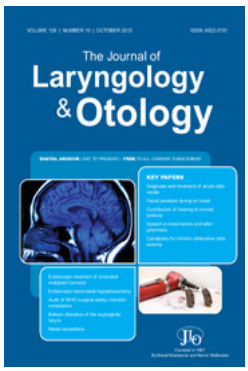

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M. W. Saunders and M. A. Birchall

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Apoptosis, matters of life and death

M. W. SAUNDERS, F.R.C.S.*†, M. A. BIRCHALL, F.R.C.S., M.D.†

Introduction

Cell death and apoptosis

The number of cells in a population is regulated not only by the rate of cell division but also by the rate of cell loss. Although this seems a relatively straightforward concept it was not until programmed cell death (PCD or apoptosis) was described in 1972 by Kerr *et al.* (Kerr *et al.*, 1972; 1994) that it was generally appreciated that cell numbers and hence physiological and pathological processes may be affected by affecting the rate of cell loss as much as by cell division. In the last two decades the mechanisms and methods of induction of apoptosis have been the subject of enormous research interest, particularly the role of apoptotic mechanisms and their malfunction in carcinogenesis.

Apoptosis and necrosis

Necrotic cell death represents an uncontrolled destruction of the cell in response to an external stimulus resulting in cellular anoxia and eventual disruption of the plasma membrane with loss of cell contents. By comparison, apoptosis is a controlled active process which may be initiated from within the cell or in response to a variety of extra-cellular stimuli. Cellular components are systematically broken down without disruption of the cell membrane and the cell is divided up into membrane-bound apoptotic bodies which are then phagocytosed by neighbouring cells or macrophages. By avoiding release of intracellular matter, the intense peri-cellular inflammation associated with necrosis is avoided, and cells may be removed from populations

TABLE I
SUMMARY OF DIFFERENCES BETWEEN APOPTOSIS AND NECROSIS

	Apoptosis	Necrosis
Occurrence	As part of physiological or pathological processes	Always pathological
Initiating factors	Range of extracellular and intracellular factors	As a direct result of injury and cellular anoxia
Distribution	Isolated apoptotic cells scattered widely throughout tissues	Occur in groups/whole areas of tissue
Mechanism	Active process – ATP dependent-regulated mechanism	Passive loss of regulation
Cytoplasmic changes	Cytoplasmic shrinkage Blebbing of cell membrane into apoptotic bodies Many organelles preserved and bound into apoptotic bodies	Swelling of cell membrane Rupture of cell membrane with loss of contents to extracellular space Organelles lost
Nuclear changes	Endonuclease activation, division of DNA into *oligosomal fragments*	Random digestion of DNA
	Chromatin condenses into specific 'crescents' visible on light microscopy	Chromatin flocculates
Effect on surrounding tissue	Little	Intense inflammation

From the Departments of Otolaryngology, Great Ormond Street Hospital*, London and University of Bristol, Southmead Hospital†, Bristol, U.K.

without affecting adjacent tissue. The principal differences between apoptosis and necrosis are summarized in Table I.

Induction of apoptosis

It is a key feature of apoptosis that it may be triggered by intracellular stimuli and hence provides a means whereby a cell having recognized itself as irreparably damaged may destroy itself; this is termed 'deliberate cell suicide'. At the centre of this process is the nucleoprotein product of the *p53* gene which has been dubbed the 'guardian of the genome' (Lane, 1992). In response to DNA damage (e.g. by radiation or virus) p53 may lead to cell cycle arrest to allow damaged DNA to be repaired, and if repair is not possible, *p53* may initiate apoptosis, preventing the replication of abnormal genetic material. *p53* interacts with a complex family of other nucleoproteins (e.g. Bax, BCL-2 and *p21*), the exact molecular mechanisms of apoptosis induction remain the subject of intense research activity. It follows that failure of normal *p53* function may lead to replication of cells with abnormal DNA and hence to malignant transformation. At least half of all malignant tumours are found to have abnormalities of the *p53* gene resulting in abnormal 'mutant' *p53* (Carson and Lois, 1995). Gene therapy for neoplasia relies on replacing mutant *p53* genes with normal or wild type genetic material to prevent further replication of abnormal DNA.

Extra-cellular influences on apoptosis are not necessarily dependent on *p53*. They include hormonal influences such as growth hormone withdrawal and the effects of drugs, notably chemotherapeutic agents for cancer (*vide infra*).

Dexamethasone induces apoptosis in thymocytes (Wyllie, 1980). and this is often used as a control in studies of programmed cell-death. Apoptosis is thought to be an important process in the reduction of inflammatory cell numbers and resolution of inflammatory processes (Haslett, 1992). In acute asthma treated with corticosteroids the induction of apoptotic cell death in eosinophils in sputum has been observed to accompany resolution of the acute attack (Woolley *et al.*, 1996). Cytokines such as GM-CSF and IL-5 which act as intercellular messengers have been found to prevent apoptosis in eosinophils (Stern *et al.*, 1992) and production of these cytokines may be suppressed by steroids (Cox *et al.*, 1991). In addition, apoptosis is induced in the inflammatory layer of human nasal polyps by *in vitro* culture with dexamethasone (authors 1997, unpublished data) and it is possible that this is one of the mechanisms by which corticosteroid treatment leads to regression of nasal polyps.

Apoptosis in non-malignant disease

Even in the developing embryo, apoptosis has a fundamental role to play in tissue differentiation and structural change. The re-absorption of interdigital webs *in utero* is a widely quoted example of this. Apoptosis has been demonstrated in the mouse inner ear at an early stage of normal development (Nishizaki *et al.*, 1998) and abnormalities of apoptotic mechanisms may be implicated in the pathogenesis of cleft palate.

Although apoptosis research is commonly associated with the study of cancer and cancer therapy, it is inevitable that such a fundamental biological process will be implicated in some way in the majority of human pathology.

Human disease may be associated with inhibition of normal apoptosis, or an abnormal rise in apoptotic rate (Thompson, 1995). Reduced lymphocyte sensitivity to normal cell death signals has been implicated in auto-immune disease (Mountz *et al.*, 1994). Viral infection may prevent cell suicide by the expression of gene products such as BCL-2 which mitigate against a cell's ability to induce apoptotic death: a cell with normally functioning apoptotic mechanisms might recognize the cellular and genetic damage associated with viral infection, but by preventing cell death from occurring, the virus keeps the cell alive long enough for viral replication to take place (Henderson *et al.*, 1991).

Abnormally elevated rates of cell death have been associated with degenerative diseases such as Alzheimer's, heart failure and retinitis pigmentosa, in which specific gene mutations lead to apoptosis of photoreceptor cells. Loss of retinal cells may be predisposed to by up to three completely separate gene mutations.

There is particular interest in abnormal cell death in human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS) (Gougeon and Montaigner, 1993). The immunodepletion of CD4 positive T cells that is a fundamental part of this disease is caused by programmed cell death. Interestingly, it appears that infection with the HIV virus can also lead to apoptosis in non-infected T lymphocytes by interference with cell surface signalling mechanisms.

Experimental evidence suggests that apoptotic cell death in the labyrinth is responsible for several forms of sensorineural deafness. Usami (Usami *et al.*, 1997) detected apoptosis in the cochlear hair cells of senescence accelerated mice, hair cell apoptosis has been detected after noise trauma (Nakagawa *et al.*, 1997) and aminoglycoside antibiotics have been shown to induce apoptosis in hair cells in experimental animals.

Measurement and identification of apoptosis

Identification of programmed cell death takes advantage of the highly characteristic biochemical and morphological changes that occur in the apoptotic cell. These are summarized in Table II. Some techniques are applicable only to single cell suspensions, others may be used to identify cell death in whole tissues such as sections of pathological specimens.

Morphological identification

Electron microscopy represents the most reliable method for qualitative identification, but to estimate the proportion of cells undergoing apoptosis in a

TABLE II
BIOCHEMICAL AND MORPHOLOGICAL FEATURES OF APOPTOSIS

Early/Biochemical Changes	*Applicable method of detection*
Rise in intracellular calcium	Flow cytometery, gel electrophoresis (single suspension)
Activation of endonuclease	End labellingof 3-OH termino of oligosomal
DNA divided into 180 base pair fragments (oligosomal fragments)	(fragments-ISEL/TUNEL (single cells or tissue sections)
Late/Nuclear Morphological Changes	
Chromatin condenses	Conventional histological stains (whole sections)
Nuclei pyknotic/small	Electron microscopy (single cells and whole sections)
hallmark 'crescents' visible in nucleus	
Late/Cytoplasmic Morphological Changes	
Cytoplasmic shrinkage away from neighbouring cells	Conventional histological stains (whole sections)
Preservation of some organelles	Electron microscopy (single cells and whole sections)
Cell divides into membrane bound apoptotic bodies	Acridine orange staining (single cell suspension)
Phagocytosis by neighbouring cells/macrophages	

section it is necessary to use high magnification (× 100) light microscopy with a variety of staining techniques. A disadvantage of morphological identification is that cells remain apparently normal to inspection some while after the nuclear biochemical events have started, so that biochemical techniques of identification allows a greater and more representative number of apoptotic cells to be identified.

Biochemical identification

After apoptosis is initiated, a calcium-dependent endonuclease splits cellular DNA into fragments of specific base pair length. These *oligosomal fragments* are a biochemical hallmark of apoptosis and may be identified by flow cytometry or by agarose gel electrophoresis where a characteristic 'DNA ladder' is obtained. These techniques are only possible with single cell suspension, however, it is possible to label the 3-OH terminal of the oligosomal fragment with a labelled nucleotide which is then identified by labelling with a secondary antibody. This *in situ* (ISEL) or *TdT mediated* (TUNEL) end-labelling may be used on sections of whole tissues to identify areas of apoptosis and is hence of more use in clinical research.

Apoptosis in head and neck cancer

Cancer cell death

The biggest challenge in modern oncology is the removal of cancerous cells at the same time as maintaining the normal cell population. Cells may be lost from a tumour in three ways: necrosis, shedding or apoptosis. Whilst some strategies, such as tumour embolization, may induce necrosis, this is of necessity a messy affair with local and systemic effects, and is not tumour-specific. Inducing abnormal epithelial cells to shed onto surfaces or into lumina where they may no longer cause harm is an attractive idea, but one unlikely to allow removal of any but the most superficial layers of cells (Rew, 1993). This leaves us with apoptosis, or programmed cell death. Even 'mitotic cell death', the title given to the cell arrest seen with radiotherapy and some forms of chemotherapy, ultimately, leads to apoptosis (Joiner, 1993), whilst this induction is more direct with most chemotherapy, such as cisplatinum. Hence, an understanding of apoptosis in head and neck cancer is vital to the development of more effective, and less toxic, therapies.

Apoptosis in the development of cancer

There is some evidence that apoptosis is disordered in pre-malignant tissues of the head and neck. As one progresses from normal, through dysplastic to frankly malignant oral epithelium, apoptosis fails to rise in proportion to mitosis (Birchall *et al.*, 1995) (Figure 1). There is also a topographic switching of mitosis and apoptosis in premalignant epithelium, with peak apoptosis moving more superficially and peak mitosis moving to the basal layer (Birchall *et al.*, 1996). These two events are likely to lead to a loss of normal policing of aberrant cells, particularly in the basal, putative stem-cell, layer where mutations are most likely to have an impact on the eventual development of invasive disease. The underlying molecular events presaging malignancy are unclear, but there appears to be a paradoxical fall in expression of *bcl-2*, a key gene in the cellular control of apoptosis, in premalignant and malignant epithelia of the head and neck (Birchall *et al.*, 1997). The importance of pinning down the defects in apoptosis present in pre-malignant tissue lies in the

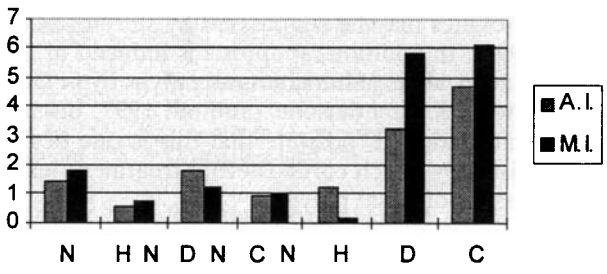

FIG. 1

Apoptotic and mitotic indices for oral epithelium. Apoptotic (AI) and Mitotic (MI) indices for oral epithelium. N = normal, H = hyperplasia, D = dysplasia, C = invasive carcinoma. HN = normal tissue from people with hyperplasia, DN = normal tissue from people with dysplasia, CN = normal tissue from people with invasive carcinoma.

development of effective strategies for preventing the occurrence of metachronous cancers, a particular problem in head and neck cancer (Jones *et al.*, 1995).

Apoptosis in untreated tumours

Studies of the difference between actual and potential doubling time of tumours (T_{pot}) (Wilson, 1991), show that more than 90 per cent of active tumour cells must survive for only a brief time before dying, probably of apoptosis. The measurement of apoptosis in tumours *in vivo*, however, is far from straightforward. Direct counts of apoptotic cells in tumour sections give a reasonable intra-tumour variability, but there is a long learning curve and this process is very laborious, even with image-analysis. The use of end-labelling techniques (Wijsman *et al.*, 1993) to stain apoptotic cells has been questioned, and it has been demonstrated that, for head and neck cancer, it was a less accurate method than simple counts (Saunders, 1997, unpublished data). Provided it is possible to verify light microscopic observations with electron microscope appearances this should remain the gold standard, and recent advances in histological techniques have simplified matters (Amal *et al.*, 1998). However, recent confirmation of impressive morphological diversity within programmed cell death, which has been hitherto described in terms of the stereotyped features seen in cells of lymphoid lineage threatens even this method of measurement (Amin and Bowen, 1997).

Another complication is the presence of tumour-infiltrating lymphocytes. These too may undergo apoptosis, and the differentiation between separate cell lines undergoing apoptosis can be difficult. In fact, this phenomenon is important in itself: squamous carcinoma can induce apoptosis in white cells, and this may be a potent means by which tumours escape the immune surveillance that might otherwise clean them out (O'Mahony *et al.*, 1993).

Measuring head and neck cancer cell line apoptosis *in vitro* is a simpler matter, and is usually done in a number of ways in every experiment to increase validity. Typically, morphological counts will be performed along with tests of dye uptake (acridine orange) and DNA laddering (Sarraf and Bowen, 1986). However, even here, observations of more than one type of programmed cell death have recently been made on a tongue squamous cell carcinoma line (Amal A., personal communication). Each type of apoptosis may require different triggers and have separate sub-cellular mechanisms (Clarke, 1990). Thus, much work remains to be done to unravel the normal patterns of apoptosis in head and neck tumours, both at the cellular and sub-cellular levels, before we can begin to understand what makes a tumour cell kill itself, and therefore how we may persuade more to do so.

The control of apoptosis in squamous carcinoma cells is known to be disordered. The most consistent gene-alteration seen in head and neck cancer is that of the *p53* gene. Normally, this promotes apoptosis in response to various stimuli, and may be regarded as the cellular 'policeman'. It is involved in a reciprocal arrangement with dimers of the *bcl-2* family, the resolution of which determines whether a cell commits suicide (Raff, 1992). Thus, an aberrant *p53* gene will tip the balance in the favour of cell survival, even in the presence of a disordered genome, with progression of cancer the likely result. There are many other genes and gene products involved in the apoptosis pathway, which is becoming increasingly complex. Many of them have identified abnormalities in head and neck cancer (Ogden *et al.*, 1992), and are therefore potential therapeutic targets.

Apoptosis as a result of therapy

Whenever we touch a tumour, be it with knives, radiation or drugs, we alter its biological behaviour. Again, this may be measured in terms of an increase in T_{pot} (Wilson, 1991). Unfortunately, the result, for example after open biopsy or recurrence after radiotherapy, may be a more aggressive tumour. Conversely, however, the main mode of action of the main chemotherapeutic agents used against head and neck cancer seems to be via apoptosis, and it is likely that mitotic cell death after radiotherapy is ultimately along apoptotic pathways. Nonetheless, these strategies are blunderbuss ones, causing death (by apoptosis, necrosis, or simple excision) of many normal cells necessary for the maintenance of local and systemic functional integrity.

What is required is a better directed means of apoptosis-induction in tumour cells. A number of approaches to this are currently being explored, particularly via gene therapy. One strategy is to insert copies of normal *p53* into tumours where aberrant expression has been detected. This may be achieved using an adenovirus vector, and has been shown to cause regression of tumours *in vivo* (Clayman *et al.*, 1996). However, the effects seem to be short-lived, and complete regression in man has not been seen. An alternative form of gene therapy is to try and stimulate local immune cells to recognize and kill tumour cells *in vitro* and then return them to the circulation. This is a reasonable route where one can identify a definite target antigen, such as that for EBV in EBV-related nasopharyngeal carcinoma, but is less practical for most squamous cancer (R. Hawkins, personal communication). An alternative to gene therapy is the activation of tumour-cell receptors that initiate apoptosis, particularly via the growth factor receptor-tyrosine kinase cascade. This is currently being pursued in a number of centres world-wide, with several agents in phase I/II trials in head and neck cancer patients (e.g. Bier *et al.*, 1995).

Conclusions

Apoptosis and cell death is as fundamental a part of life as mitosis and cell division. It is a key process in development and also a way in which abnormal and effete cells can be removed from the host before they cause problems.

Such an important part of normal cellular activity is inevitably implicated in many pathological conditions, and it is only in recent years that it has become apparent by how much. The implication of understanding and hence being able to influence this process is enormous: if we wish to kill tumours, we need to know what makes them die. Most head and neck cancer cells die via apoptosis and this is a highly-controlled process with consequent potential for manipulation. Therefore, research into types and mechanisms of apoptosis of head and neck cancer cells is a vital pre-requisite to the development of tomorrow's effective therapies for the victims of this, in many ways the most devastating of cancers.

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Address for correspondence:
Mr M. Saunders,
Department of Otolaryngology – Head and Neck Surgery,
Southmead Hospital,
Bristol BS10 5NS.