volumes greater than 1 tonne per year has led to predictions that as many as four million animals will be used in testing unless suitable alternative methods become available (Clemedson, 2008).

The use of in vitro methods in regulatory toxicity testing has conventionally been limited to defining the potential for in vivo toxicity and verifying negative results. The effort to further develop and validate better in vitro alternatives has become increasingly urgent following the enactment of the REACH regulations in June 2007 and the 7th Amendment in March 2009. In particular, the 7th Amendment has prohibited animal testing of cosmetic ingredients, including the use of animals for skin irritation, corrosion, and genotoxicity testing, since March 2009. Furthermore, the use of animals for absorption, distribution, metabolism and excretion (ADME) testing of cosmetic ingredients has been banned since March 2013.

The conventional view is that in vitro tests are suitable for hazard identification but not for the other aspects of risk assessment, and the use of in vitro tests has tended to comply with these perceived limits. In the pharmaceutical industry, however, strenuous efforts have been made to overcome these limitations and predictions of bioavailability, pharmacokinetics and pharmacodynamics are now integrated into the early stages of drug development, being conducted concomitantly with efficacy tests. The results obtained are used to refine the process of selection of drug candidates and this approach has been found to save time, money and resources in the early stages of drug development.

The development of integrated testing strategies is key to the implementation of the 3Rs in risk assessment. This entails using a range of different types of information (of the kinds described in the remainder of this book) and combining such different inputs rationally in regulatory decision-making. Animal testing should be a final resort, carried out only in cases where insufficient pre-existing information is available. Before doing so, a number of questions should be addressed:

- Can a TTC approach be justified?
- Is there scope for the use of an integrated testing strategy or an in silico approach?

http://iccvam.niehs.nih.gov/
Is it possible to extrapolate from pre-existing information, including data from the literature or other information which is in the public domain? While this may not have been generated according to GLP standards, it can be used in some settings; for example, the REACH regulations do not require all the information in a dossier to meet GLP criteria.

It is important to optimise *in vivo* assays in order to obtain the best possible data without causing undue pain and suffering.\textsuperscript{25} Annexes VII to X in the REACH regulations provide specific examples of situations in which animal testing can be avoided by taking advantage of pre-existing information. The sequence in which tests are performed is critical: it is important to give thought to planning a sequence of tests which will generate the maximum amount of information with the lowest severity and least suffering to animals.

In order to identify the chronic effects of a test compound, suitably long-lived *in vitro* systems, representing all cell types of each organ/tissue are needed. These must be capable of providing an estimate of the potential impact of repeated exposure on those organs/tissues. Any effects in these *in vitro* systems will have to be assessed by reference to kinetic and modelling data which allow the prediction of potential tissue and organ interactions which may be important in risk assessment. The final stage of risk assessment is the evaluation of risk to the consumer from exposure which may arise from the intended use of the chemical. This requires establishment of a quantitative link between the results of the *in vitro* assays and human exposure.

### 1.5.6 Use of human tissues in toxicology

The use of human tissue for *in vitro* toxicology is becoming increasingly popular because it is considered to provide more relevant data than do animal models. Human tissue-based methods also avoid the animal welfare concerns inherent in conventional animal-based toxicology studies, although they have their own problems relating to the legal, ethical and informed consent issues surrounding human tissue acquisition.

Three main sources of human tissue are typically used in drug development:

- **Tissues residual to surgery**: tissues not required for diagnosis or which are generated by cosmetic procedures can be accessed rapidly and stored as fresh, fixed or frozen tissues.
- **Tissues and organs from transplant procedures**: organ donation rightly takes precedence over research; however, many organs cannot be used in a transplant procedure and may be consented for use in medical research.
- **Tissues retrieved post-mortem**: these tissues are most often frozen or fixed and used in target discovery or identification.

The regulatory environment surrounding the use of human tissues in UK research has become much more demanding following the implementation of the Human Tissue Act (2004) and the Human Tissue (Scotland) Act (2006). In particular there is now a requirement for all end users to register and have a designated

\textsuperscript{25} For a detailed discussion of this issue, see Madden et al. (2012).
person to deal with approved procedures and record keeping. Key facts relating to the Human tissue Act (2004) are summarised in Box 1.1.

**Box 1.1 Human Tissue Act 2004**


The Human Tissue Act 2004 covers England, Wales and Northern Ireland. It established the HTA to regulate activities concerning the removal, storage, use and disposal of human tissue. Consent is the fundamental principle of the legislation and underpins the lawful removal, storage and use of body parts, organs and tissue. Different consent requirements apply when dealing with tissue from the deceased and the living. The Human Tissue Act 2004 lists the purposes for which consent is required (these are called Scheduled Purposes).

There is separate legislation in Scotland - the Human Tissue (Scotland) Act 2006. While provisions of the Human Tissue (Scotland) Act 2006 are based on authorisation rather than consent, these are essentially both expressions of the same principle.

Further information about the Human Tissue (Scotland) Act 2006

**The key points of the Human Tissue Act 2004**

The Human Tissue Act 2004 regulates the removal, storage and use of human tissue. This is defined as material that has come from a human body and consists of, or includes, human cells.

The Human Tissue Act 2004 creates a new offence of DNA ‘theft’. It is unlawful to have human tissue with the intention of its DNA being analysed, without the consent of the person from whom the tissue came.

The Human Tissue Act 2004 makes it lawful to take minimum steps to preserve the organs of a deceased person for use in transplantation while steps are taken to determine the wishes of the deceased, or, in the absence of their known wishes, obtaining consent from someone in a qualifying relationship.

**Offences under the Human Tissue Act 2004**

- Removing, storing or using human tissue for Scheduled Purposes without appropriate consent.
- Storing or using human tissue donated for a Scheduled Purpose for another purpose.
- Trafficking in human tissue for transplantation purposes.
- Carrying out licensable activities without holding a licence from the HTA (with lower penalties for related lesser offences such as failing to produce records or obstructing the HTA in carrying out its power or responsibilities).
- Having human tissue, including hair, nail and gametes (i.e. cells connected with sexual reproduction), with the intention of its DNA being analysed.

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26 [http://www.hta.gov.uk/legislationpoliciesandcodesofpractice/legislation/humantissueact.cfm](http://www.hta.gov.uk/legislationpoliciesandcodesofpractice/legislation/humantissueact.cfm), Downloaded with the permission of the Human Tissue Authority.

without the consent of the person from whom the tissue came or of those close to them if they have died. (Medical diagnosis and treatment, criminal investigations, etc. are excluded).

The first four offences only apply in England, Wales and Northern Ireland, although the Human Tissue (Scotland) Act 2006 has similar offences and penalties. The offence of DNA theft applies UK-wide. To find out more about the offences you can download the Human Tissue Act 2004\(^\text{28}\) and Human Tissue (Scotland) Act 2006.\(^\text{29}\)

(Source: Reproduced with permission of the Human Tissue Authority)

The remaining issues surrounding the routine use of human tissues in toxicology include:

- **The need to ensure a regular, reliable supply:** In particular, suitable infrastructure must be in place if using fresh tissues and it may be necessary to have an on-call rota of individuals trained to receive and process tissue at short notice.

- **Inter-individual variability:** This may be perceived to be both a limitation of the use of human tissues (since it will increase the variability of the results of *in vitro* studies) and an advantage (since this represents the true range of responses within the general population).

- **Regulatory acceptability:** Regulatory bodies are seeing more and more data from *in vitro* studies with human tissue; this is therefore becoming less of an issue as time moves on.

The expert view is that *in vitro* human tissue has great potential as an experimental system in various aspects of drug development as well as in other aspects of toxicology, but that there is still room for improvement and expansion of its application (Clotworthy, 2012).

1.6 **Summing up**

This chapter should have provided sufficient background to allow the readers to put into context the molecular and cellular processes described in subsequent chapters. The further reading recommended here is intended to help readers who have found any of the concepts unfamiliar, and therefore focuses on key pathology and toxicology textbooks containing background information relevant to the remaining chapters of this book.

**Self-assessment questions**

- It could be said that ‘Apoptosis is a double-edged sword’. Do you agree with this statement?

- What are the ethical issues involved in the use of human tissues for research purposes?

\(^{28}\) http://www.legislation.gov.uk/ukpga/2004/30/contents

Background reading


References

Benford, D., Bolger, P.M., Carthew, P. et al. (2010) Application of the margin of exposure (MOE) approach to substances in food that are genotoxic and carcinogenic. *Food and Chemical Toxicology*, 48 (Suppl 1), S2–S24.


