Mechanism of Cell Death: Early Morphological Changes and DNA Degradation of Apoptosis in Rat Thymocytes

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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July 1994
Abstract

Apoptosis is an active process of gene-directed cellular self-destruction. It serves a biological meaningful and homeostatic function in diverse circumstances, including embryonic development, physiological involution or pathological atrophy of adult organs, normal tissue turnover, immune cell ontogeny and immune killing.

A new flow cytometric method using Hoechst 33342/propidium iodide staining has been established in this study. The basis of this method is the change of apoptotic thymocytes both in cell size and membrane permeability. This method has been used to study glucocorticoids or DNA topoisomerase inhibitor-induced apoptosis in immature rat thymocytes both in vitro (dexamethasone, etoposide, m-AMSA and camptothecin) and in vivo (etoposide).

During establishment of the flow cytometric method, it was found that induction of apoptosis by dexamethasone was not inhibited by zinc as assessed by flow cytometry. Further studies indicated that apoptotic thymocytes induced by dexamethasone in the presence of zinc showed distinct morphology and other cellular changes characteristic of apoptosis but no DNA laddering. Thus, internucleosomal cleavage of DNA was, for the first time, dissociated experimentally from early nuclear morphological changes, as well as other cytoplasmic changes. Identification of a transitional preapoptotic population of thymocytes further confirmed this observation. In this population of cells, early nuclear morphological changes and other cytoplasmic changes were observed in the absence of DNA laddering.

By using field inversion gel electrophoresis, it was found that formation of kilobase pair fragments of DNA preceded internucleosomal cleavage of DNA. Formation of large fragments of DNA was particularly visualised when thymocytes were treated with dexamethasone in the presence of zinc, due to an inhibitory effect of zinc on the internucleosomal cleavage of DNA. These large fragments of DNA were also present in the transitional preapoptotic population of thymocytes in the absence of oligonucleosomal fragments. Further studies with isolated thymocyte nuclei showed that formation of these large fragments of DNA was a Mg^{2+}-dependent process that can be facilitated by Ca^{2+}. The nature of these large fragments, the mechanism involved in their formation and their relevance to apoptosis require further investigation.
Acknowledgements

I am indebted to Dr. Gerry Cohen for his supervision and financial support throughout the work for this thesis, as well as for providing a stimulating and exciting environment to work in. I also wish to thank Dr. Lewis Smith for offering me a place to do my PhD study. I would also like to use this opportunity to thank the Chinese educational authority and the British Council for initiating this study.

My thanks also go to Dr. David Dinsdale and Mr. Matthew Lee for all the help and advice in ultrastructural observation, Dr. Michael Ormerod for help in establishing the flow cytometric method, Dr. Philip Carthew and Mrs. Jennifer Edward for help with slide preparation and reading, Drs. Ludmila Kokileva and Reg Davies for help in making thymocyte nuclei, Mr. Roger Snowden for all the work on the flow cytometer and Mr. David Brown for establishing FIGE.

I am grateful to Dr. Marion MacFarlane, Mr. Roger Snowden and Mr. David Brown for proof-reading of my manuscript. They have had a difficult job to do. I could not present this thesis without their efforts.

In addition, I would like to thank friends and colleagues at the MRC Toxicology Unit, particularly those in this laboratory, Drs. Michael Butterworth and Jane Holley, Gareth Bicknell, Howard Fearnhead, James Wolfe, Liz Slee, Salmaan Hussain, Drs. Kelvin Cain and Mehran Magsoudloo for their stimulating discussion, sense of humour and friendship.

My greatest thanks go to my parents, my wife, Chao-Ying, and my son, Yu-Jia for their understanding and support during these years, especially when I was absent. I would like to dedicate this thesis to them.
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>BCECF-AM</td>
<td>2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
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<tr>
<td>CD</td>
<td>Cluster designation</td>
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<tr>
<td>CFDA</td>
<td>5,6-carboxyfluorescein diacetate</td>
</tr>
<tr>
<td>DiCCFDA</td>
<td>5,6-carboxy-4',5'-dimethyl fluorescein diacetate</td>
</tr>
<tr>
<td>DN</td>
<td>Double negative</td>
</tr>
<tr>
<td>DP</td>
<td>Double positive</td>
</tr>
<tr>
<td>EB</td>
<td>Ethidium bromide</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
<td>Ethyleneglyco-bis-(β-aminoethyl)-N,N',N'-tetraacetic acid</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>FBS</td>
<td>Fetal bovines serum</td>
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<td>FDA</td>
<td>Fluorescein diacetate</td>
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<td>FIGE</td>
<td>Field inversion gel electrophoresis</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>FLS</td>
<td>Forward light scatter</td>
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<td>H&amp;E</td>
<td>Hematoxylin and eosin staining</td>
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<td>H33342</td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td>ip</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilobase pair</td>
</tr>
<tr>
<td>m-AMSA</td>
<td>4'-(9-acridinylamino)methanesulfon-m-anisidide</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium Eagle</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>SDS</td>
<td>sodium deodecyl sulfate</td>
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<tr>
<td>SP</td>
<td>Single positive</td>
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Chapter 1. Introduction
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1.1. Types of cell death

Cell death is still an unclear biological phenomenon from the point of view of the mechanisms involved. Defined as an irreversible loss of cell structure and function, cell death has been considered for more than a century as a primary degradative or degenerative phenomenon, and an essentially passive 'running down' process as a consequence of primary interference with the structure and function of chemical components essential for cell integrity. This classic concept of the pathogenesis of cell death was challenged by experiments that showed that cells, at least in the liver and a number of other cell types, did not die when protein and RNA synthesis were almost completely inhibited. Cells could also survive even when polysomes were extensively desegregated. Cells still appeared viable even when intracellular concentrations of K+ and Na+ were changed and cellular ATP was decreased to 20% or less of the control value, as well as the nucleolus and many cytoplasmic membranes were severely disorganised for up to 48 h (McLean et al, 1965 and Farber et al, 1971). Cell death can occur as a controlled event in healthy tissues or organs, e.g. focal cell death in the morphogenesis of normal embryos (Glucksmann, 1951 and Saunders, 1966). By different approaches (see Wyllie et al, 1980 for a review of these approaches), two commonly occurring, distinct patterns of morphological changes in cell death have been identified, i.e. necrosis and apoptosis (Kerr et al, 1972), which are intimately related to the mechanisms leading to changes in cellular homeostasis and finally to the loss of cell viability. These two patterns can actually cover most cell death found in higher animals under natural and experimental conditions. It is now clear that cell death plays an important role in normal development and metamorphosis, in a wide variety of diseases including many of toxicological origin and also in the chemotherapy and radiotherapy of diseases of abnormal growth, i.e. cancer. Cell death, together with proliferation and differentiation, maintains the balance between cell populations and manifests pathological status in a multicellular organism.
### Table 1.1 Landmarks in cell death research

<table>
<thead>
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<th>Year</th>
<th>Researchers</th>
<th>Contents</th>
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<tr>
<td>1850's</td>
<td>Virchow</td>
<td>Cellular pathological description of cell death</td>
<td>Virchow, 1858</td>
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<td>Glucksmann</td>
<td>Classification of cell death based on evolutionary significance</td>
<td>Glucksmann, 1951</td>
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<td>1960's</td>
<td>McLean</td>
<td>Experimental description of cell death by non-physiological, chemical insults</td>
<td>McLean et al, 1965</td>
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<td>Saunders</td>
<td>Experimental description of cell death in development</td>
<td>Saunders, 1966</td>
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<td></td>
<td>Lockshin</td>
<td>Concept of programmed cell death</td>
<td>Lockshin and</td>
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<td></td>
<td>Williams, 1964</td>
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<tr>
<td>1970's</td>
<td>Farber</td>
<td>Concept of cell suicide</td>
<td>Farber et al, 1971</td>
</tr>
<tr>
<td></td>
<td>Kerr, Wyllie</td>
<td>Morphological description of different types of cell death, concept of apoptosis</td>
<td>Kerr et al, 1972</td>
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<tr>
<td></td>
<td>and Currie</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1990's</td>
<td>Raff</td>
<td>Social control of cell survival and death</td>
<td>Raff, 1992</td>
</tr>
</tbody>
</table>
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1.1.1. Necrosis

Necrosis is a morphological description of the progressive degradation of cell structure that occurs after sudden environmental changes such as severe hypoxia and ischemia or cell injury by direct damage of the plasma membrane (McLean et al, 1965). Cells undergo death in two stages basically, one reversible and the other irreversible. At the first stage, cell swelling is seen as an increase in cell size, a loss of specialised surface structures such as microvilli and the appearance of surface blebs. Other morphological characteristics of necrosis at this stage are mild swelling of the mitochondria and cell sap, dilatation of the endoplasmic reticulum (ER), depletion of glycogen, dissociation of ribosomes from the ER, and irregular clumping of loosely textured chromatin (flocculation of chromatin). These initial responses are reversible upon removal of the stimulus. If the stimulus is continuous, irreversible changes in the cellular constitution take place. At this later stage, cytoskeletal structures are disrupted. There is a further gross swelling of the mitochondria and appearance of flocculent substances. In some instances, granular densities appear in the cell matrix and these are generally regarded as being the earliest reliable ultrastructural markers of necrosis. Ribosomes are disintegrated and discontinuities in the plasma, organelle and nuclear membranes are developed. The irregularly clumped chromatin eventually disappears to leave ghostlike nuclei.

Histologically, necrosis typically affects groups of contiguous cells, in a synchronous manner, and its occurrence in tissue is usually associated with the development of inflammation. Necrotic cells appear swollen with eosinophilic cytoplasm and have indistinct cell boundaries. The overall nuclear outline tends to be retained initially, but soon shows uniformly condensed chromatin or dispersed chromatin masses inside swollen cells. In the later stages, dissolution of chromatin results in the disappearance of nuclear ghosts.
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Biochemically, it is commonly accepted that direct injury to the cell membrane, e.g. by trauma or by membrane active toxins, or as a result of failure of membrane pumps secondary to cellular energy depletion, causes cations (e.g. Na\(^+\), K\(^+\), Ca\(^{2+}\) and Mg\(^{2+}\)) to move across the membrane along concentration gradients. The accompanying fluid shifts cause cellular swelling, which is the process occurring at the earlier reversible stage of necrosis. However, if the disturbances are severe or persistent, the increase in free cytosolic Ca\(^{2+}\) levels results in an extensive activation of membrane-bound phospholipase that degrades membrane phospholipids to fatty acids and lysophospholipids. This causes further widespread disruption of membranes. The increased cytosolic Ca\(^{2+}\) level is also responsible for releasing lysosomal enzymes, e.g. hydrolases, which cause a rapid acceleration of cellular disintegration in the later stage of the evolution of necrosis. A dramatic fall in intracellular pH results from releasing free fatty acids, free amino acids and inorganic phosphates as a result of degradation of phospholipid, triglyceride, proteins, RNA and DNA. This acidic environment facilitates DNA (already exposed by proteolytic digestion of histones) cleavage by lysosomal deoxyribonuclease into fragments displaying a continuous spectrum of size. The cellular contents released into the extracellular space invoke an inflammatory response.

1.1.2. Apoptosis

Apoptosis is a distinct type of morphological description of cell death that differs fundamentally from necrosis both in its nature and biological significance (Kerr et al, 1972, Wyllie et al, 1980, Walker et al, 1988, Arends and Wyllie, 1991, Cohen, 1991, Raff, 1992 and Schwartzmann and Cidolowski, 1993). Apoptotic cell death is an active process of gene-directed cellular self-destruction, which serves a biologically meaningful, homeostatic function in most circumstances. Apoptosis is named from the ancient Greek word for the 'falling off' of petals from
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flowers, or leaves from trees with the highlight of its kinetic significance as an opposite role to mitosis in the regulation of cell populations.

Apoptosis involves a complex and stereotyped sequence of morphological events. The ultrastructural description is the best way to define the histological manifestation of the process, as some details of this process are difficult to discern with light microscopy. Electron microscopy shows that the structural changes in apoptosis take place in two direct stages: formation of apoptotic bodies and the phagocytosis and degradation of apoptotic bodies by other cells.

In contrast to necrosis, apoptosis tends to affect scattered individual cells rather than groups of contiguous cells. At an early stage, the nuclear outline becomes convoluted and heterochromatin aggregates in dense, sharply circumscribed masses that abut on the nuclear membrane. At the same time, mitochondria are conspicuously normal in structure, but the endoplasmic reticulum dilates and a series of crater-like cavities appear where the dilated cisternae fuse with the cell surface. Otherwise, cytoplasmic organelles are largely intact although side-to-side aggregates of cytoskeletal filaments and semicrystalline arrays of ribosomes may be seen, which are associated with the overall condensation of cytoplasm and accompanied focal vacuolation. Protuberances develop on the cell surface, which can be visualised by scanning electron microscopy as a loss of specialised surface elements such as microvilli and cell-cell junctions. In later stages of the process, the cell further shrinks and the cell outline extraordinarily convolutes and then segregates the cell into a series of membrane-bounded, condensed apoptotic bodies. Some of the nuclear fragments appear uniformly dense whilst others contain more lucent areas. The closely packed organelles in newly formed apoptotic bodies are essentially intact. Individual bodies vary in content, and many lack a nuclear component. The occurrence of apoptosis does not evoke an acute, exudative inflammatory response in the surrounding tissues, even when present in large numbers. Apoptotic cells and bodies are targets of immediate phagocytosis, either by macrophages already present nearby, or by
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other adjacent viable cells (see Savill et al, 1993 for review). Within these phagocytic cells, the compacted organelles and condensed chromatin of the apoptotic cells or bodies may be visible for a few hours but eventually reduce to large, nondescript lysosomal residual bodies. Sometimes, apoptotic cells which have escaped from phagocytosis, such as those that are lost from the epithelial surface into lumina, eventually swell and rupture, undergoing a process called secondary necrosis. Similarly, when apoptosis occurs in a cultured monolayer or suspension in vitro, some of the dying cells are phagocytosed by their neighbours, whilst the majority float off into the supernatant medium or stay in suspension without being phagocytosed and then undergo secondary necrosis. Evidently the characteristic structure of apoptosis persists for only an hour or two.

Programmed cell death. The concepts of programmed cell death and apoptosis have been considered to be related or even interchangeable, but the two terms are definitely not synonymous (Lockshin and Zakeri, 1991). The reason for this is that programmed cell death is an operational definition of a functional relationship in development, whereas apoptosis is an original morphological, but now somewhat biochemical, description of a type of cell death. Alternatively, programmed cell death in development is a scheduled process that will inevitably happen in normal development, while apoptosis may not be a scheduled (programmed) process but rather a cell's response to changes in its local environment. Cells dying for developmental reasons resemble apoptosis far more than they resemble necrosis, especially in many of the morphological aspects, such as controlled shrinkage of the cell, condensation of chromatin and lack of an inflammatory response. However, morphologically and biochemically, programmed cell death and apoptosis differ in several notable respects. For example, cells undergoing programmed cell death frequently manifest involvement of lysosomes, and they rarely show evidence of a laddering pattern of DNA degradation. Practically, it appears useful to consider programmed cell death to be a type of apoptotic (controlled) cell death, recognising that the sequence of events may
differ according to whether the death is planned in a developmental sequence or is a potential solution to an inadvertent but dangerous situation.

1.2. Biochemical and molecular mechanisms of apoptosis

Although apoptosis is a morphological description of a type of cell death, insights into the biochemical and molecular mechanisms have been difficult to obtain, mainly because a very small number of cells within a tissue is affected by this process and condemned cells are intermingled with healthy ones. On the basis of the stereotyped morphology of apoptosis, it has naturally been suggested that there exists a series of intracellular events common to many different situations. A few experimental systems, in which a sufficiently large amount of apoptotic cells can be induced to make biochemical studies possible, have been used in order to answer those basic questions relating to the mechanisms of apoptosis. Among those experimental systems are immature thymocytes and other lymphoid cell lines (for review see Schwartzman and Cidlowski, 1993). Studies on the genetic control of apoptosis (or programmed cell death) have been mainly carried out in insects, e.g. nematode and moths, and transformed lymphoid or leukaemia cell lines (for review see Schwartz, 1991 and 1992 and Vaux, 1993). The major areas of interest have focused on: (1) biochemical and molecular events related to characteristic changes of morphology; (2) signal transduction and (3) its genetic regulation in apoptosis.

1.2.1. Biochemical and molecular events associated with morphological changes

In general, cellular structural changes in apoptosis are intimately related to a cascade of biochemical and molecular events whose alterations (activation or inactivation) can lead to changes in cellular homeostasis and ultimately produce those apoptotic morphological characteristics. Identification of those events
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responsible for the morphological changes will throw light on the mechanistic study of apoptosis.

**Chromatin condensation.** Nuclear chromatin condensation is considered as one of the most distinct morphological features of apoptosis, which is initially associated with a special pattern of DNA degradation, i.e. internucleosomal cleavage of DNA by endonucleases, visualised as a DNA ladder on gel electrophoresis (Skalka, 1976, Wyllie, 1980 and Arends et al, 1990). A selective activation of nuclease(s) within dying cells results in structural changes in the nucleus of apoptotic cells, i.e. chromatin condensation and its margination towards the nuclear membrane. This forms the basis of a universal hypothesis that internucleosomal fragmentation of DNA is an important part of the cell death mechanism. Recently a number of endonucleases, including NUC-18 (Caron-Leslie et al, 1991 and Gaido and Cidlowski, 1991), DNase I (Ucker et al, 1992, Peitsch et al, 1993) and DNase II (Barry and Eastman, 1993) have been identified as potential candidates responsible for this internucleosomal cleavage of DNA. Among those candidates, DNase I and II are actually the constitutive components in a large number of tissues and cells, and are primarily known for their DNA-degradative functions in the digestive tract and the lysosome, respectively. Therefore, the notion that DNA degradation during apoptosis is mediated by a novel nuclease synthesised during cell death may in fact be logically incorrect (Peitsch et al, 1994).

The importance of DNA degradation during apoptosis is obvious because, serving as a protective function, it limits the possibility that transfers genes, in a potentially active state, from dying cells to the nuclei of their viable neighbours (Arends et al, 1990) and additionally prevents an autoimmune reaction triggered by undigested chromatin (Peitsch et al, 1994). Three inter-related questions remain controversial. Firstly, what role does DNA fragmentation play in the death process? Is it the cause of death or only one of the results of death? Secondly, is internucleosomal cleavage of DNA the only early detectable marker of DNA...
fragmentation in apoptosis? Lastly, is there a true correlation between DNA fragmentation and chromatin condensation? Previous studies showed that internucleosomal cleavage of DNA is one of the earliest changes denoting irreversible commitment to cell death, and that cells do not die when endonuclease is inhibited by nuclease inhibitors, e.g. zinc and aurin (Cohen and Duke, 1984 and Waring, 1990). Based on these observations, internucleosomal cleavage of DNA was generally suggested to be the cause of death in some cells (Schwartzman and Cidlowski, 1993). This was also unlikely to be a universal hypothesis by the facts that in oligodendrocytes (Barres et al, 1992), hepatocytes (Oberhammer et al, 1993a) and some epithelial cell lines (Oberhammer, 1993b), no evidence of internucleosomal cleavage of DNA was obtained. In recent studies of our own and others, some cytoplasmic and nuclear morphological changes typical of apoptosis were still evident in the absence of internucleosomal cleavage of DNA (Cohen et al, 1992, Barbieri et al, 1992 and Tomei, et al, 1992). In this context, DNA degradation may thus be limited to a clean-up function (Peitsch et al, 1994). To address the second question, recent studies indicate that the cleavage of DNA into 30-50 and 200-300 kilobase pair fragments occur prior to or even in the absence of internucleosomal cleavage of DNA (Walker et al, 1991, Brown et al, 1993, Oberhammer et al, 1993b, Cohen et al, 1994 and Sun and Cohen 1994), which means that internucleosomal cleavage of DNA is neither the earliest nor sole detectable marker of DNA degradation in apoptosis. Concerning the correlation between chromatin condensation and internucleosomal cleavage of DNA, it is possible that internucleosomal cleavage of DNA is neither necessary nor sufficient to induce chromatin condensation, and that DNA fragmentation and chromatin condensation may be triggered through separate pathways during apoptosis (Cohen et al, 1992, Barbieri et al, 1992, Oberhammer et al 1993a, Sun et al, 1994b and Sun and Cohen, 1994).

Cell shrinkage and cytoplasmic condensation. In apoptosis, another morphological feature of interest is the shrinkage of the cell, which led to
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apoptosis being initially called shrinkage necrosis (Kerr, 1971). An inter-related phenomenon to cell shrinkage is cytoplasmic condensation, in which cytoplasmic organelles become compacted. The physiological significance of cell shrinkage and cytoplasmic condensation may be that they facilitate the formation of apoptotic bodies and further facilitate the phagocytosis of these bodies by their neighbours. In this way provocation of inflammation by cellular components from dying cells can be avoided. The mechanisms related to these phenomena are not well understood. If apoptotic cells are capable of exerting some normal function during the commitment phase of apoptosis, then a controlled contraction of the cytoskeleton in response to special intracellular signals (e.g. Ca$^{2+}$) may be expected. Some early studies indicate a redistribution of cytoplasmic microfilaments in apoptosis (Clouston and Kerr, 1979), which is likely to be associated to changes in the spatial organisation of cytoplasmic components. Recent studies have shown that disruption of the cytoskeleton leads to apoptosis or interferes with the formation of apoptotic bodies (Cotter et al, 1992), while cytoskeleton stabilisation inhibits apoptosis (see Cohen, 1991 for review).

An alternative explanation for cell shrinkage and cytoplasmic condensation is that during apoptosis, cells passively lose water (Lockshin and Beaulaton, 1981). In thymocytes, this is associated with an increase in buoyant density, suggesting loss of water and ions without corresponding loss of macromolecules or organelles (Wyllie and Morris, 1982). In apoptotic cells, the endoplasmic reticulum dilates, forming vesicles that fuse with the plasma membrane, voiding their contents extracellularly (Morris et al, 1984). It has been suggested that this rapid and selective export of fluid and intracellular ions into the endoplasmic reticulum (ER) may be mediated by an ionic transport system, e.g. Na$^+$-K$^+$-Cl$^-$-cotransporter (Arends and Wyllie, 1991).

1.2.2. Signal transduction

It is now well accepted that many instances of apoptosis occur as a result of the response of cells to changes in their local environment. There are a wide
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range of environmental stimuli, both external and internal, which can induce apoptosis in different cell types. Based on their properties, those environmental stimuli can be practically divided into three major categories: (1) those inducing apoptosis by binding to their respective receptors (either membrane-bound or intracellular), including hormones, growth factors, cytokines and those not yet identified factors responsible for regulating cell death during development, metamorphosis and differentiation, as well as those responsible for cellular turnover in neoplastic tissues; (2) those present in cytotoxic cells and passed to target cells during CTL-mediated apoptosis; and (3) those actually triggering apoptosis indirectly by mild damage of intracellular homeostasis, including radiation, hypoxia and hyperthermia, as well as some antitumour agents and other toxins. The fact that a variety of morphological and biochemical characteristics of apoptosis are identical in different tissues and experimental systems implies that the mechanism of apoptosis may be common to all cell types up to some extent. Diverse stimuli with completely different properties can induce the unique morphological changes characteristic of apoptosis in the same or different cell types, which supports the hypothesis that there may exist individual upstream pathways to transduct diverse initial signals to a final downstream common pathway. This is further supported by the fact that in cells sensitive to multi stimuli-induced apoptosis, inhibition of one stimulus-induced apoptosis does not necessarily block other stimuli-induced apoptosis in the same cell type. Meanwhile, inhibition of one of the downstream common pathways does not necessarily block the appearance of other biochemical or morphological features, which implies that those downstream pathways are somewhat independent on each other. All these raise a series of questions: (1) Is there a junction point that leads to the common pathway? (2) What is the relationship between individual upstream pathways and existing intracellular signal transduction pathways? (3) Can individual downstream pathways share only one junction point? (4) What are the places, if any, of gene-directed events in this kind of network.
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**Ca^{2+} and cyclic AMP** function as ubiquitous intracellular messengers to mediate cell surface receptor activation. Their roles in apoptosis have been widely studied but are not yet clear.

Ca^{2+} plays an important role in apoptosis (see Orrenius et al, 1992 for review). A rapid, sustained increase in intracellular Ca^{2+} concentration is the earliest detectable change in cells undergoing apoptosis. Inhibition of the Ca^{2+} increase blocks stimuli-induced apoptosis and increasing intracellular Ca^{2+} by treating cells with Ca^{2+} ionophores such as A23187 or ionomycin leads to apoptosis in several cell types (for review see Schwartzman and Cidlowski, 1993). A number of mechanisms have been considered to be responsible for this increased intracellular Ca^{2+}. It is a predominant hypothesis that the increased Ca^{2+} comes from the extracellular medium, as incubation of cells in Ca^{2+}-free medium prevents the elevation of intracellular Ca^{2+} level and then also apoptosis (McConkey et al, 1989a and Kizaki et al, 1989 and Kizaki and Tadakuma, 1993). Existing Ca^{2+} channels (Martikainen and Isaacs, 1991 and Aw et al, 1990) and a glucocorticoid-inducible cytosolic factor (McConkey et al, 1989a) are among the candidates for transferring Ca^{2+} into the cells. Receptor-tyrosine kinase(s) or the G-protein -IP_{3} pathway is responsible for a transient increase in the cytosolic Ca^{2+} concentration by facilitating Ca^{2+} release from the endoplasmic reticulum. Its relevance to apoptosis remains to be evaluated. However, it was reported that an increase in inositol triphosphate level in macrophages precedes apoptosis induced by gliotoxin (Waring, 1990).

As a second messenger, Ca^{2+} controls the activity of a range of Ca^{2+}-binding and Ca^{2+}-regulatory proteins, while the specific events that are resulted from the increase in Ca^{2+} levels that are directly related to apoptosis are not clear. Activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease might be one of these examples although its biochemical characteristics are not fully understood yet. The requirement of Ca^{2+} in apoptotic DNA degradation is still subjected to argument (Alnemri and Litwack, 1990 and Kawabata et al, 1993). Expression of high levels
of Ca\(^{2+}\)-dependent transglutaminase may play a part in cytological degeneration in apoptosis (Fesus et al, 1987). Increasing Ca\(^{2+}\) level-induced apoptosis is inhibited by calmidazolium, an inhibitor of calmodulin, without changing intracellular Ca\(^{2+}\) levels (Zheng et al, 1991 and McConkey et al, 1989b). Calmidazolium inhibits the ATP- and calmodulin-dependent uptake of Ca\(^{2+}\) by the nucleus (Nicotera et al, 1989), which implies at least some of the Ca\(^{2+}\)-related apoptotic processes occur within the nucleus. Cyclosporine A blocks anti-CD3 mAb induced apoptosis in T cell clones (Shi et al, 1989). Recently, it was found that cyclosporine A and FK-506, a compound with identical biological effects to cyclosporine, exert their activity by binding to their respective intracellular receptors. This binding further affects the activity of calcineurin, a Ca\(^{2+}\)- and calmodulin-dependent serine-threonine protein phosphatase that is an important component of the signal transduction pathway resulting in IL-2 expression, thereby inhibiting early Ca\(^{2+}\)-associated events involved in cytokine expression, apoptosis and degranulation (Wiederrecht et al, 1993).

A sustained (or sometimes, transient) increase in intracellular Ca\(^{2+}\) is not necessarily associated with apoptosis (Lennon et al, 1992 and Iseki et al, 1993). In contrast, it was found that increased intracellular Ca\(^{2+}\) levels protect sympathetic ganglion cells from apoptosis following nerve growth factor deprivation (Koike et al, 1989) and protect the BAF-3 cell line from apoptosis after IL-3 removal (Rodriguez-Tarduchy et al, 1990). Some studies support the hypothesis that a third factor(s), besides the initial trigger and Ca\(^{2+}\), is needed to decide the destiny of the cells. Those factors include the activation of protein kinase C in diverting a Ca\(^{2+}\) signal generated by T cell mitogen Con A from a cell death pathway to a proliferative pathway (for review see McConkey and Orrenius, 1991) and the CD28 pathway in switching enhanced TCR/CD3 signals by CD4 or CD8 cross-linking from apoptosis or negative selection to proliferation and positive selection(Turka et al, 1991 and Couez et al, 1994).
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Cyclic AMP (cAMP) exerts its effect in cells mainly by activating a cAMP-dependent protein kinase (A-kinase), which catalyses the transfer of the terminal phosphate group from ATP to specific serine or threonine residues of selected proteins in target cells, in turn regulating the activity of a cascade of enzymes. An increase in intracellular cAMP levels, by cAMP analogues (e.g. dibutyl cAMP) or agents that induce an increase in endogenous cAMP levels (e.g. PGE, adenosine and forskolin), causes apoptosis in lymphoid cells and is associated with glucocorticoid-induced apoptosis in thymocytes (for review see McConkey and Orrenius, 1991). In contrast, cAMP analogues prevent activation-induced T cell death in T-cell hybridomas (Lee et al, 1993). Similarly, treatment with anti-class II MHC antibodies, or other agents, e.g. dibutyl cAMP or isoproterenol, results in apoptosis in resting B cells rather than in those activated by anti-Ig or IL-4 (Newell et al, 1993). Regulation of the cAMP-dependent second messenger system is cell type-dependent. Activation of PKC by phorbol esters, e.g. PMA, or ligating surface CD40 rescues germinal centre (GC) B cells from apoptosis by decreasing cAMP levels, while the same treatment causes an increase in cAMP levels and cell death in resting B cells (Knox et al, 1993a). These results imply that cAMP, like Ca^{2+}, plays a dual role in deciding the destiny of a cell, i.e. controlled by another factor, cAMP switches intracellular signals from survival to death, and vice versa. Another explanation of why the effects of cAMP vary depending on the cell types is that many of A-kinase substrates differ in different cell types therefore accounting for the effects of cAMP in those cells. cAMP-induced death does not involve an increase in intracellular Ca^{2+} levels but nevertheless is a Ca^{2+}-dependent process, because the presence of intracellular Ca^{2+} chelators inhibits the cAMP effect (McConkey et al, 1990a).

**Kinases** are important components in intracellular signal transduction. Some kinases function as trans-membrane receptors, e.g. receptor tyrosine kinases, whereas some of them act as the mediators in signalling transduction pathways, e.g. cytoplasmic protein tyrosine kinase, protein kinase A and C, and others
function as effectors, e.g. those proteins with kinase activity in cell cycle regulation. At present, the overall role(s) of kinases in apoptosis remain unclear.

Tyrosine phosphorylation of cellular proteins plays an important role in the activation of signal transduction pathways. These signalling pathways begin at the cell surface, where they are initiated by growth factors, hormones or antigens, and end either in the cytoplasm to control metabolic processes, or in the nucleus to regulate gene expression. General biochemical events downstream of receptor protein-tyrosine kinases include dimerization and autophosphorylation of receptors themselves promoted by ligand binding via an intermolecular mechanism, which creates high affinity binding sites for the SH2 domains of cytoplasmic signalling proteins that regulate the following phospholipid metabolism, Ras or Ras-like GTPase and protein phosphorylation and dephosphorylation (for review see Pawson and Hunter, 1994). The possible involvement of protein tyrosine kinases (PTK) in apoptosis has drawn some attention and in this context, preliminary and contradictory results have been obtained. Ionising radiation stimulates PTK in human B-lymphocyte precursors, triggering apoptosis and clonogenic cell death, which is inhibited by genistein and herbimycin A, both known as PTK inhibitors (Uckun et al, 1992). Antisense oligos for the blk tyrosine kinase, a component in signal pathways that is essential for growth inhibition, effectively prevents anti-Ig (anti-μ-chain)-mediated growth inhibition and subsequent apoptosis in a murine B-cell lymphoma model (Yao and Scott, 1993). CD3/Thy-1 cross-linkage delivers a PTK-dependent negative signal for inhibition of early and late nuclear events, e.g. c-fos transcription, of both immature thymocytes and mature T lymphocytes and induces apoptosis, which can be inhibited by herbimycin A (Nakashima et al, 1993). In contrast, PTK inhibitors, e.g. herbimycin A, genistein, tyrophostins AG490 and AG814 abrogate or inhibit anti-CD40-mediated rescue of GC B cells from apoptosis (Knox and Gordon, 1993). It has also been shown that herbimycin A and methyl-2,5-dihydroxycinnamate cause an inhibition of tyrosine phosphorylation of nuclear proteins in parallel with apoptosis in mouse thymocytes.
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(Azuma et al, 1993). Herbimycin A also blocks the ability of IL-2 and IL-3 to up-regulate bcl-2 mRNA levels and induces apoptosis in an IL-2- or IL-3-dependent hematopoietic cell line even in the presence of IL-2 or IL-3 (Otani et al, 1993).

Protein kinase C (PKC) is a family of closely related intracellular serine/threonine-specific protein kinases which consist of Ca^{2+}-dependent (PKC-\(\alpha, \beta, \beta II \text{ and } \gamma\)) and Ca^{2+}-independent (PKC-\(\delta, \epsilon, \eta, \theta, \zeta \text{ and } \lambda\)) isozymes. The roles of PKC in regulating apoptosis are complicated and are dependent on the involvement of different isozymes, cell physiological conditions, cell types and different stimuli. PKCs are ordinarily related to negative regulation of cell death, based on results that PKC activators or factors upstream to PKC activation rescue different cell types from apoptosis. These include spontaneous apoptosis in the following cell types: vascular endothelial cells (Araki et al, 1990), mesenchyme during their conversion to epithelial cells (Koseki et al, 1992), hematopoietic cells (Rajotte et al, 1992), GC B cells (Knox et al, 1993b), spleen T (Perandones et al, 1993) and B cells (Illera et al, 1993), sheep ileal Peyer's patch B cells (Motyka et al, 1993) and chicken bursa cells (Asakawa et al, 1993). Activation of PKC also rescues cells from apoptosis in group I Burkitt's lymphoma cell lines and B104 lymphoma cell lines induced by the crosslinking of Ig receptor or exposure of cells to Ca^{2+} ionophore (Knox et al, 1992 and BonnefoyBerard et al, 1994). IL-2 withdrawal-induced apoptosis in CTLL cells and glucocorticoid-induced apoptosis in mature \(T_h\) cells can also be inhibited by activation of PKC (Zubiaga et al, 1992 and Walker et al, 1993). In contrast, phorbol ester mimics the Ig crosslinking-induced apoptosis in WEHI-231 B lymphocyte lines (Haggerty and Monroe, 1994). Phorbol ester also induces apoptosis in HL-60 promyelocytic leukaemia cells, which is associated with phorbol ester induced differentiation in this cell line (MacFarlane and O'Donnell, 1993). There is disagreement about the role of PKC in thymocyte apoptosis. In their early studies, McConkey and co-workers found that activation of PKC protects thymocytes from apoptosis induced by glucocorticoids, Ca^{2+}-ionophores, cAMP and T cell receptor activation.
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(McConkey et al, 1989b and c). However, Ojeda's group reported that the PKC inhibitor H-7 or staurosporine rescue thymocytes from glucocorticoid- and irradiation-induced apoptosis (Ojeda et al, 1990 and 1992). Activation of PKC together with an increase in intracellular Ca\(^{2+}\) are essential for TCR/CD3-mediated apoptosis in T cell hybridomas (Iseki et al, 1991). These inconsistencies remain to be resolved. Generally, Ca\(^{2+}\)-dependent PKCs are involved in cells undergoing apoptosis. For example, PKC-\(\beta\) II immunostaining is increased in the neuronal cell body and neurophil of Alzheimer's disease samples (Saitoh et al, 1993) and PKC-\(\alpha\) and \(\beta\) expression is most intense in tonsil epithelial cells undergoing apoptosis in vivo (Knox et al, 1993b). In addition, a translocation of PKC-\(\epsilon\), a Ca\(^{2+}\)-independent isozyme, from the cytosolic fraction to the particular fraction is found in thymocytes undergoing glucocorticoid-induced apoptosis (Iwata et al, 1994).

1.2.3. Genetic regulation

Protein synthesis. Although total protein and RNA synthesis are shut down in apoptotic cells (Wyllie and Morris, 1982), the synthesis of special individual proteins still plays an important role in this process. The finding that inhibitors of protein synthesis or transcription prevent cells from stimuli-induced apoptosis or programmed cell death (see Wyllie et al, 1980 for review) provides evidence supporting the hypothesis that apoptosis is an active, genetically controlled process, or at least indicates a requirement for continuing macromolecular synthesis in this process (see Wyllie et al, 1980 for review). However, this appears not to be a universal finding, as in some situations macromolecular synthesis is not required for apoptosis, e.g. regression of mammary tumours following endocrine ablation (Gullino, 1974), and in others protein synthesis or transcription inhibitors per se induce apoptosis, e.g. in S49 cells (Vedeckis and Bradshaw, 1983), HL-60 cells (Martin et al, 1990), macrophages (Warring, 1990), CEM-C7 cells (Bansal et al, 1991) and B-chronic lymphocytic leukaemia cells (Collins et al, 1991). J.J. Cohen has categorised the
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dearth mechanisms based on their susceptibility to inhibition by cycloheximide and other protein synthesis inhibitors as follows: (1) an induction mechanism which needs an activation of death genes and thus is protein synthesis dependent, (2) a transduction mechanism which represents the destruction of target cells by cytotoxic T lymphocytes and does not need protein synthesis in either killer or target, and (3) a release mechanism which implies that there exists a suicide inhibitor protein that has a short life time and has to be synthesised continuously in order to maintain cell survival, thus in the presence of protein synthesis inhibitors the death program is released (Cohen, 1991). Besides this explanation, all the inhibitors used may be capable of affecting apoptosis by mechanisms other than their well-defined effects on protein synthesis. It is not easy to assess whether the direct effects of those inhibitors on protein synthesis or indirect (secondary) effects on other processes are responsible for their role in apoptosis. Moreover, the complexity of drug metabolism in vivo makes particularly difficult the interpretation of results of experiments that involve the use of protein synthesis inhibitors in whole animals (Wyllie et al, 1980).

Gene expression. A number of groups have endeavoured to identify genes associated with apoptosis or so called "death genes" by using a variety of strategies, including direct mRNA measurement and subtractive or differential hybridisation screening. Some genes for known proteins have been identified, whose expression increases in apoptosis induced by different stimuli in different cell types (Table 1.2), although their direct involvement, for at least some of them, in apoptosis has not yet been determined. These genes are expressed in response to cell stress or damage, and sometimes account for some features of apoptosis that are characteristic but are not necessarily essential for the process, i.e. it is unlikely that the expression of such genes is a requirement for death, per se. Meanwhile, a number of novel genes with no apparent homology to established genes have also been found. These include RP-2 and RP-8 in thymocyte (Owens et al, 1991), RVP1 in rat ventral prostate epithelial cells (Briehl and Miesfeld, 1991), clone 32 and
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310 in human T cell leukaemia line CEM-C7 (Goldstone and Lavin, 1993), and egr-1, nur-77 and apt-4 in thymocytes (Schwartz and Osborne, 1993). The role of those genes in apoptosis remains to be established.

**Death related genes in C. elegans.** The nematode *Caenorhabditis elegans* has the best genetically characterised cell-death pathway. In the nematode, among 1090 somatic cells formed during development, 131 cells die in a process resembling apoptosis (Sulston and Horvitz, 1977, Sulston et al, 1983 and Hedgecock et al, 1983). Fourteen genes have been identified that take part in various cell death programs. Some genes are involved in commitment, others carry out the death program, and the remainder are required for engulfment and disposal of the dead cells (Ellis and Horvitz, 1986, Ellis et al, 1991 and Hengartner et al, 1992) (Fig.1.1). Of interest is observation that the mammalian homologue of death suppressing gene of *C.elegans*, ced-9, is bcl-2 (Hengartner et al, 1994). Expression of the human bcl-2 in *C.elegans* prevents cell death that is normally mediated by ced-3 and ced-4 (Vaux et al, 1992). Recent studies have shown that ced-3 encodes a protein, CED-3, which shares functional and sequence similarity with interleukin-1β-converting enzyme (ICE), a cysteine protease that appears to play some role in mammalian cell death (Yuan et al, 1993 and Miura et al, 1993).

![Fig.1.1 Genes involved at different stages of apoptosis in C. elegans](image-url)

Fig.1.1 Genes involved at different stages of apoptosis in *C. elegans*
## Table 1.2 Genes of known proteins expressed in apoptosis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cell type</th>
<th>Stimulus</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Ubiquitin</td>
<td>Tobacco hawkmouth</td>
<td>Metamorphosis</td>
<td>Schwartz et al, 1990</td>
</tr>
<tr>
<td></td>
<td>Human lymphocytes</td>
<td>γ-irradiation</td>
<td>Delic et al, 1993</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Lymphocytes</td>
<td>Glucocorticoid</td>
<td>Dowd et al, 1991</td>
</tr>
<tr>
<td></td>
<td>Prostatic glandular cells</td>
<td>Androgen ablation</td>
<td>Furuya and Issacs, 1993</td>
</tr>
<tr>
<td>TRPM-2 (SGP-2, Clusterin)</td>
<td>Prostate cells</td>
<td>Castration</td>
<td>Buttyan et al, 1989</td>
</tr>
<tr>
<td></td>
<td>Thymocytes</td>
<td>Glucocorticoid</td>
<td>Bettuzzi et al, 1991</td>
</tr>
<tr>
<td></td>
<td>L929 tumour cells</td>
<td>TNF+TopoII drugs</td>
<td>Kyprianou et al, 1991a</td>
</tr>
<tr>
<td></td>
<td>MCF-7 human mammary adenocarcinoma cells</td>
<td>Estrogen ablation</td>
<td>Kyprianou et al, 1991b</td>
</tr>
<tr>
<td></td>
<td>Prostatic glandular cells</td>
<td>Androgen ablation</td>
<td>Furuya and Issacs, 1993</td>
</tr>
<tr>
<td></td>
<td>Retinitis pigmentosa retinas</td>
<td>Unknown</td>
<td>Jones et al, 1992</td>
</tr>
<tr>
<td></td>
<td>Liver cells</td>
<td>Cycloheximide</td>
<td>LeddaColumbano et al, 1992</td>
</tr>
<tr>
<td></td>
<td>Rat mammary gland luminal epithelial cells</td>
<td>Cessation of lactation</td>
<td>Guenette et al, 1994</td>
</tr>
<tr>
<td>Cathepsin D (lysosomal protease)</td>
<td>Prostate cells</td>
<td>Castration</td>
<td>Sensibar et al, 1990</td>
</tr>
<tr>
<td>β-galactoside binding protein</td>
<td>CEM-C7(human T cell leukaemia line)</td>
<td>Glucocorticoid</td>
<td>Goldstone and Lavin, 1991</td>
</tr>
<tr>
<td>Transglutaminase</td>
<td>Hepatocytes</td>
<td>hyperplasia</td>
<td>Fesus et al, 1987</td>
</tr>
<tr>
<td></td>
<td>Thymocytes</td>
<td>glucocorticoids</td>
<td>Fesus et al, 1987</td>
</tr>
<tr>
<td></td>
<td>Rat mammary gland luminal epithelial cells</td>
<td>Cessation of lactation</td>
<td>Guenette et al, 1993</td>
</tr>
</tbody>
</table>
Proto-oncogenes and tumour suppressor genes have been shown to play a role in the regulation of apoptosis. Proto-oncogenes have a well established role as genes encoding components of signal transduction pathways, promoting cell proliferation and differentiation. The surprising observation is that activation of those mitogenic proto-oncogenes can be a potent trigger of apoptosis. In fact, a number of oncogenes exert the apparent growth/death dual function, including c-myc, E1A, E2A-PBX1 and c-fos. Generally, expression of those genes in cells can cause them to enter either an apoptotic pathway or to proliferate. The final decision depends on additional survival signals that comprise growth factors or other oncogene products. There are important implications of this interplay between apoptotic and anti-apoptotic signals in cell transformation and the resistance of some tumour cells to therapeutic agents capable of inducing apoptosis.
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The c-myc proto-oncogene encodes a short-lived, sequence-specific DNA-binding protein, C-Myc. Higher levels of expression of C-Myc correlate both with increased proliferative rate and with increased sensitivity to apoptosis (Askew et al, 1991, Shi et al, 1992 and Evan et al, 1992). Studies show that induction of Myc expression in the absence of growth factors is still capable of driving cells into the cell cycle, but concomitantly induces apoptosis, unless specific survival cytokines are present in the culture medium, e.g. insulin-like growth factors (IGFs) and (platelet derived growth factor (PDGF) (Marcu et al, 1992, Evan and Littlewood, 1993 and Harrington et al, 1994). Furthermore, the induction of cell proliferation and apoptosis by Myc in non-transformed cells requires dimerization with Max, a partner to all Myc proteins (Amati et al, 1993), which implies that Myc/Max heterodimers function as sequence-specific transcription regulators and control the alternative cell fates between mitosis and apoptosis.

The DNA virus adenovirus infects human cells, recruits them into a proliferative state, and borrows elements of the host cell transcription, translation and DNA replication machinery to reproduce viral proteins and DNA. The adenovirus E1A oncogene is one of the viral genes required for oncogenic transformation. The E1A protein is responsible for initiating a proliferative response and an indirect consequence of this required function of E1A is the induction of apoptosis (White et al, 1991). In order to allow the virus to gain a replicative capacity, adenovirus has evolved two independent anti-apoptotic mechanisms, both encoded by the E1B gene, i.e. p55^E1B and p19^E1B (White et al, 1992). Thus, E1A expression and subsequent growth deregulation can occur unimpeded by cell death in the presence of E1B expression, which suggests that inhibition of apoptosis is likely to be an important step in the progression of the transformed state.

A further example of the coupling between growth and apoptotic pathways by oncogenes is the chimaeric homeobox oncogene E2A-PBX1, generated during t(1;19) chromosomal translocations in childhood leukaemia. Transgenic mice that
constitutively express E2A-PBX1 in lymphocytes show a high incidence of lymphoma, as well as massive lymphocyte apoptosis in the pre-malignant phase (Dedera et al, 1993).

There is a rapid and transient expression of \textit{c-fos} gene following stimulation of cells by mitogenic growth factors. Recent studies have shown that sustained expression of \textit{c-fos} coincides with regions that will undergo apoptosis in mouse embryos (Smeyne et al, 1993). Like \textit{c-myc}, deregulated expression of \textit{c-fos} induces apoptosis in serum-deprived primary fibroblasts.

On the other hand, oncogenes have also been identified which are now known to suppress apoptosis, e.g. \textit{bcl-2} proto-oncogene (Henderson et al, 1991 and 1993, Allsopp et al, 1993 and Cuende et al, 1993). \textit{bcl-2} was identified as a gene whose expression is greatly increased due to a chromosomal translocation in human follicular B cell lymphoma (Tsujimoto et al, 1984). It encodes a membrane associated protein, Bcl-2 that is present in the endoplasmic reticulum, and in nuclear and outer mitochondrial membranes. \textit{bcl-2} expression in myeloid precursors and pro-B cells dramatically enhances the survival of the cells (without proliferation) from growth factor withdrawal or glucocorticoid induced cell death (Hockenbery et al, 1990 and 1993). There is also an oncogenic synergy between the \textit{c-myc} and \textit{bcl-2} oncogenes due to the ability of Bcl-2 to block C-Myc induced apoptosis without affecting its mitogenic capacity (Bissonnette et al, 1992, Fanidi et al, 1992a and Wagner et al, 1993). Bcl-2 is one of a growing family of related proteins that have been conserved throughout multicellular evolution (Table 1.3).

Among them, Bax and the small splice variant of Bcl-X, Bcl-X\textsubscript{S}, antagonise the anti-apoptotic activity of Bcl-2. Bax heterodimerizes with Bcl-2 (Oltavai et al, 1993), while Bcl-X\textsubscript{S} antagonises Bcl-2 by interfering with upstream or downstream effectors of Bcl-2 (Biose et al, 1993).

The biochemical functions of Bcl-2 remain unknown, although there are some data to support a role in the control of oxidative damage (Hockenbery et al, 1993 and Kane et al, 1993). Bcl-2 has also been shown to interact with the R-Ras
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23KDa protein, so believed to be involved in some aspect of signal transduction (Fernadez-Sarabia and Bischoff, 1993).

<table>
<thead>
<tr>
<th>Prevent apoptosis</th>
<th>Promote apoptosis</th>
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</thead>
<tbody>
<tr>
<td>Members</td>
<td>Origin</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>mammalian</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>mammalian</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>mammalian</td>
</tr>
<tr>
<td>CED-9</td>
<td>C. elegans</td>
</tr>
<tr>
<td>BHRF1</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>p19E1B</td>
<td>adenovirus</td>
</tr>
</tbody>
</table>

The tumour suppressor gene *p53*, in addition to implementing a cell cycle check point, might also be an important component of the apoptosis pathway. This suggestion was supported by the observation that the re-introduction of wild-type *p53* into *p53*-negative tumour cells triggers apoptosis (Yonish-Rouach et al, 1991, and Shaw et al, 1992). It was further confirmed by studies with *p53* knockout mice (Donehower et al, 1992, Clarke et al, 1993 and Lowe et al, 1993a). It is now obvious that *p53* plays a pivotal role in mediating the cellular response to DNA damage based on the observation that thymocytes obtained from *p53* negative mice are markedly radiation- and drug induced-DNA damage resistant compared with those from normal isogenic animals. Data from two experimental systems in which DNA damage is not directly involved indicate that this observation is not universal. Firstly, *p53* null thymocytes are still sensitive to glucocorticoid induced apoptosis. Secondly, the induction of apoptosis by either C-Myc (Harrigton et al, 1994) or E1A (Debbas and White, 1993) does require *p53*, although neither oncogene
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directly induces DNA damage. The obvious deduction from these observations is that the role of p53 in mediating apoptosis may not be restricted solely to responding to DNA damage.

1.3. Involvement of apoptosis in medical science:

Apoptosis and disease

Apoptosis is a normal process by which cells are eliminated during normal embryonic development and in adult life. Disruption of this normal process, resulting in illegitimate cell survival or unnecessary tissue damage, can cause certain types of diseases. Meanwhile, understanding the involvement of apoptosis in those pathological process will not only offer the insight into the mechanism of those diseases but also offer new approaches for preventing and treating these diseases.

1.3.1 Cancer

Spontaneous apoptosis in a variety of malignant neoplasms results in a substantial cell loss (Walker et al, 1988). This may be a consequence of intrinsic autoregulatory mechanisms, which begin to operate at an early stage in the sequence of events leading to malignant transformation, although environmental factors, e.g. mild ischaemia in malignant tissue, can not be completely excluded. This phenomenon is coincident with the involvement of the c-myc oncogene in carcinogenesis. High levels of expression of c-myc correlates both with increased proliferative rate and with increased sensitivity to apoptosis, while serum (GFs) starvation causes apoptosis in the presence of c-Myc (Askew et al, 1991 and Evan et al, 1992). During the early malignant process, a rapid proliferation exhausts those factors necessary for survival from the local environment, which results in apoptosis if there exists an expression of mitogenic oncogene.

Abnormal apoptosis, for example by either introducing a death suppressing gene bcl-2 or mutating a oncosuppressor gene p53, can promote cancer
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development, both by allowing accumulation of dividing cells and by obstructing removal of genetic variants with enhanced malignant potential (Strasser et al, 1991 and Yonish-Rouach et al, 1991).

Although the primary cellular targets of many anticancer agents have been identified, less is known about the processes leading to the selective cell death of cancer cells or the molecular basis of drug resistance. Cytotoxic action of many anticancer agents involves processes subsequent to the interaction between drug and cellular target. Divergent stimuli can activate a common cell death program. Identification of the interaction between these traditional anticancer agents and those oncogenes related to apoptosis will increase clinical efficiency in chemotherapy. Studies show that expression of the apoptosis-inducing gene E1A can sensitise fibroblasts to apoptosis induced by ionising radiation, 5-FU, etoposide and adriamycin, while p53-deficient mouse embryonic fibroblasts acquire cross-resistance to anticancer agents (Lowe et al, 1993b and McCarthy et al, 1994). Transfection of bcl-2 increases the resistance of diverse tumour cell lines to chemotherapeutical drugs (Miyashita and Reed, 1992 and 1993).

1.3.2 Acquired immune deficiency syndrome (AIDS)

It is now postulated that the loss of T-cell function in AIDS is a result of the induction of apoptosis in CD4+ and/or CD8+ lymphocytes by gp120 HIV envelope protein (Banda et al, 1992 and Meyaard et al, 1992). It is hypothesised that ongoing apoptosis in T-cells is related to AIDS pathogenesis. This requires not only HIV infection, but also further deleterious interactions with the immune system. Excessive triggering of apoptosis may be one of the pathogenic mechanisms underlying the collapse of the immune system in HIV-related disorders. Other than apoptosis, there are mechanisms involved in HIV induced AIDS, including anergy, superantigen-induced cell proliferation and depletion, defective signalling, molecular mimicry and autoimmunity (Gougeon et al, 1993 and Martin et al, 1993). For example, signalling by HIV through the T cell
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receptor could initiate the markedly different responses among activation, anergy or apoptosis. It is likely that more than one of these mechanisms are involved in cell depletion (or at least functional depletion) during different phases of the disease. Understanding these mechanisms and their role in HIV pathogenesis would be important in new vaccine and therapeutical approaches.

1.3.3 Autoimmune disease

Fas/APO-1 is a cell-surface protein that mediates apoptosis in various tissues including the thymus. Mice carrying the lymphoproliferation (lpr) mutation have defects in the Fas antigen gene, which causes a defect in the deletion of those autoantigen binding, self-reactive lymphocytes by apoptosis. The lpr mice develop lymphadenopathy and suffer from a systemic lupus erythematosus (SLE)-like autoimmune disease, indicating an important role for Fas antigen in both thymic selection and T-cell survival in the periphery (WatanabeFukunaga et al, 1992 and Drappa et al, 1993). The link between this experimental animal model and the clinical situation remains to be established.

Clinically, SLE is a multisystem autoimmune disease in which autoantibody targets a variety of autoantigens of diverse subcellular location. Lymphocytes from SLE patients demonstrate a high rate of apoptosis in vitro, which might provide extracellular nuclear autoantigens to trigger an immune response, and also raises the possibility that abnormalities of apoptosis may contribute to the pathogenesis of SLE (Emlen et al, 1994). When lymphocytes from lpr mice are cultured in vitro, they also show accelerated apoptosis compared with cells from normal strain (Van Houten and Budd, 1992). To resolve this apparent discrepancy between decreased apoptosis in vivo due to lack of Fas/APO-1 and accelerated apoptosis in vitro, Van Houten and Budd have postulated that lpr/lpr lymphocytes are "primed" for apoptosis, but are prevented from dying in vivo. Once these primed cells are placed in culture ex vivo, they die rapidly as a result of cytokine withdrawal or non-specific perturbation of an
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immunological balance maintained *in vivo* (Van Houten and Budd, 1992). Research also shows that autoantigens in SLE formed by cell fragments are clustered on the surface of apoptotic keratinocytes, where an increased generation of reactive oxygen species is observed (CasciolaRosen et al, 1994).

### 1.3.4 Alzheimer's disease (AD)

The molecular mechanism responsible for the neurodegeneration in AD is not known, however, accumulating evidence suggests that β-amyloid contributes to this process. β-amyloid deposition in brain parenchyma and vessel walls is a major pathological feature of AD. It has been reported that synthetic β-amyloid peptide triggers the degeneration of cultured neurones through activation of an apoptotic pathway (Loo et al, 1993 and Forloni et al, 1993). Another major neuropathological finding is that the death of cholinergic cell bodies originating in the nucleus basalls of Mynert is involved in AD. This cholinergic neuronal death has been associated with Ca\(^{2+}\)-activated cytotoxic mechanisms which may lead to the activation of a putative apoptotic gene in AD (Branconnier et al, 1992). β-amyloid peptide also destabilises Ca\(^{2+}\) homeostasis and renders neurones vulnerable to the cytotoxic effect of excitatory amino acids (Mattson et al, 1992).

### 1.3.5 Involvement of apoptosis in xenobiotic related diseases

Cell death is an important aspect of cell toxicity. Traditionally, chemical induced cell death was regarded as a passive degenerative process. The concept of apoptosis renders cell death a new significance, i.e. a cell can die from the inside or actively, and cell death can take part in a variety of developmental, physiological and pathological processes. From the toxicological point of view, cell death plays a role in a diverse xenobiotic (drugs or other chemicals) originated diseases.
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The importance of apoptotic elimination of excessive cells in mammalian embryonic development has been recognised for a few decades (Glucksmann, 1951). Improper induction or inhibition of apoptosis during embryonic development by chemicals will cause malformations. This might explain the toxicological mechanisms of some teratogenic chemicals, including phenytoin, retinoic acid and its derivatives, and triazenes (see Bursch et al, 1992 for review).

Apoptosis may also play a role in different stages of chemical carcinogenesis (see Schulte-Hermann et al, 1993 for a review). Chemicals that interfere with the apoptotic process may initiate or promote carcinogenesis. So far, there is little direct evidence to associate chemical carcinogenesis with the effect of xenobiotics on the apoptotic process. Possibly, chemicals that are able to mutate key tumour suppressor gene, e.g. p53 or to stimulate the expression of apoptosis protective genes, e.g. bcl-2, will be involved in carcinogenesis.


1.4. The thymus and thymocytes

1.4.1. The thymus and cell populations within the thymus

The thymus is one of the generative lymphoid organs of mammals, where T cells mature and reach a stage of functional competence. In the lymphoid system, the thymus is considered as the privileged site of T-lymphocyte generation and
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The thymus is a bilobed organ situated in the anterior mediastinum (Kendall, 1988). Each lobe is divided into multiple lobules by fibrous septa, and each lobule consists of a subcapsular (outer) cortex, cortex and medulla. These compartments differ in the population of both stationary (stroma) and passenger cell (leukocytes). Scattered throughout the thymus are non-lymphoid epithelial cells (reticular epithelium), which form a unique frame-work in the thymus, as well as bone marrow-derived dendritic cells and macrophages (Table 1.4). Epithelial cells in the cortex and medulla differ in morphology and marker expression, which may be related to the different embryonic origin of these cells. The passenger leukocyte populations definitely belong to different lineages. Macrophages occur in the cortex and medulla. In addition, the medulla harbours a special cell type belonging to the monocyte lineage, the so called interdigitating reticulum cell (IDC). The main function of macrophages is phagocytosis, whereas that of the IDC is the presentation of antigens. Also, the lymphocyte populations have different characteristics depending on their compartmental location (Table 1.4).

1.4.2. Thymocyte population dynamics (see Boyd and Hugo, 1991 for review)

The ultimate thymocyte precursors are probably derived from multipotential haemopoietic precursors in the fetal liver and, thereafter, in the bone marrow. Individual bone marrow progenitor cells are able to give rise to very large numbers of thymocytes (Scollay et al, 1986). Around 10-20% of adult thymocytes are blast cells and a large fraction of thymocytes is replaced every day. However, measurements of the rate of migration to the periphery suggest only a few percent are exported from thymus. This apparent discrepancy is accounted by intra-thymic cell death (Smith et al, 1989).
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Table 1.4 Cell populations in the thymus

<table>
<thead>
<tr>
<th>Cells</th>
<th>Location</th>
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</thead>
<tbody>
<tr>
<td><strong>Lymphocytes</strong></td>
<td></td>
</tr>
<tr>
<td>Immature blasts (DN)</td>
<td>Outer cortex</td>
</tr>
<tr>
<td>Small thymocytes (DP)</td>
<td>Cortex</td>
</tr>
<tr>
<td>Intermediate lymphocytes (SP)</td>
<td>Medulla</td>
</tr>
<tr>
<td><strong>Epithelial cells</strong></td>
<td></td>
</tr>
<tr>
<td>Cortex (endoderm)</td>
<td>Cortex</td>
</tr>
<tr>
<td>Medulla (ectoderm)</td>
<td>Medulla</td>
</tr>
<tr>
<td><strong>Dendritic cells</strong></td>
<td>Medulla</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td></td>
</tr>
<tr>
<td>IDC</td>
<td>Medulla</td>
</tr>
<tr>
<td></td>
<td>Cortex, medulla</td>
</tr>
</tbody>
</table>

DN: double negative thymocytes (CD4⁻CD8⁻),
DP: double positive thymocytes (CD4⁺CD8⁺),
SP: single positive thymocytes (CD4⁺CD8⁺ or CD4⁺CD8⁻),
IDC: interdigitating reticulum cell

Under the influence of thymic epithelium-derived chemoattractants, e.g. β₂-microglobulin, adult pro-T cells initially locate to the thymic corticomedullary junction or subcapsule. While within the thymus, the cells rearrange their T cell receptor (TCR) genes, express the accessory molecules CD4 and CD8, and are subjected to selection processes based on receptor specificity.

The induction of αβ TCR gene rearrangement and expression occurs exclusively in the thymus and is a major function of the thymic stroma. Rearrangement of the β chain occurs in pre-T cells, accumulating as CD4⁻CD8⁻ CD3⁻ cells in the subcapsule (Crispe et al, 1987 and Fowlkes et al, 1987). Three phenotypically and functionally distinct forms of these cells exist: CD4⁺CD25⁻, CD4⁺CD25⁺ and CD4⁺CD25⁻. The first two differentiate only in the presence of thymic stroma, while the third group, which are CD4⁻CD8⁻CD3⁻ cells, are pre-
programmed to become CD4+CD8+CD3- and later CD4+CD8+CD3lo. This is an important stromal cell-mediated control point in thymocyte development. The interactions between thymocytes and epithelial cells or macrophages are mediated via adhesion molecules. Many thymic pathological conditions are associated with abnormalities in the outer cortex/subcapsular region.

The pre-programmed differentiation of the CD4loCD8loCD3 cells into CD4+CD8+CD3- cells is mediated by a transient immature single-positive stage, either CD4loCD8+CD3- or CD4+CD8loCD3+. Among CD4+CD8+CD3lo cells there is positive selection of those recognising self MHC-peptide complexes expressed primarily on the cortical epithelium. The positive selection process has two consequences; the cells with self-MHC restriction potential preferentially survive, and the cells with MHC class I specificity develop into the CD8+ mature subset, while class II restricted cells become CD4+. The cells that have undergone positive selection upregulate their CD3 to intermediate (int) the high (hi) levels. This process is another key stromal-cell-dependent control point in thymocyte development.

The positive selection and further maturation of immature T cells also depends on their avoidance of cell death in two situations. One is the negative selection: the removal of immature cells whose high-avidity receptors for self-determinants react too efficiently (Fowlkes et al, 1988, MacDonald et al, 1988 and Kisielow et al, 1988). The other death is by default: the slow disappearance of cells that fail to be positively selected because of the expression of a defective TCR or one inappropriate for binding the MHC structures present in the thymus. The timing of negative selection depends on thymocyte accessibility to antigen, the combined avidity of its TCR and accessory molecules for the antigen, and the intra-thymic localisation of appropriate deleting cells. This deleting population certainly includes bone-marrow-derived macrophage/dendritic cells, and perhaps also contains intrathymic B cells. Thymic nurse cells may also play a role in the negative selection (Aguilar et al, 1994). Hence, depending on the model, negative
selection can be manifested at the CD4⁺CD8⁺CD3⁻ (Murphy et al, 1990) or the
CD4⁺CD8⁺CD3⁻ thymocyte stages, the direct consequence being induction of
apoptosis.

Thymocytes that progress beyond negative selection have continued
upregulation of CD3, loss of CD4 or CD8 and modulation of several other plasma
membrane markers. Further maturation includes altered signal transduction
through the CD3-TCR complex and the acquisition of antigen responsiveness and
cortisone resistance. The CD4⁺CD8⁻CD3⁺ or CD4⁻CD8⁺CD3⁺ thymocytes are
localised predominantly in the medulla. Heterogeneous medullary epithelial cells
might be the candidates for the stromal cells mediating or supporting progressive
maturation, since they form complexes with thymocytes. They do not effect MHC
restriction and/or positive selection but may be involved in tolerance induction,
most likely through anergy rather than clonal deletion.

1.4.3. Thymocyte death: a model system in the study of
apoptosis

Intra-thymic cell death is responsible for the involution of the thymus, while
apoptosis seems to be the predominant mechanism of thymocyte death. Thymocyte
apoptosis possesses most of the morphological and biochemical characteristics of
apoptosis. Elucidation of the mechanism of thymocyte apoptosis may help us to
understand the apoptotic cell death mechanism in the other biological systems. In
fact, radiation- and steroid-induced thymocyte apoptosis served as one of the most
important experimental models in the late 70's and 80's and did facilitate studies of
the mechanism of apoptosis (Wyllie, 1980, Umansky et al, 1981 and Cohen and
Duke, 1984). One of the most important findings in apoptosis studies, coming
from the thymocyte model, was the recognition of internucleosomal cleavage of
DNA, a specific DNA degradation pattern characteristic of this type of cell death
(Wyllie, 1980). A large portion of thymocytes are primed to die via apoptosis or
have the machinery for the death program, thus *in vitro* culture of both the thymus

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organ or thymocytes in suspension provides a sufficiently large amount of sample for biochemical and morphological studies. This is the advantage that other model systems do not have, especially in vivo systems, due to the fact that very small numbers of cells within a tissue are affected by apoptosis and condemned cells are intermingled with healthy ones. However, the observations made in thymocytes may not account for the mechanisms of apoptosis in other cell types, due to the existence of potentially different individual pathways in the induction of apoptosis. Experiments have shown that some biochemical characteristics of thymocytes do not fit in with those of the other cell types. For example, the prevention of apoptosis in thymocytes by inhibitors of protein synthesis does not occur in some other cell types (Gullino, 1974). In contrast, they actually induce apoptosis in some tumour cell lines (Vedeckis and Bradshaw, 1983, Martin et al, 1990 and Collins et al, 1991). The extrapolation of observations made in thymocytes to other experimental system should be made with caution.

1.5 Aims of this study

1. To establish a flow cytometric method for separation and quantification of apoptotic thymocytes;

2. To detect apoptosis induced by various agents both in vitro and in vivo by using the methods established in conjunction with other methods routinely used in assessing apoptosis;

3. To investigate the relationship between DNA degradation and the morphological changes characteristic of apoptosis;

4. To identify possibly early morphological and biochemical changes in apoptosis in thymocytes and associate these changes with the mechanism of apoptosis.
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Agents used in this study to induce apoptosis in rat thymocytes in vitro or in vivo were divided into following two major categories:

1. Glucocorticoids

Dexamethasone (9α-fluoro-16α-methyl-prednisolone) and corticosterone (11β,21-dihydroxy-4-pregnene-3,20-dione) were obtained from Sigma (Poole, UK). Methylprednisolone hemisuccinate sodium salt was from Upjohn (Crawley, UK).

2. DNA topoisomerase-reactive agents

Etoposide (VP 16) was from Sigma (Poole, UK) or kindly provided by Bristol Myers Company (Evansville, IN, USA). Camptothecin, m-amsacrine (m-AMSA), o-amsacrine (o-AMSA) were kindly provided by National Cancer Institute (USA). Novobiocin was from Sigma (Poole, UK).

Other chemicals used in this study as pharmacological active agents (e.g. inhibiting apoptosis or activity of endonuclease) were: cycloheximide, actinomycin D, aurintricaboxylic acid, spermine and spermidine (Sigma, Poole, UK), zinc diacetate (BDH Chemical Ltd., UK).

2.1. Preparation and incubation of rat thymocyte suspensions

Male Fischer 344 rats (4-5 weeks), bred mainly at the MRC Toxicology Unit, Carshalton, Surrey, were killed by an overdose of pentobarbital (Sagatal*, Rhône Mérieux Ltd, Harlow, Essex, UK, 600 mg/kg, ip). Thymi were removed and diced in a McIlwain tissue chopper (Mickle Labs, Gomshall, UK). The tissue minces were scraped into ice-cold carbogen (O2: CO2 =95 : 5)-gassed Krebs-Henseleit buffer (pH 7.4), filtered first through a tea strainer then nylon gauze. The resulting thymocytes were suspended in ice-cold, carbogen gassed RPMI 1640 (Gibco, Paisley, UK) supplied with 10% fetal bovine serum (FBS, Gibco, Paisley, UK).

Thymocyte suspensions were adjusted to a final suspension of 2 x 10^7 cells/ml with RPMI 1640 containing 10% FBS. Incubations were carried out at 37°C under an atmosphere of air : CO2 = 95 : 5.
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2.2. Isolation and autodigestion of rat thymocyte nuclei

Hypotonic buffers containing either magnesium or polyamines (spermine and spermidine) were used to lyse thymocytes in order to isolate nuclei. Thymocyte suspensions were prepared either in RPMI 1640 containing 10% FBS or TPEE buffer (50 mM Tris-HCl, pH 7.5, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.1 mM EGTA and 0.25 M sucrose). Thymocytes were pelleted and resuspended in either TM buffer (10 mM Tris-HCl, pH 7.5, 5 mM MgCl₂) or TPEE* buffer (TPEE minus sucrose), well mixed and kept on ice for 20 min. Resulting nuclei were pelleted, resuspended in TMS buffer (TM buffer plus 60 mM KCl and 15 mM NaCl) or TPEE buffer and layered on top of 5 ml of 2.25 M sucrose and purified by centrifugation at 40,000 g for 1 h at 4°C using a Beckman L8-80M ultracentrifuge with SW 40Ti rotor (Beckman Instruments Inc, Palo Alto, CA, USA). Pellets were collected from the bottom of centrifuge tubes and washed once with TMS or TPEE.

Autodigestions of nuclei were carried out at 37°C in TMS, TPEE or TPEE* (TPEE minus EDTA and EGTA) buffers with the supplements of various cations or nuclease inhibitors.

2.3. Flow cytometry

The flow cytometer used in all studies was an Ortho Cytofluorograph 50H linked to a 2150 computer system (Ortho Diagnostic System Ltd, High Wycombe, UK).

2.3.1. Ethidium bromide staining

Thymocytes (2×10⁶) were vigorously mixed with 2 ml of 70% ethanol (pre-cooled to -20°C), and maintained at 4°C for at least 30 min, pelleted at 400 g for 5 min and washed in phosphate-buffered saline (PBS, Dulbecco'A', Unipath Ltd, Basingstoke, Hampshire, UK) prior to be resuspended in 2 ml filtered (0.22 μm) DNA fluorochrome stain (containing 0.1% sodium citrate, 0.005% ethidium bromide (Sigma Chemical Co., Poole, UK) and 0.1% Triton X-100). RNase A (Sigma Chemical Co., Poole, UK) was added to a final concentration of 0.25 mg/ml and the mixtures were
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maintained at room temperature for 1 hr prior to flow cytometric analysis of $1 \times 10^4$ cell nuclei using argon laser excitation at 488 nm and emission at above 630 nm. The data was displayed by the instrument as a cell cycle phase histogram depicting $G_0/G_1$, $S$ and $G_2/M$ regions and in some cases with an additional sub-$G_0/G_1$ (hypodiploid) peak. The percentage values of the regions were calculated using the Ortho program DNADISC.

2.3.2. Hoechst 33342/propidium iodide staining

Thymocytes ($2 \times 10^6$ cells/ml) were incubated with Hoechst 33342 (H33342, Sigma Chemical Co., Poole, UK) (1 µg/ml) in RPMI 1640 containing 10% FBS at 37°C for 10 min, chilled to 4°C to stop uptake of the dye, pelleted at 400 g for 5 min, resuspended in PBS containing propidium iodide (PI, Sigma Chemical Co., Poole, UK) (5 µg/ml), and examined by flow cytometry.

Hoechst 33342 and propidium iodide were excited using the 352 nm UV line of a krypton laser and resultant blue (400 - 500 nm) versus red (> 630 nm) fluorescence was recorded using linear amplification. The blue fluorescent cells were gated and displayed as a two-dimensional cytogram of fluorescence intensity versus forward light scatter. Cell sorting was carried out at a flow rate of 800 cells/sec on the basis of above parameters.

2.3.3. Fluorescein staining

The following fluorescein dyes used in the study of membrane permeability were purchased from Molecular Probes (Junction City, OR, USA): fluorescein diacetate (FDA), 2',7'-bis(2-carboxyethyl)-5,6-carboxyfluorescein acetoxyethyl ester (BCECF-AM), 5,6-carboxyfluorescein diacetate (CFDA), 5,6-carboxy-2',7'-dichlorofluorescein diacetate (DiCCFDA), 5,6-carboxy-4',5'-dimethyl fluorescein diacetate (DiMCFDA).

Thymocytes ($2 \times 10^6$ cells/ml) were incubated with one of following dyes for 10 min at 37°C in RPMI 1640 containing 10%FBS: FDA (0.2 µM), BCECF-AM (0.2 µM), CFDA (1 µM), DiMCFDA (5 µM), DiCCFDA (5 µM), pelleted at 200 g for 5 min at 4°C, resuspended in PBS containing propidium iodide (5 µg/ml) and stored on ice until analysis.
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For fluorescein measurement, an argon-ion laser tuned to the blue line (488 nm) was used and forward and orthogonal light scatter and red (> 630 nm) and green (520 nm) fluorescences were recorded.

2.3.4. Anti CD4 and anti CD8 antibody staining

Thymocytes (1×10^6 cells) were washed with PBS containing 2% FBS, resuspended in a mixture of anti CD4 and anti CD8 antibodies (Serotec Ltd., Oxford, UK), incubated on ice for 30 min, washed once, resuspended in PBS containing 2% FBS and 12.5%(v/v) streptavidin-phycoerythrin conjugate (Serotec Ltd., Oxford, UK), further incubated on ice for 30 min and washed, then resuspended in 0.5 ml PBS containing 2% FBS.

An argon-ion laser tuned to 488 nm was used for flow cytometric analysis of CD4/CD8 expression. Forward and orthogonal light scatter and orange(>570 nm) and green (>520 nm) fluorescences were measured using linear amplification. After gating on a cytogram of light scatter to exclude clumps of cells and debris, a cytogram of green versus orange fluorescence was recorded.

2.4. Microscopy

2.4.1. Light microscopy

Tissues (thymus, spleen, intestine, etc.) were fixed in 10% neutral buffered formalin, dehydrated with ethanol, cleared in chloroform and embedded in paraffin wax. Paraffin sections (5 μm) were cut and stained with haematoxylin and eosin.

2.4.2. Electron Microscopy

Thymus cubes (1 mm³) or thymocyte pellets (1 mm in thickness) were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight, rinsed with the cacodylate buffer, postfixed for 1 hr in 1% osmium tetraoxide in 0.1 M sodium cacodylate buffer containing 0.04 M potassium ferrocyanide, stained en bloc, for 30 min in 2% aqueous uranyl acetate then dehydrated through a series of ethanols and embedded in Araldite Cy212 (Agar Scientific Ltd., Stansted, UK). Semi-thin (1 μm) sections were cut, axially, through all layers of each pellet to select area for
ultrmicrotomy. Ultrathin sections were stained with lead citrate and examined in a Jeol 100-CX electron microscope (JEOL, Akishima, Japan).

2.5. Percoll fractionation of thymocyte subpopulations

Discontinuous Percoll (Sigma, Poole, UK) gradients were used in this study (Wyllie and Morris, 1982). One part of the Percoll was mixed with nine parts of the 10 × minimum essential medium (MEM, Flow Laboratories, Irvine, UK) to make an arbitrary 100% Percoll. The osmolarity of this arbitrary 100% Percoll is controlled at 373 ± 2 mOsm as measured by the Advanced™ Micro-osmometer (Model 3MO plus, Advanced Instrumentals, INC., MA, USA). This 100% Percoll was diluted with 1× MEM (290 ±2 mOsm) to produce 60%, 70% and 80% Percoll. 100% Percoll (1 ml) was put on the bottom of a test tube (17×100 mm) above which 3 ml of 80%, 70% and 60% Percoll was added respectively (Fig.2.1). The density of the gradients was further calibrated by density marker beads (Pharmacia Biosystem, Milton, UK). The buoyant densities at the top, 60% to 70%, 70% to 80% and 80% to 100% interfaces were 1.063, 1.075, 1.099 and 1.119 g/ml, respectively. Thymocytes (2 - 4 ×10⁸ cells) were resuspended in 0.5
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ml PBS and layered on the top of gradients. The gradients were centrifuged at 2000 g, for 10 min, at 4°C using a Beckman GPR centrifuge with SW 3.7 rotor (Beckman Instruments Inc., Palo Alto, CA, USA). Cells were removed from the interfaces, mixed with five volumes of RPMI 1640, washed once with RPMI 1640, and resuspended in RPMI 1640 with 10% FBS.

2.6. Analysis of DNA fragmentation

2.6.1. Diphenylamine colorimetry (Burton method) (Burton, 1956).

Thymocytes (1×10^7 cells) were pelleted, resuspended gently in 1 ml of lysis buffer (10 mM Tris, 20 mM EDTA, 0.5% Triton-X 100, pH 8.0), left on ice for 15 min, centrifuged at 13,000 rpm, 4°C for 20 min. Supernatants were transferred to tubes containing an equal volume of 20% (v/v) perchloric acid. 10% (v/v) perchloric acid (1 ml) was added to the pellets. These were transferred to the tubes containing of 10% perchloric acid (1 ml). Samples in the tubes containing either supernatants or pellets were hydrolysed at 80°C for 15 min. The reaction was terminated by allowing tubes to cool on the bench for at least 15 min. Freshly prepared diphenylamine reagent (2 ml, containing 0.01%(v/v) paraldehyde, 4%(w/v) diphenylamine in glacial acetic acid) was added. The reaction was carried out at 30°C overnight in the dark. Calf thymus DNA (Sigma Chemical Co., Poole, UK) was used to make a standard curve. The level of DNA fragmentation was expressed as percentage of DNA in the supernatants.

2.6.2. Conventional agarose gel electrophoresis

Internucleosomal cleavage of DNA was assessed by conventional agarose gel electrophoresis in whole thymocytes or nuclei (Sorenson et al, 1990). Thymocytes or nuclei (1×10^6) were pelleted, resuspended in 15 μl ultraclean water to which 6 μl of RNase (50 mg ml⁻¹) were added, left at room temperature for 20 min and mixed with DNA loading buffer. Two kinds of agarose gel were made separately. The running gel was made of 1.8% Agarose 10 (BDH Chemicals Ltd., Poole, UK) or Ultra-pure DNA agarose (Gibco BRL, Paisley, UK) in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). The digestion gel was made of 0.8% Agarose 2206-105 (Pharmacia
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Biosystem, Milton Keynes, UK) or Ultra-pure DNA agarose in TBE buffer containing 2% SDS. Proteinase K (Sigma Chemical Co., Poole, UK, about 10 mg per 10 ml digestion gel) was added when gel had cooled below 50°C. A section of the running gel, 1 cm in width, was removed just above the sample wells and the digestion gel was poured to fill this gap (Fig. 2.2).

![Diagram of agarose gel electrophoresis](image)

**Fig. 2.2 Illustration of one step agarose gel electrophoresis for detecting DNA ladders.**

Electrophoresis was carried out in TBE buffer, initially at 30 V for 45 min then at 100 V for 4 hrs. The gel was washed in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing RNase (20 µg/ml) overnight and then stained with ethidium bromide (1 µg/ml) for 40 min. After being destained for 4 hrs, the gel was visualised for DNA fragmentation under UV light. Lambda DNA Hind III fragments and 123 base pair DNA ladders (both from Gibco, Uxbridge, UK) were used as standards.

**2.6.3. Field inversion gel electrophoresis** (Anand and Southern, 1990)

Thymocytes or nuclei (0.5 - 1×10⁶) were used to make DNA plugs (1 mm³) in 0.5% agarose L (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden). Briefly, thymocytes or nuclei were resuspended in PBS (0.5 ml/10⁶ cells or nuclei, prewarmed to 43°C) and agarose L (1%, 0.5 ml/10⁶ cells or nuclei, 43°C) was added. This mixture was dispensed to each well of the plug mould (100 µl/well) and the mould was
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cooled on ice for 30 min. DNA plugs were processed at 50°C in NDS solution (1% N-lauryl sarcosine, 0.5 M EDTA, 10 mM Tris, pH 9.5) containing Pronase (Boehringer Mannheim, East Sussex, UK, 1 mg/ml) for 48 h in a gently shaking incubator (NDS solution was replaced once during this period) and then stored in NDS (no Pronase) at 4°C.

The running gel was made of 1% Agarose NA (Pharmacia LKB, Biotechnology AB, Uppsala, Sweden) in 0.5× TBE buffer. DNA plugs were washed three times in TE buffer, loaded in the sample wells and cemented with molten Agarose NA.

Electrophoresis was carried out vertically in 0.5× TBE initially at 200 V with a forward pulse for 15 min followed by a 2.4 sec forward to 0.8 sec reverse (3:1 ratio) constant pulse generated by a PC 750 pulse controller (Hoefer Scientific Instruments, San Francisco, CA, USA) for 1 hr. After this step, a ramp factor of 1.5 was applied to increase the forward pulse interval to 24 sec and reverse interval to 8 sec during a total running time of 7 hrs. Two sets of standards were used: *Saccharomyces cerevisiae* chromosomes (243 - 2200 kilobase pairs, Clontech, Cambridge, UK) and pulse markers (0.1 - 200 kilobase pairs, Sigma Chemical Co., Poole, UK).

2.7. Other methods

2.7.1. Coulter counter

Size and number of thymocytes or nuclei were quantified by using a model ZM Coulter Counter plus 256 Channelizer (Coulter Electronics Ltd., Luton, UK). Thymocytes or nuclei (approx. 1×10⁶) were suspended in 20 ml of Isoton II (Coulter Electronics Ltd., Luton, UK) and measured by using settings of lower and upper thresholds of 2.486 and 9.046 μm, respectively, and an attenuation of 2. The instrument was calibrated with size marker beads supplied by Coulter Electronics Ltd..

2.7.2. Radioimmunoassay of corticosterone in rat serum
Chapter 2: Material and methods

Rat corticosterone -3H kit was purchased from ICN Biomedicals Inc. (ICN Plaza, Costa Mesa, CA, USA). Radioimmunoassay of rat serum corticosterone was based on the commercially supplied procedure. Rat whole blood was left at room temperature for at least 1 hr prior to centrifugation at 1500 rpm for 5 min. Serum was taken and stored at 4°C until assay. Rat serum was diluted 1:500 with steroid diluent. Duplicates of both corticosterone standards and diluted sample serum (0.5 ml) were incubated at 98°C for 10 min, cooled to room temperature. Anti-corticosterone antibody (0.1 ml) and "working" corticosterone-3H tracer (0.1 ml, 10^5 CPM/ml) was added to each tube. All tubes were incubated at 4°C for 3 hrs. Ice-cold charcoal dextran (0.2 ml) was added to each tube that was then shaken vigorously, incubated at 4°C for 20 min and centrifuged at 2500 rpm for 15 min. The entire supernatants were decanted into scintillation vials containing Hydrofluor (Pational Diagnostics, Manvelle, NJ, USA) and counted on a Wallac 1410-001 Liquid Scintillation Counter (Pharmacia Diagnostics, Milton Keynes, UK). The serum concentration of corticosterone was calculated as described below.

1. Preparation of standard curve:

The percent bound (B/B₀ %) was obtained according to

\[
\frac{\text{CPM}_1 - \text{CPM}_0}{\text{CPM}_2 - \text{CPM}_0} \times 100
\]

here:

CPM₀ = average counts of non-specific binding tube (blank tube)

CPM₁ = average counts of duplicated standards (e.g. 0.05, 0.1, 0.2 ... ng/ml)

or sample tubes

CPM₂ = average counts of 100% binding tube (i.e. 0 ng/ml tube)

Then a standard curve was made by plotting B/B₀ % against log concentrations of standard tubes.

2. Calculation of sample concentration:
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Average CPMs of sample tubes were used to calculate each sample $B/B_0 \%$ according to the formula shown above. Using these data, corresponding log concentrations were found from standard curve. Serum corticosterone concentrations were converted to molar concentration (μM).

2.8. Statistics

One way ANOVA was used to study the effects of the treatment on the incidence of apoptosis and corticosterone levels in Chapter 3. Analysis of covariance was used to correlate apoptosis levels to a standard corticosterone level. The combined effects of etoposide and cycloheximide were analysed using a two way analysis of variance. All analyses were carried out using MINITAB statistical package (version 8.1).
Chapter 3. Separation and quantification of normal and apoptotic thymocytes by flow cytometry
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3.1. Introduction

3.1.1 Flow cytometry

Flow cytometry is a technique which utilises the principles of fluidics, optics and electronics to analyse the cells. The fluidic system used in a flow cytometer makes it possible to deliver cells from a random, three-dimensional suspension, one by one, to an interrogation point so the measurements are made separately on each cell in turn rather than an average value for the whole population. The detection and measurement units, made up of a laser or arc lamp (as light source), optical collection and filtration and signal processing systems, make it possible to measure multiple cellular parameters based on light scatter and fluorescence. The former is representative of cell size or granularity. The latter is concerned with quantifying the amount of fluorescence probe in the specific population of cells according to physiological, biochemical and biophysical characteristics of cell. On the basis of these parameters and combined with an electrostatic deflecting system, flow cytometric sorting of cell subpopulations can be achieved (Carter and Meyer, 1990).

3.1.2 Applications of flow cytometry in apoptosis research

The main purposes for using flow cytometry in apoptosis research are to identify and separate apoptotic cells from normal and necrotic cells in a cell suspension. Regardless of cell type, apoptotic cells basically have a number of features different from normal and necrotic cells that can be used by flow cytometry to achieve this purpose.

DNA degradation pattern. Genomic DNA is degraded by endonuclease(s) in a controlled manner during apoptosis, which enable us to use flow cytometry to detect apoptotic cells by the following methods: measuring nuclear DNA stainability with DNA-specific fluorochromes (Nicoletti et al, 1991, Telford et al, 1991 and 1992, and Afanasyev et al, 1993) or labelling ends of cleaved DNA by in situ end labelling (Gavrieli et al, 1992, Gorczyca et al, 1993 and Gold et al, 1993). The former can be used to detect cell cycle specificity of apoptotic cell death in conjunction with the
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bromodeoxyuridine (BrdU) incorporation technique, while the latter can do so independently (Wolfe et al, unpublished data). In conjunction with cell surface antibodies, immunophenotype specificity of certain cell types for apoptosis can be identified (Swat et al, 1991a and Bruce-Lyons et al, 1992).

Cell membrane integrity. It is well known that the integrity of the cell membrane of cells undergoing apoptosis, in contrast to necrotic cells, is preserved and most functions of the membrane remain unchanged (Wyllie et al, 1980). However, some changes in membrane structure (Fadok et al, 1992, Ramakrishnan et al, 1993 and Mower et al, 1994) and permeability (Ormerod et al, 1993) have been found, which, in conjunction with other features (e.g. cell size), provide the basis for the new flow cytometric method used mainly in this study.

Gene products related to apoptosis. A number of genes have been suggested as the candidates for death related genes (as described in chapter 1), the expression of a few of them, e.g. bcl-2, c-myc and p53 have been detected by flow cytometry (Gazitt et al, 1993, Veis et al, 1993 Zhu et al, 1993 and Campana et al, 1993). Further exploration of flow cytometric methodology to detect death genes or their expression will facilitate apoptosis research.

Others. Intracellular pH (Barry et al, 1993), transmembrane potential of mitochondria, ATP-dependent lysosomal proton pump, protein or RNA contents have been proposed to be the useful features of apoptotic cells for flow cytometric analysis (reviewed by Darzynkiewicz et al, 1992). Changes in intracellular free Ca^{2+} in apoptosis precede a series of the other biochemical changes (as discussed in chapter 1). In my preliminary studies, Indo-1 was used to separate apoptotic cells from normal cells on the basis of the difference in the content of intracellular free Ca^{2+} between apoptotic and normal cells. I was not able to achieve this purpose, possibly due to either the limited increase in intracellular free Ca^{2+} during thymocyte apoptosis or inadequate sensitivity of the probe used in the study.

In conjunction with the other routinely used biochemical and morphological techniques, two flow cytometric methods, ethidium bromide staining and Hoechst
3342/propidium iodide staining, were used in this study to quantify apoptotic thymocytes induced by a variety of compounds both in vivo and in vitro.

3.2. Use of flow cytometry in the study of dexamethasone-induced apoptosis in rat thymocytes in vivo: ethidium bromide staining

It is a well-known phenomenon that thymic atrophy is induced by treatment with glucocorticoid hormones (Cowen and Sorenson, 1964 and Clayman, 1972). The mechanism of this hormone-induced cell deletion was not clear until recently when the involvement of apoptosis was proposed (Wyllie, 1980, Cohen and Duke, 1984 and Compton et al, 1987). The morphological and biochemical characteristics of apoptotic thymocytes have been primarily established by treating isolated immature rodent thymocytes with various glucocorticoids in vitro (Thomas and Bell, 1981, Wyllie and Morris, 1982, and Smith et al, 1989). This study was carried out to determine if the cellular changes produced in immature thymocytes, following in vivo administration of dexamethasone, were similar to those identified previously following the treatment of cells in vitro. Flow cytometry was used as one of the methods to detect and quantify apoptotic thymocytes induced by dexamethasone in vivo based on the formation of a sub G₀/G₁ (hypodiploid) peak in the DNA histogram during apoptosis.

3.2.1. Dexamethasone-induced thymic atrophy

In preliminary studies, dexamethasone (0.1-5 mg/kg) caused a dose-dependent thymic atrophy. On the basis of these studies, a dose of 1 mg/kg was chosen, which caused a 50% loss in thymic weight after 24 h (Fig.3.1). Thymocytes, isolated 8 hrs after dosing, were 75-80% viable as assessed by trypan blue exclusion (Fig.3.1).
3.2.2. Dexamethasone-induced changes in cell size and buoyant density

Discontinuous Percoll gradients were used to separate thymocyte subpopulations into four fractions named F1 to F4, respectively. The cells in both F1 and F2 apparently exhibited features of normal thymocytes. In contrast, cells in F4

![Graph showing time-dependent loss of thymus weight and thymocyte viability](image)

**Fig. 3.1** Time-dependent loss of thymus weight (-□-) and thymocyte viability (-△-) in rats treated with dexamethasone (1 mg/kg). The relative thymus weight was expressed as mg of thymus per 100 g body weight. Thymocyte viability was assessed by trypan blue (0.1%) exclusion. Results were mean ± SEM (n ≥ 3).

![Graph showing time-dependent changes in thymocyte subfractions](image)

**Fig. 3.2** Time-dependent changes in thymocyte subfractions isolated from rats dosed with dexamethasone. Thymocytes were isolated at 0, 2, 4, 8, 16 and 24 h after treatment with dexamethasone (1 mg/kg) and separated by Percoll gradient into fractions (F1-F4) with modal densities of 1.063 (-○-), 1.075 (-□-), 1.087 (-△-) and 1.119 (-◇-) g/ml, respectively.
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illustrated the characteristics usually associated with apoptosis (Wyllie and Morris, 1982). Treatment of rats with dexamethasone in vivo resulted in very marked changes in the distribution of cells among different fractions (Fig.3.2). The most striking change was the rapid fall of cells in F2 observed during the first 8 h, which was accompanied by an increase in the number of cells with high density in F4 (Fig.3.2). Over the same period, a small increase was also observed in the number of cells with intermediate density (F3). After 8 h, the number of cells recovered from the thymus declined markedly (data not shown). In contrast to the marked loss of cells from F2, very few cells were lost from F1 during the first 16 h (Fig.3.2).

Size analysis of thymocytes, isolated from untreated rats, showed that most of the cells had a modal diameter of 5.83 µm. Treatment with dexamethasone resulted in the appearance of a second population of cells with a modal diameter of 4.89 µm. The number of these small cells increased markedly up to 8 h (Fig.3.3) and, from 8-24 h, these smaller cells formed the majority of the population of cells recovered from the thymus (Fig.3.3). Examination of cell fractions separated by Percoll showed that the small diameter cells were found exclusively in F4 during first 4 h (Table 3.1). However, by 8 h the smaller cells were present in all Percoll fractions, notably in F3.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Unfractionated thymocytes</th>
<th>% small diameter cells in</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>62</td>
<td>5</td>
</tr>
<tr>
<td>16</td>
<td>54</td>
<td>16</td>
</tr>
<tr>
<td>24</td>
<td>72</td>
<td>25</td>
</tr>
</tbody>
</table>

3.2.3. Dexamethasone-induced changes in the DNA profile

The application of discontinuous Percoll gradients and Coulter counting offered the possibility to quantify apoptotic cells, but both techniques are time
Chapter 3: Detection of apoptosis in thymocytes

consuming and inconvenient, therefore are not ideal for a large number of samples. The appearance of a sub $G_0/G_1$ peak (hypodiploid cells) in the DNA histogram during apoptosis means that flow cytometry can be used to detect and quantify apoptotic cells. A variety of DNA-specific binding dyes with diverse primary binding mechanisms have been evaluated for their ability to detect glucocorticoid or ionising radiation-induced apoptosis in thymocytes, these include phenanthridinium (propidium iodide and ethidium bromide) (Nicoletti et al, 1991 and Telford et al, 1991 and 1992), acridine orange, actinomycin (7-aminoactinomycin), chromomycinone (mithramycin A and chromomycin A$_3$), anthracycline (daunomycin) and bisbenzimidazoles (H33342 and H33258) (Telford et al, 1992). All those compounds demonstrated clearly defined sub $G_0/G_1$ peaks with constant percentages, which indicated that the appearance of the sub $G_0/G_1$ peak is not dependent on a particular dye binding characteristic and may be the consequence of extensive changes in chromatin structure resulting in a significant decrease in the stainability of dyes to DNA (Telford et al, 1992).

Ethidium, as well as propidium, intercalates stoichiometrically between the bases in double-stranded nucleic acid and is excited either by UV or blue light giving red fluorescence and can be used for quantitative measurement of cell ploidy and visualisation of the cell cycle. In this study, a flow cytometric method with ethidium bromide staining was used to quantitatively assess dexamethasone-induced apoptosis in conjunction with gel electrophoresis and the Burton method.

The DNA histogram of normal unfractionated thymocytes comprised of $G_0/G_1$, S and $G_2$ regions. Dexamethasone administration caused a time-dependent formation of a sub $G_0/G_1$ peak (Fig.3.5), which was coincidental with the appearance of DNA ladders and DNA fragmentation as assessed by the Burton method.

Little or no DNA laddering was observed in thymocytes at 2 h, but a gradual time-dependent increase in internucleosomal cleavage was of DNA observed which reached a maximum 8 h after dexamethasone administration (Fig.3.4, panel b). DNA fragmentation measured by the Burton method also showed a maximum at 8 h (Table 3.2). The appearance of hypodiploid peak on DNA histogram in total thymocytes was
well correlated to DNA fragmentation as assessed by Burton method (Table 3.2, \( r = 0.9941 \)). Flow cytometric analyses showed that very small numbers of hypodiploid cells were present at 2h but by 8 h this population had enhanced markedly (Table 3.2 and Fig.3.5). These results were confirmed by detection of DNA ladders in Percoll fractions F3 and F4, at both 2 and 4 hrs (Fig.3.4), as well as a hypodiploid peak (Table 3.2). However, at 8 h there was an increase in both the hypodiploid peak and DNA laddering in F2 (Table 3.2 and Fig.3.4, panel e). And by 16 h hypodiploid cells and DNA laddering were found in all fractions (F1-F4) (Fig.3.4, panel f). Interestingly, thymocytes isolated by Percoll fractionation 8 h after administration of dexamethasone (0.1 mg/kg) showed DNA laddering only in F3 and F4 (Fig.3.4, panel g), whilst after a higher dose (5mg/kg), DNA laddering was found in all four fractions (Fig.3.4, panel h). Thus, the extent of DNA fragmentation in different Percoll fractions was both time

Table 3.2 Flow cytometric and DNA analysis of thymocytes from dexamethasone-treated rats

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% of DNA fragmentation in total thymocytes</th>
<th>% Hypodiploid</th>
<th>Total thymocytes</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>1.7 ± 0.4</td>
<td>2.0 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>7.0 ± 1.9</td>
<td>11.2 ± 2.2</td>
<td>1.5 ± 0.6</td>
<td>3.5 ± 1.2</td>
<td>48.5 ± 11.4</td>
<td>68.4 ± 6.7</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>15.3 ± 1.6</td>
<td>17.1 ± 4.4</td>
<td>2.1 ± 0.9</td>
<td>3.6 ± 0.7</td>
<td>44.4 ± 4.5</td>
<td>66.1 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>36.0 ± 8.4</td>
<td>47.7 ± 6.5</td>
<td>5.3 ± 1.5</td>
<td>24.0 ± 1.0</td>
<td>63.9 ± 8.0</td>
<td>89.4 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>24.5 ± 3.5</td>
<td>33.3 ± 9.1</td>
<td>9.0 ± 1.8</td>
<td>39.8 ± 7.8</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>29.6 ± 7.2</td>
<td>41.2 ± 13.7</td>
<td>11.8 ± 3.8</td>
<td>27.6 ± 8.4</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Rats were dosed with dexamethasone (1 mg/kg) and thymocytes were isolated at indicated times. The % of DNA fragmentation and % of hypodiploid cells were determined. The remaining thymocytes were fractionated on Percoll and the % of hypodiploid cells in F1-F4 were determined. The values are mean ± SEM for at least four separated determinations. ND: not determined due to insufficient cells being available for analysis.
Fig. 3.3 Dexamethasone-induced increase in a population of small cells in vivo. Thymocytes were isolated from 2 - 24 h after treatment with dexamethasone (1 mg/kg). Peak A and B indicate cells estimated by Coulter counter analysis to have a mean diameter of 4.89 and 5.83 μm, respectively.

Fig. 3.4 Time course of DNA fragmentation in thymocytes isolated from rats treated with dexamethasone 1 (a - f), 0.1 (g) or 5 (h) mg/kg. Relative molecular mass standards showing multiples of 123 and single 500 bp band (a). Thymocytes were isolated at indicated times after dosing analysed by agarose gel electrophoresis either unseparated (b) or fractionated by Percoll (c - f).
Chapter 3: Detection of apoptosis in thymocytes

and dose dependent. At 16-24 h, a high percentage of hypodiploid cells was found in all fractions (Table 3.2) and all Percoll fractions showed evidence of DNA laddering (Fig. 3.4).

3.2.4. Dexamethasone-induced changes in cell morphology

Changes in the DNA profile correlated with the morphological characteristics of the thymocytes, which appeared normal at 2 h but by 4 h a significant number showed condensation of chromatin, a characteristic feature of apoptosis. The proportion of these apoptotic cells had increased substantially by 8 h (Fig. 3.6a). At early times (up to 8 h), typical apoptotic morphology was primarily observed in F4. At 16-24 h, the number of morphologically distinct apoptotic thymocytes had decreased considerably and the populations of isolated cell in the Percoll fractions were very heterogeneous. Macrophages, most of which contained apoptotic remnants, were common primarily in F1 (Fig. 3.6, b).

Fig. 3.5 Dexamethasone-induced formation of hypodiploid cells. Rats were dosed with dexamethasone (1 mg/kg) and thymocytes were isolated either 2 or 8 h after dosing, fixed and analysed by flow cytometry. Cells in different phases of the cycle are indicated on DNA histogram. A hypodiploid peak (A₀) indicative of apoptotic cells is also shown.
Fig. 3.6 Ultrastructural changes induced by dexamethasone (1 mg/kg) in vivo. (a) Many of the cells, isolated 8 h after dosing, show condensation of chromatin and other characteristic signs of apoptosis (asterisks). (b) One of the many macrophages present in Percoll F1, prepared 24 h after dosing, showing the partially digested remnants of phagocyted apoptotic bodies/cells (asterisks).

Bars = 5 μm.
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3.2.5. Discussion

A range of morphological, physical and biochemical criteria used in this study showed that the cellular changes involved in dexamethasone-induced thymic atrophy \textit{in vivo}, particularly at early times (up to 4 hrs) after dosing, closely resemble those observed previously following treatment of thymocytes with glucocorticoids \textit{in vitro}. The apoptotic thymocytes were characterised ultrastructurally by condensed nuclear chromatin, coupled with other evidence, e.g. an altered DNA structure as revealed by flow cytometry, a smaller size as assessed by Coulter counting, an increased buoyant density on Percoll gradients and the presence of internucleosomal cleavage of DNA as assessed by gel electrophoresis. The application of the flow cytometric method facilitated the quantification of apoptotic cells. There was a good correlation between the appearance of the hypodiploid peak on the DNA histogram with DNA fragmentation as assessed by both the Burton method and agarose gel electrophoresis. It was interesting to note that the apoptotic thymocytes were primarily derived from the Percoll fraction F2 (Fig.3.2), the population that is mainly composed of quiescent immature thymocytes as assessed by flow cytometry (data not shown), rather than those from F1, the population that contains more proliferative cells (thymoblasts). A similar differential sensitivity of these cells to glucocorticoids was observed \textit{in vitro} (Wyllie and Morris, 1982) and may relate to their immunological status. Recent studies showed that most thymocytes deleted in response to glucocorticoids were the immature double positive (CD4$^+$/CD8$^+$) rather than double negative (CD4$^-$/CD8$^-$) populations (Screpanti et al, 1989, Swat et al, 1991b and Lundberg, 1991). Under physiological condition, the elimination of autoreactive thymocytes by negative selection is believed to occur as a consequence of apoptosis (Smith et al, 1989 and Jenkinson et al, 1989), therefore it is possible that dexamethasone, like native existing glucocorticoids, plays a role in the negative selection and speeds this process up at pharmacological levels.

It has also been found that following dexamethasone administration \textit{in vivo}, the number of apoptotic cells (in F4) increased up to 8 h and then decreased substantially.
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This decline was probably caused by their phagocytic removal from the tissue, as a result of a relatively short half-life (2-3 h), rather than by their decreased induction. The first suggestion was supported by the presence of macrophages containing remnants of apoptotic cells (apoptotic bodies), particularly at later times. Meanwhile, a time-dependent redistribution of apoptotic cells was revealed by the appearance of DNA laddering and hypodiploid cells in all Percoll fractions at later times. This also implied that the decline of F4 cells may have resulted from changes in the buoyant density of this population due to metabolic disturbances or uncontrolled re-entry of water into apoptotic thymocytes at later stage of apoptosis. Results after higher doses of dexamethasone, which lead to a more rapid association of DNA laddering with all four fraction, suggested that the level of apoptosis and the rate of consequent events was dose dependent.

In summary, the data shown in this study suggested that the widely used \textit{in vivo} model of glucocorticoid-induced thymocyte apoptosis closely mimics the \textit{in vivo} situation at early times after glucocorticoid administration. However, at later times the fate of apoptotic cells \textit{in vivo}, such as phagocytosis, is obviously not mirrored \textit{in vitro}, Our results highlight the particular susceptibility of the F2 subpopulation of thymocytes to dexamethasone-induced apoptosis.

3.3. Flow cytometric method to separate and quantify apoptotic thymocytes: H33342/propidium iodide staining

In apoptosis research, it is important to separate and quantify apoptotic cells in a cell suspension. Traditionally, detection of apoptosis has involved determination of fragmented DNA in whole cell lysates either colorimetrically or electrophoretically, based on the characteristic degradation pattern of genomic DNA during apoptosis. A major drawback of these methods is they do not quantify apoptosis on the basis of individual cells rather give qualitative or semi-quantitative values for the total cell population. Flow cytometric analysis based on the formation of a hypodiploid or sub
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G₀/G₁ peak in DNA histogram during apoptosis has been described in the above section. Due to the use of fixed cells, no discrimination between viable and nonviable cells could be observed. A flow cytometric method using H33342/propidium iodide has been established in this study to separate and quantify the normal and apoptotic thymocytes.

Hoechst 33342 (H33342) is one of the bisbenzimidazole probes for nucleic acid. It binds preferentially to AT-rich regions in the minor groove of the DNA helix and fluoresces blue when excited by UV light. H33342 is also actively transported in and out of the cells so that the concentration of dye in the cell and its fluorescent spectrum will depend largely on the conditions of incubation (Watson et al, 1985). A proper control of incubation conditions will allow one to distinguish between different cells in a suspension.

Propidium iodide (PI), in contrast to H33342, can not cross an intact plasma membrane freely, which is very useful in distinguishing between viable and nonviable cells.

3.3.1. Characterisation of an apoptotic population separated by flow cytometry

Isolated thymocytes were incubated for up to 6 h either alone or in the presence of dexamethasone (0.1 μM). The cells were then incubated for a further 10 min with H33342 (1 μg/ml), centrifuged, and resuspended in PBS containing PI (5 μg/ml). In both control and dexamethasone-treated thymocytes, two distinct populations were observed in the cytogram of blue vs red fluorescence (Fig.3.7). A small number of cells (12.2 ± 2.4% and 12.7 ± 2.8% for control and dexamethasone-treated cells, respectively, mean ± SEM, n = 4) which fluoresced red, was observed (region 2, Fig.3.7.a and b). These were identified as nonviable cells, which in contrast to viable cells, no longer exclude PI. When the nonviable cells were gated out electronically and the region of blue fluorescence (region 1 in Fig.3.7, a and b) was examined, two distinct populations were observed in the cytogram of blue fluorescence vs forward light scatter (Fig.3.7, c and d), one of high (region 3) and one of low
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(region 4) fluorescence intensity, the former being significantly greater in the dexamethasone-treated than in control thymocytes (compare Fig. 3.7, c and d). The cells in region 3 exhibited a lower forward light scatter than those in region 4, suggesting that they were smaller.

Fig. 3.7 Flow cytometric separation of rat thymocyte subpopulations. Rat thymocytes were incubated either alone (A and C) or with dexamethasone (0.1 μM) (B and D) for 4 h. The cells were then incubated with H33342 and propidium iodide. Cytograms of blue versus red fluorescence (A and B) showed the separation of viable (region 1) and nonviable cells (region 2). When the nonviable cells were excluded by electronic gating, cytograms of blue fluorescence versus forward light scatter showed two distinct populations of normal (region 4) and apoptotic (region 3) cells.

Biochemical and morphological characteristics of apoptotic cells sorted by flow cytometry. To identify the two viable populations, the cells in these two populations were sorted by flow cytometry and examined by gel electrophoresis (Fig. 3.8). The smaller cells with high blue fluorescence (region 3 in Fig. 3.7, d) showed extensive internucleosomal cleavage of DNA (Fig. 3.8, lane 3), whilst cells in region 4 (of Fig. 3.7,d) showed no detectable DNA laddering. This result suggested that the cells with high blue fluorescence and low forward light scatter were apoptotic whereas the cells with low blue fluorescence and high forward light scatter were normal.
Further support for this suggestion was obtained on examination of these sorted populations by electron microscopy (Fig.3.9). The high blue cells (from region 3 of Fig.3.7, d) demonstrated all the characteristic morphological changes of apoptotic thymocytes, including a reduced volume of both the cells and their nuclei (Fig.3.9, a). The nuclei contained zones of condensed chromatin, often associated with one pole of the nucleus. The cell membrane was intact and smooth, except for regions where they appeared to have fused with slightly dilated cisternae of smooth endoplasmic reticulum. The cytoplasm was more electron-dense than that in normal thymocytes but contained mitochondria of normal appearance. The low blue cells (from region 4 of Fig.3.7, c or d) showed irregular profiles with numerous small cytoplasmic processes (Fig.3.9, b). The nuclei exhibited the normal distribution of euchromatin and heterochromatin without any signs of condensation.

Fig.3.8 Agarose gel electrophoresis of cells exhibiting low (lane 2) or high (lane 3) blue fluorescence with H33342. Following incubation with dexamethasone (0.1 μM) and further incubation with H33342 and propidium iodide, cells were sorted by flow cytometry into cells either high or low blue fluorescence and examined for DNA laddering by gel electrophoresis. Molecular weight standards of multiples of 100 bp are shown in lane 1.
Fig. 3.9 Electron micrographs of thymocytes sorted by flow cytometry. (a) Cells with high blue fluorescence show condensed chromatin and fusion (arrow) of dilated cisternae of the smooth endoplasmic reticulum with the plasma membrane. (b) Cells with low blue fluorescence show normal morphology including numerous cytoplasmic processes (arrowheads). Bars = 5 μm.
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Flow cytometric profile of Percoll fractionated apoptotic thymocytes.
Discontinuous Percoll gradients were used to obtain purified normal and apoptotic thymocytes. These purified cells were then subjected to flow cytometric analysis. After incubation with H33342, apoptotic cells from Percoll fraction F4 exhibited high blue fluorescence with low forward light scatter (Fig. 3.10.a) and the normal cells from Percoll fraction F2 exhibited low blue fluorescence with high forward light scatter (Fig. 3.10.b).

![Cytograms of blue fluorescence versus forward light scatter for (A) apoptotic and (B) normal thymocytes. Purified populations of normal (F2) and apoptotic cells (F4) were prepared by Percoll separation, incubated with H33342 and propidium iodide, and analysed by flow cytometry.](image)

Effects of cycloheximide and actinomycin D on the formation of apoptotic population. It was well documented that glucocorticoid-induced apoptosis in thymocytes can be prevented by inhibitors of macromolecular synthesis, e.g. cycloheximide and actinomycin D (reviewed by Arends and Wyllie, 1991). In this study, after 6 hrs incubation, 6.3 ± 1.6% and 36.0 ± 3.6% apoptotic cells were induced in control and dexamethasone (0.1 μM) treated thymocytes, respectively. Co-incubation of thymocytes with cycloheximide (10 μM) and actinomycin D (0.9 μM) inhibited the induction of apoptosis by dexamethasone to 9.5 ± 0.6% and 12.0 ± 0.7% (mean ± SEM, n = 4), respectively.

In summary, a flow cytometric method has been described in this study to separate viable apoptotic thymocytes from normal thymocyte and quantify them. A
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variety of biochemical and morphological criteria was used to verify the method. It has been proved that the thymocytes in the population with high blue fluorescence and low forward light scatter are apoptotic thymocytes. The use of this method should facilitate studies on the mechanism of induction of apoptosis in thymocytes.

3.3.2. Basis of the method: increased membrane permeability to H33342 in apoptotic thymocytes

In this study, as well as others (Sun et al, 1992b, Dive et al, 1992 and Ormerod et al, 1992), it has been observed that apoptotic and normal cells can be distinguished by a brief incubation with the bisbenzimidazole dye, H33342. In these studies, apoptotic cells fluoresced more brightly on excitation of the dye-DNA complex by UV light.

It has been established that once H33342 has bound to DNA helix, the fluorescence is an order of magnitude greater than its fluorescence in aqueous solution. At high concentrations of dye, there is a second type of binding which leads to fluorescence quenching and a red shift of the fluorescence spectrum (Bontemps et al, 1975, Smith et al, 1985 and Stokke and Steen et al, 1985). H33342 is taken up by viable cells although the rate of uptake into the nucleus is strongly dependent on the type of cell studied and this property has been used to distinguish different types of lymphoid cells (Lalande et al, 1981 and Watson et al, 1985). The dye is also actively pumped out of the cells by an energy dependent pump that is probably the p-glycoprotein pump responsible for multiple drug resistance in some tumours. The pump can be blocked by verapamil (Krishan, 1981, Morgan et al, 1989 and Smith et al, 1991).

Two possibilities have been considered to be responsible for the mechanism by which differences in H33342-DNA fluorescence distinguish normal and apoptotic thymocytes: (1) an alteration in the structure of the DNA, or (2) a change in the permeability of the plasma membrane causing either an increased rate of uptake or a decreased rate of efflux of the dye.
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**Time-dependent uptake of H33342 by thymocytes.** Thymocytes were incubated with etoposide for 4 h to induce apoptosis, then were further incubated with H33342 (1 μg/ml) for the indicated times (5 - 60 min), PI (5 μg/ml) was added afterwards, and subjected to flow cytometric analysis. When cells fluoresced red were excluded, cytogram of blue fluorescence vs forward light scatter of remaining thymocytes showed two populations: one with high light scatter, the other with low light scatter (Fig.3.11). The latter population has been identified as apoptotic thymocytes based on biochemical and morphological criteria as described before. Initially, the fluorescence of the apoptotic thymocytes increased more rapidly (compare Fig.3.11, a, b and c, also Fig.3.12). After longer incubation, the fluorescence from both normal and apoptotic cells was equally intense (Fig.3.11, d). When thymocytes were

![Fig 3.11 Time dependence of uptake of H33342 by normal and apoptotic thymocytes. Rat thymocytes were incubated with etoposide (10 μM) for 4 h and then further incubated with H33342 (1 μg/ml) for various times, i.e. (A) 5, (B) 10, (C) 20 and (D) 40 min. At early times, two populations can be seen, normal cells with high light scatter, low blue fluorescence, apoptotic cells with low light scatter, high blue fluorescence. Linear scales.](image-url)
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incubated with H33342 in PBS instead of RPMI 1640 containing 10% FBS, a small increase in blue fluorescence intensity was noticed (Fig.3.12). Verapamil, which blocks the p-glycoprotein pump, increased slightly and equally the rate of uptake of H33342 in both untreated and apoptotic thymocytes (Fig.3.12).

![Figure 3.12: Effect of verapamil on the uptake of H33342 by normal and apoptotic thymocytes in different medium.](image)

*Fig.3.12 Effect of verapamil on the uptake of H33342 by normal and apoptotic thymocytes in different medium.* Normal cells (closed symbols) or apoptotic cells (open symbols) were incubated with H33342 in (-•-) PBS, (-□ - or -■-) medium or (-Δ- or -▲-) medium plus verapamil (100 µM)

The normal and apoptotic cells were distinguished on the basis of light scatter.

Extended times of incubation with H33342 resulted in a shift in the emission spectrum towards the red that was manifested as a change in the ratio of red to blue fluorescence (Fig.3.13). A similar shift was observed if blue fluorescence was

![Figure 3.13: Extended incubation of thymocytes with H33342 caused a red shift in the fluorescence of H33342/DNA complex.](image)

*Fig.3.13 Extended incubation of thymocytes with H33342 caused a red shift in the fluorescence of H33342/DNA complex.* Rat thymocytes treated with etoposide (10 µM) for 4 h and then stained with H33342 for (A) 10 min or (B) 40 min at 37° in the presence of 100 µM verapamil. Cytograms showed the normal cells (n) and apoptotic cells (a). The red shift in the fluorescence of the H33342/DNA complex can be seen in the heavily stained apoptotic cells (arrowhead in panel B).
compared to green (520 nm) or orange (570 nm) fluorescence and was also seen in the absence of propidium iodide. This spectral shift, which has been observed in other cells, is caused by self quenching of H33342-DNA fluorescence as the dye/DNA ratio increases. The effect demonstrated that, at longer times of incubation, the amount of dye bound to the DNA in apoptotic thymocytes continued to increase although the total fluorescence had reached a plateau.

**Thymocyte nuclei stainability with H33342.** An alternative possibility responsible for the difference in fluorescence between normal and apoptotic thymocytes is the affinity of H33342 to the chromatin DNA. In order to eliminate the influence of the cell membrane on controlling the accessibility of dye to chromatin, isolated nuclei were used. Thymocytes were incubated for 4 h either with or without etoposide (10 µM), fractionated by discontinuous Percoll gradients into four fractions as described before. Nuclei were isolated from normal (F2 of control) or apoptotic (F4 of etoposide-treated) thymocytes and then incubated with H33342. It was shown in DNA histogram that normal nuclei (from F2 cells) were nearly all in the G_0/G_1 region, while apoptotic nuclei gave a peak below G_0/G_1 region (Fig.3.14), which meant that, under this condition, the apoptotic nuclei actually stained less dye than normal nuclei.

![Fig.3.14 Stainability of normal and apoptotic thymocyte nuclei](image-url)

*Fig.3.14 Stainability of normal and apoptotic thymocyte nuclei.* Normal and apoptotic thymocytes were purified on Percoll gradients. The nuclei were prepared using buffer containing 10 mM Tris-HCl, 100 mM NaCl, 1.5 mM MgCl\_2, 0.15% Nonidet-P40, 1 mM PMSF (pH 7.4) and stained with H33342. (A) normal cells from F2 of untreated cells. (B) apoptotic cells from F4 of thymocytes incubated with etoposide (10 µM) for 4 h.
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did. It is therefore unlikely that the increased affinity of apoptotic thymocyte chromatin DNA to H33342 is responsible for the increase in fluorescence observed in intact cells.

Thymocyte stainability with fluorescein derivatives. It is widely accepted that apoptotic cells maintain the integrity of their plasma membrane and exclude vital dyes for a few hours after the morphological changes in the nucleus and DNA degradation (reviewed by Wyllie et al, 1980 and Cohen, 1991). However, a few lines of evidence have shown that during apoptosis, the plasma membrane of cells does undergo multiple changes, including surface alterations revealed by electron microscopy (Morris et al, 1984), less abundant in lectin binding sites, reduced expression of CD4 and CD8 of thymocytes (Swat et al, 1991a) and loosely packed membrane lipids (Fadok et al, 1992, Ramakrishnan et al, 1993 and Mower et al, 1994).
The question has therefore been addressed to whether there were also subtle changes in membrane permeability that could account for the increased stainability of apoptotic cells by H33342. To substantiate this possibility, fluorescein diacetate (FDA) and its derivatives have been used in this study.

FDA, as well as its derivatives, diffuses into cells where it is hydrolysed by intracellular esterase to give a charged, fluorescent product, e.g. in the case of FDA, fluorescein (Thomas et al, 1979). The rate of loss of fluorescent products from the cells is dependent on both the chemical structure of the analogues and the ability of the membrane to retain them inside the cell, i.e. membrane permeability (Kolber et al, 1988).

Normal and apoptotic thymocytes were fractionated by Percoll gradients as described before and then loaded with dyes. Marked differences were observed between the normal and apoptotic cells (Fig3.15). The fluorescence intensity of apoptotic relative to normal thymocytes depended on the dye used (Fig.3.15). After thymocytes were loaded with different dyes for certain times, washed out dye and resuspended in dye-free buffer, the decline of fluorescence from thymocytes was measured at room temperature up to 60 min. Under this experimental condition, only
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Figure 3.15 Fluorescence histograms from normal (a - e) and apoptotic (f - j) cells stained with various analogues of FDA. Thymocytes were incubated for 4 h with dexamethasone (0.1 μM) and fractionated by Percoll gradient to give normal (F2) or apoptotic (F4) cells. The fractionated cell were further with either FDA (a, f), CFDA (b, g), BCECF-AM (c, h), DiMCFDA (d, i), or DiCCFDA (e, j). The photomultiplier settings were different for each dye, being adjusted so that the normal and apoptotic cells could be recorded on scale. Linear scales.

Figure 3.16 Efflux of fluorescein at room temperature from normal and apoptotic thymocytes. Thymocytes were incubated for 4 h with dexamethasone (0.1 μM) and separated on Percoll gradient to give normal (F2, ●) and apoptotic (F4, □) thymocytes. The cells were then incubated for 10 min with FDA (0.2 μM) at 37°, centrifuged, and resuspended in PBS.
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thymocytes loaded with FDA showed a measurable loss of dye. The rate of loss of dye from apoptotic thymocytes was significantly greater than that from the normal thymocytes (Fig. 3.16).

The production of an intracellular fluorescent product depends on the rates of uptake and hydrolysis of the ester, while retaining the fluorescence depends on the efflux of fluorescent product. The rates of both uptake and efflux of dye could be affected by alteration in the plasma membrane during apoptosis. Such a change is clearly demonstrated by the greater rate of efflux of fluorescein from apoptotic compared to normal thymocytes.

In this study, we have basically excluded the possibility that the alteration in apoptotic thymocytes in either chromatin structure or the p-glycoprotein is responsible for the increase in the fluorescence intensity. Our data suggested that there is a change in the membrane permeability of apoptotic thymocytes and that it is this change that leads to a more rapid entrance of H33342 in apoptotic thymocytes.

3.3.3. Application of the method in analysis of etoposide-induced apoptosis in rat thymocytes in vitro and in vivo

DNA topoisomerases manage the superhelicity of DNA in a living cell. They participate in several processes in which DNA is involved, including replication, recombination, and chromosome condensation and decondensation during mitosis. Two major topoisomerases, i.e. type I and II, have been found in eukaryotic cells. They function by forming transient enzyme-bridged DNA breaks on one (type I) or both DNA strands (type II). DNA topoisomerase inhibitors interfere with the breakage-rejoining reaction of topoisomerase to stabilise so called cleavable complexes, which appears to be responsible at least in part for a number of cellular effects of topoisomerase inhibitors, including cytotoxicity and elevated levels of sister chromatid exchange and chromosome aberrations (see Liu, 1989 for review). The mechanism of cell killing by topoisomerase inhibitors remains unclear, but it has been related to apoptosis (Chow et al, 1988, Walker et al, 1991, Bertrand et al, 1991, Fanidi
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et al, 1992 and Clark et al, 1993). By using this new flow cytometric method in conjunction with other methods, it has been demonstrated in this study that etoposide and other topoisomerase inhibitors induced apoptosis in thymocytes both in vitro and in vivo.

Etoposide and other topoisomerase inhibitors-induced apoptosis in rat thymocytes in vitro. The following DNA topoisomerase inhibitors were used in the study: etoposide (VP 16, epipodophyllotoxin derivative, nonintercalative topoisomerase II inhibitor), m-AMSA and its structurally relative but nonactive analogue, o-AMSA (acridine derivative, intercalative topoisomerase II inhibitor) and camptothecin (topoisomerase I inhibitor). Thymocytes were incubated with or without these compounds for up to 6 h and then further incubated with H33342/PI. Flow cytometric analyses were carried out as described before. The apoptotic thymocytes were defined as the cells with high blue fluorescence and low forward light scatter (i.e. region 3 of Fig.3.7). A time- and concentration-dependent induction of apoptosis was observed in etoposide, m-AMSA and camptothecin treated thymocytes, but not with o-AMSA at same concentration as m-AMSA (Fig3.17). It was noticed that within a

Fig.3.17 DNA topoisomerase inhibitor-induced apoptosis in rat thymocytes in vitro as assessed by flow cytometry. The results are mean ± SEM (n ≥ 3).

considerable range of concentrations, etoposide and camptothecin induced apoptosis in thymocytes without dramatically changing cell viability, whilst m-AMSA, as well as o-
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AMSA, at higher concentrations (up to 25 μM) caused a notable loss of viability (data not shown). A good correlation between the induction of apoptosis and DNA fragmentation as assessed by both Burton's method and gel electrophoresis was also found in etoposide-treated thymocytes (Table 3.3). The induction of apoptosis and DNA fragmentation by etoposide were inhibited by co-incubation with the macromolecular synthesis inhibitors, cycloheximide and actinomycin D (Table 3.3).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells (%)</th>
<th>DNA fragmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.5 ± 0.3</td>
<td>5.7 ± 1.7</td>
</tr>
<tr>
<td>Etoposide</td>
<td>27.7 ± 0.7</td>
<td>32.3 ± 6.4</td>
</tr>
<tr>
<td>+Cycloheximide (10 μM)</td>
<td>9.1 ± 0.6</td>
<td>11.3 ± 2.4</td>
</tr>
<tr>
<td>+Actinomycin D (1 μM)</td>
<td>11.1 ± 0.7</td>
<td>8.6 ± 2.4</td>
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</table>

Thymocytes were incubated for 4 h with etoposide (10 μM) either alone or in the presence of inhibitors. The results represent the mean ± SEM (n ≥ 3).

Involvement of apoptosis in etoposide-induced thymic atrophy. Chemical-induced thymic atrophy often precedes an alteration in immune function, particularly T-cell dependent immune responses (Snoeij et al, 1989 and Schuurman et al, 1992). Chemicals may induce thymic atrophy by a number of mechanisms including depletion of lymphoid cells (Pieter et al, 1992 and Lundberg, 1991) or damage to the epithelial stroma of the thymus (Thomson et al, 1991, Beschorner et al, 1991 and De Waal et al, 1992), as well as reduction of migration of prothymocytes by affecting the T-lymphocyte stem-cell population in the bone marrow (Fine et al, 1990). Depletion of lymphoid cells may be due either to damage to a proliferative thymoblast subset (Pieter et al, 1992) or deletion of immature thymocytes (Lundberg, 1991). Although apoptosis has been proposed to be involved in the thymic atrophy induced by some chemicals (Comment et al, 1992 and Raffray and Cohen, 1993), to our knowledge, only with glucocorticoids has apoptosis been shown to be a major mechanism involved in the
induction of thymic atrophy in vivo (Compton and Cidlowski, 1986). In a comprehensive preclinical toxicity study, etoposide induced thymic atrophy (Takahashi et al, 1986). Whilst the induction of apoptosis by etoposide in vitro has been well documented, this study was carried out in order to ascertain if apoptosis was involved in the mechanism of etoposide-induced thymic atrophy.

Relative thymus weight expressed as mg of thymus per 100 g body weight was measured in all studies to assess thymic atrophy. Slight loss of relative thymus weight was seen in untreated control animals most likely due to the increase in body weight in young animals over the experimental time studied (Fig.3.18). A slightly greater loss in relative thymus weight was observed in control animals dosed with vehicle alone at 24 h, whereas at 48 h no differences were observed (Fig3.18). Etoposide (10, 30 and 100 mg/kg, ip) caused a dose-and time-dependent thymic atrophy with no sign of recovery up to 72h after dosing (Fig3.18). The dose dependence was statistically significant (p < 0.01).

![Graph showing time- and dose-dependent thymic atrophy](image)

**Fig.3.18 Etoposide causes a time- and dose-dependent thymic atrophy.** The thymus was removed from untreated animal (-O-) or animal dosed with vehicle (-•-) or etoposide 10 mg/kg (-○-), 30 mg/kg (-Δ-) or 100 mg/kg (-□-) intraperitoneally. The results were mean ± SEM (n = 4).

Pathological changes in the thymus first appeared 4 h after etoposide injection (Figs.3.19, a and b). The peak incidence of cells undergoing apoptosis in the thymus...
Fig. 3.19 Histopathology of the thymus following dosing with etoposide (100 mg/kg, ip). (a) Etoposide induced apoptosis of thymocytes (arrow) in vivo 4 h after dosing. H&E x 320. (b) Higher magnification of etoposide induced apoptosis shown in (a), note apoptotic cells and apoptotic bodies with condensed hyperchromatic chromatin (arrows). H&E x 800. (c) 24 h after dosing, nearly all of the cortical thymocytes are shrunken with condensed hyperchromatic chromatin. A smaller number of such cells was observed in the medulla (M) below the corticomedullary junction. H&E x 320. (d) 72 h after dosing, severe depletion of cortical thymocytes with residual apoptotic bodies present, some in phagocytic cells (arrow). H&E x 320.
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occurred at 12 h after dosing with virtually all cortical thymocytes being affected. No morphological changes were detected in either the epithelial or stromal cells. Cortical areas of the thymus were filled with massive number of apoptotic cells at 24 h (Fig.3.19, c). By 72 h, nearly all the apoptotic cells had been removed from the affected areas with only a few apoptotic bodies remaining in the cortex of the thymus (Fig.3.19, d). The increase in the number of apoptotic thymocytes induced by etoposide (100 mg/kg) was markedly inhibited by prior (1 h) injection of cycloheximide (1.5 mg/kg, ip) (data not shown).

Ultrastructure of outer thymic cortex from untreated control animals showed the normal morphology of immature and dividing thymocytes as well as background levels of the phagocytosed remnants of pyknotic nuclei (asterisk, Fig.3.20, a). Numerous apoptotic thymocytes (solid arrows, Fig.3.20, b) and apoptotic bodies (open arrows, Fig.3.20, b) were readily observed 8 h after dosing with etoposide (100 mg/kg). In contrast, few cells exhibiting necrotic morphology were observed. Many of the apoptotic bodies were being phagocytosed by macrophages, which had already ingested numerous pyknotic remnants (asterisks, Fig.3.20, b).

Internucleosomal cleavage of DNA in thymocytes isolated 4 h after dosing with etoposide (100 mg/kg) was revealed by agarose gel electrophoresis (Fig.3.21, lane 4), which was markedly inhibited by prior injection of cycloheximide (Fig.3.21, lane 5).

In conjunction with histopathological and biochemical observation, the flow cytometric method with H33342/PI staining was used to quantitatively assess etoposide-induced apoptosis in thymocytes in vivo. Thymocytes were isolated from both control and etoposide-treated animals and incubated with H33342 and PI. The cells were displayed as cytograms of blue vs red fluorescence (Fig.3.22, a, c and e). The cells fluoresced blue, region 1 of Fig.3.22, a, c and d, were then displayed as a cytograms of blue fluorescence vs forward light scatter. In contrast to thymocytes treated with either etoposide or dexamethasone in vitro, thymocytes isolated from animals 4 h after dosing etoposide (100 mg/kg) showed three rather than two populations in the cytograms of blue fluorescence vs forward light scatter. Apart from
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Fig. 3.20 Etoposide induced death of thymocytes with the characteristic ultrastructural morphology of apoptosis. The thymus was removed from (a) control animals or (b) animals dosed with etoposide (100 mg/kg) for 8 h and processed for EM. Solid and open arrows indicate apoptotic cells and bodies respectively. Phagocytosed remnants of pyknotic nuclear material are indicated by asterisks. Only one such remnant is discernible within a macrophage from normal control animal (a) but many are evident in a macrophage from a dosed animal (b). The two largest of these fragments are indicated but the remainder are unlabelled, for reason of clarity. × 3,400 (Bars = 5 µm).
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Fig. 3.21 Internucleosomal cleavage of DNA induced by etoposide treatment in vivo is inhibited by cycloheximide. Thymocytes were isolated either from untreated (lane 2) or vehicle treated (lane 3) control or from animals treated with etoposide (100 mg/kg) either alone (lane 4) or in the presence of cycloheximide (1 h prior to the administration of etoposide, lane 5), or cycloheximide (1.5 mg/kg) alone (lane 6). Lane 1 contains markers of 123 bp or multiples thereof.

Fig. 3.22 Flow cytometric analysis of thymocytes from etoposide treated animals. Rat thymocytes were isolated from untreated animals (panels a and b), or from animals dosed with etoposide (100 mg/kg, ip) either alone (panels c and d) or in the presence of cycloheximide (1.5 mg/kg, 1 h prior to etoposide, panels e and f) for 4 h. Thymocytes were incubated with H33342 and propidium iodide. Results were displayed as cytograms of blue fluorescence versus red fluorescence (panels a, c and e) or blue fluorescence versus light scatter (panels b, d and f). The numbers on the figures represent the various populations described in the text.
well-characterised normal and apoptotic populations (regions 1 and 2 of Fig.3.22, b, d and f), a population with both low fluorescence and forward light scatter was found (region 3 of Fig.3.22, b, d and f). The characteristics of this population were compatible with those of apoptotic bodies.

Thymocytes isolated from rats 2 to 24 h after dosing with etoposide (100 mg/kg) showed a time-dependent loss in viable cells accompanied by a concomitant increase in nonviable cells (Fig.3.23). Between 2 and 8 h after dosing, the decrease in normal thymocytes was accompanied by an increase in the number of viable apoptotic cells and apoptotic bodies (Fig.3.23). After 4 h, the rapid increase in the number of nonviable cells was accompanied by a slow decrease in the number of apoptotic cells and a continual small increase in the apoptotic bodies up to 16 h (Fig.3.23). Prior injection of cycloheximide markedly decreased the number of apoptotic cells and bodies induced by etoposide (100 mg/kg) (compare Figs.22, e and f, and Table 3.4.).
levels were measured by radioimmunoassay. The control levels of corticosterone (1.0 ± 0.1 μM, Table 3.4) were in good agreement with those reported previously (De Waal et al, 1992) and were not affected following dosing with the vehicle. Both etoposide and cycloheximide caused an increase in corticosterone levels albeit to different extents (Table 3.4). A concentration of corticosterone of 10 μM was required to induce a similar extent of apoptosis in vitro over the same period as that happened in vivo (i.e. 4 h, data not shown). When the data were subjected to an analysis of covariance, no correlation was observed between the corticosterone levels and the percentage of apoptosis induced in vivo as assessed by flow cytometry (Table 3.4).

Table 3.4 Lack of correlation between the induction of apoptosis and the serum level of corticosterone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Apoptosis</th>
<th>Corticosterone (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>2.3 ± 0.2</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>Vehicle control</td>
<td>7.1 ± 2.0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Etoposide (100 mg/kg)</td>
<td>31 ± 3.1*</td>
<td>2.2 ± 0.1*</td>
</tr>
<tr>
<td>Etoposide + Cycloheximide (1.5 mg/kg)</td>
<td>13 ± 1.5*</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>Vehicle + Cycloheximide (1.5 mg/kg)</td>
<td>8.3 ± 1.3</td>
<td>3.1 ± 0.1*</td>
</tr>
</tbody>
</table>

Two groups of rats were dosed with cycloheximide 1 h prior to treatment with etoposide. Animals were killed 4 h later. Serum corticosterone was measured by radioimmunoassay. The % apoptosis in total isolated thymocytes was assessed by flow cytometry and included both apoptotic cells and proposed bodies (regions 2 + 3, Fig.3.23d).

* Significantly different from corresponding untreated control value at p < 0.05.
3.3.4. Discussion

A new flow cytometric method has been described in this study, which has been verified biochemically and morphologically. The method is based on the alterations in permeability of the plasma membrane and also cell size. Therefore, this method is limited in the study of the induction of apoptosis by some chemicals that dramatically change plasma membrane integrity. In the cells that do not readily change cell volume during apoptosis, such as U937 cells, apoptotic cells can still be quantified based on the formation of a population with high blue fluorescence (Bicknell et al, 1994).

Using this method, we have studied the induction of apoptosis by etoposide and other DNA topoisomerase inhibitors in vitro and by etoposide in vivo. It was found that there existed the differences in the induction of apoptosis between DNA topoisomerase I and II inhibitors (between etoposide, m-AMSA and camptothecin), as well as between active (m-AMSA) and inactive (o-AMSA) form of compounds. This might imply that the mechanism by which this kind of chemicals induced apoptosis in thymocytes is related to DNA topoisomerase II. DNA topoisomerases have been regarded as the targets of cytotoxicity of these compounds, which was partially proved in this study by the effect of acridine derivatives (m-AMSA and o-AMSA) on the induction of apoptosis. Both m-AMSA and o-AMSA intercalates DNA equally well, but only m-AMSA has strong antitumour activity (Wilson et al, 1981). Further study showed that m-AMSA, but not o-AMSA, interferes with the break-reunion reaction of DNA topoisomerase II by forming a non-productive drug-enzyme-DNA ternary complex, therefore stabilising the cleavable complex (Nelson et al, 1984). In this study, it was shown that within the same concentration range (1-10 μM), only m-AMSA induced profound apoptosis in thymocytes (Fig.3.17). Camptothecin, a DNA topoisomerase I inhibitor, seemed not as potent as etoposide and m-AMSA in the induction of apoptosis in thymocytes (Fig.3.17), possibly due to less topoisomerase I in rat thymocytes.
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When this method was explored for the study of the induction of apoptosis by etoposide *in vivo*, some differences in the characteristics of apoptosis induced by etoposide between *in vitro* and *in vivo* studies were noticed. One is the formation of the third population with both low fluorescence and forward light scatter in the cytogram of blue fluorescence vs forward light scatter, which was not found in *in vitro* studies at the same time. The cells in this population may represent apoptotic bodies according to their smaller size and exclusion of propidium iodide. Same population was also noticed in dexamethasone-induced apoptosis *in vivo* (data not shown). The other difference was that the increase in blue fluorescence in apoptotic population *in vivo* was not as great as observed in *in vitro* studies. It is possible that these differences come from effects of the microenvironment on the fate of apoptotic thymocytes. These results highlight some limitations to the extrapolation of *in vitro* results to *in vivo* studies. It was also of interest that following *in vivo* exposure to etoposide, a time-dependent decrease in viable cells was accompanied by an increase in nonviable cells as assessed by propidium iodide (Fig.3.23). However, examination of tissue sections at both light microscopic and ultrastructural levels did not provide any evidence for the presence of necrotic cells (Fig.3.19 and 20). Similarly, no evidence for an acute inflammatory reaction was observed (Fig.3.19). These data could basically exclude the possibility that etoposide induced thymocyte death through necrosis *in vivo*. The reason these cells with apoptotic morphology include propidium iodide are not clear, but it may be partially due to the fragility of apoptotic thymocytes and further loss of membrane integrity during isolation of these thymocytes from the thymus.
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3.4. Summary

1. Flow cytometric methods were used in this study, by either ethidium bromide or H33342/propidium iodide staining, to separate and quantify apoptotic thymocytes induced various compounds either \textit{in vivo} and \textit{in vitro}.

2. It was well established that thymocyte apoptosis induced by \textit{in vivo} administration of glucocorticoid closely mimics that induced by \textit{in vitro} treatment at early times. The particular susceptibility of the F2 subpopulation of thymocytes to dexamethasone-induced apoptosis \textit{in vivo} was noticed. The flow cytometric method using ethidium bromide staining facilitated the quantification of DNA fragmentation related apoptotic thymocytes.

3. The flow cytometric method using H33342/propidium iodide staining was validated morphologically and biochemically. The separation of apoptotic thymocytes by this method is based on changes in membrane permeability and cell size. It has been used in this study to investigate DNA topoisomerase inhibitors induced apoptosis in thymocytes both \textit{in vivo} (etoposide) and \textit{in vitro} (etoposide, m-AMSA and camptothecin). The application of this method will facilitate apoptosis research.
Chapter 4. Key morphological features of apoptosis may occur in the absence of internucleosomal cleavage of DNA.
4.1. Introduction

4.1.1. Apoptosis and internucleosomal cleavage of DNA

Biochemically, cells dying via apoptosis undergo extensive genomic degradation, typically generating double-stranded oligonucleosomal fragments of DNA visualised by conventional agarose gel electrophoresis as a DNA ladder (Wyllie, 1980 and reviewed by Walker et al, 1988, Cohen, 1991 and Compton, 1992). Internucleosomal cleavage of DNA was so unequivocally associated to apoptosis, by comparison with necrosis, that DNA laddering was taken as a biochemical hallmark of apoptosis. This biochemical change was also considered to be responsible for one of the morphological changes, i.e. condensation of chromatin and its margination onto the nuclear envelope (Arends et al, 1990). It was believed that inhibition of this internucleosomal cleavage of DNA, e.g. by zinc, may stop cells dying by apoptosis (Cohen and Duke, 1984 and Waring et al, 1990). It was therefore unexpected that, in my preliminary study, zinc was not able to prevent the formation of apoptotic population induced by dexamethasone as assessed by flow cytometry. Further experiments were carried out to examine this phenomenon. Dexamethasone was used in the experiments reported here as an inducer of apoptosis, while similar results were obtained from the experiments using other compounds such as corticosterone, methylprednisolonlone and etoposide (Sun et al, 1994b).

4.1.2. Zn\textsuperscript{2+} inhibited dexamethasone induced internucleosomal cleavage of DNA, but not the formation of apoptotic cells as assessed by flow cytometry

Isolated rat thymocytes (2x10^7 cells/ml) were incubated with dexamethasone (0.1 \( \mu \)M) in the presence or absence of Zn(Ac)\textsubscript{2} (1 mM) for 4 h. Thymocytes (1x10^6 cells/ml) were then further incubated with H33342 and PI and subjected to flow cytometric analysis as described before. Thymocytes exhibiting
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high blue fluorescence and low forward light scatter (Fig. 4.1, region 1) have been characterised as apoptotic using a number of criteria, including their size, ultrastructure and the ladder pattern of DNA degradation on gel electrophoresis as discussed in the previous chapter. The cells with low fluorescence and high forward light scatter were identified as normal thymocytes. An increase in the formation of apoptotic thymocytes was observed in dexamethasone treated cells (Fig. 4.1.b), which was inhibited by coincubation with cycloheximide (10 μM) (Fig. 4.1.c) or actinomycin D (Table 4.1).

<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
</tr>
</thead>
<tbody>
<tr>
<td>c</td>
<td>d</td>
</tr>
</tbody>
</table>

**Fig. 4.1** Zn\(^{2+}\) does not prevent the formation of apoptotic thymocytes induced by dexamethasone as assessed by flow cytometry. Rat thymocytes were incubated for 4 h either alone (a) or with dexamethasone (0.1 μM) in the absence (b) or presence of either cycloheximide (10 μM) (c) or zinc diacetate (1 mM) (d). Normal (region 2) and apoptotic (region 1) thymocytes were separated by flow cytometry following incubation with H33342

Zinc has also been reported to prevent apoptosis, an effect assumed to be due to the ability of metal ion to inhibit a Ca\(^{2+}/\)Mg\(^{2+}\)-dependent endonuclease (Cohen and Duke, 1984 and Waring et al, 1990). It was therefore unexpected that zinc failed to inhibit the formation of apoptotic population, as assessed by flow
cytometry (Fig. 4.1.d), while a slight increase in the percentage of apoptotic cell was found in the presence of zinc (Table 4.1). Under the same conditions, zinc almost completely inhibited dexamethasone induced DNA fragmentation as assessed by either Burton method and gel electrophoresis, respectively (Fig. 4.2, lane 4 and Table 4.1). Further experiments were carried out to identify the apoptotic cells induced by dexamethasone in the presence of zinc.

Table 4.1 Dexamethasone-induced thymocyte apoptosis is inhibited by cycloheximide, actinomycin D and aurin, but not zinc

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apoptotic cells (%)</th>
<th>DNA fragmentation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.5 ± 1.7</td>
<td>9.1 ± 2.3</td>
</tr>
<tr>
<td>Dex</td>
<td>18.7 ± 4.3</td>
<td>33.7 ± 8.6</td>
</tr>
<tr>
<td>Dex + cycloheximide</td>
<td>6.3 ± 1.6</td>
<td>10.0 ± 1.4</td>
</tr>
<tr>
<td>Dex + actinomycin D</td>
<td>7.7 ± 1.7</td>
<td>13.1 ± 2.8</td>
</tr>
<tr>
<td>Dex + aurin</td>
<td>7.2 ± 2.2</td>
<td>18.3 ± 4.1</td>
</tr>
<tr>
<td>Dex + Zn²⁺</td>
<td>21.1 ± 3.4</td>
<td>7.2 ± 2.8</td>
</tr>
<tr>
<td>Control + Zn²⁺</td>
<td>7.2 ± 1.2</td>
<td>4.0 ± 1.2</td>
</tr>
</tbody>
</table>

Thymocytes were incubated for 4 h either alone or with dexamethasone (Dex, 0.1 μM) in the absence or presence of various putative inhibitors of apoptosis. The percentage of apoptotic cells was determined by flow cytometry following incubation of cells with H33342 and PI. The percentage of DNA fragmentation was measured by Burton method.

4.2. Dissociation of apoptosis from DNA laddering

4.2.1. Cells sorted by flow cytometry

Thymocytes treated with dexamethasone (0.1 μM) either alone or in the presence of zinc (1 mM) were sorted by flow cytometry into two viable populations, one with high blue fluorescence and low forward light scatter (apoptotic, Fig. 4.1.region 1) and the other with low blue fluorescence and high forward light scatter (normal, Fig. 4.1.region 2). These cells were examined by conventional agarose gel electrophoresis. No DNA laddering was found in normal populations of dexamethasone treated thymocytes either alone or in the presence
Chapter 4: Early morphological changes

of zinc (Fig.4.2, lanes 7 and 9). The apoptotic population from dexamethasone alone treated cells showed clear DNA laddering (Fig.4.2, lane 8), whereas no DNA laddering was found in the same population in the presence of zinc (Fig.4.2, lane 9). Apparently, the formation of the apoptotic population was dissociated from DNA laddering.

Fig.4.2 Inhibits dexamethasone-induced DNA laddering in thymocytes. Thymocytes were incubated for 4 h either alone or with dexamethasone (0.1 μM) in the absence or presence of Zn²⁺ (1 mM). Cells (1 x 10⁶) were taken for measurement of internucleosomal cleavage of DNA by agarose gel electrophoresis. Lane 1 contains molecular size standards of multiples of 123 bp. In unsorted thymocytes, dexamethasone (lane 3) caused an increased DNA laddering compared with control cells (lane 2). This increase was totally inhibited by Zn²⁺ (lane 4).

Apoptotic thymocytes were obtained from Percoll gradient F4 of thymocytes treated with dexamethasone either alone (lane 5) or in the presence of Zn²⁺ (lane 6). Alternatively, normal (lanes 7 and 9) and apoptotic thymocytes (lanes 8 and 10) were sorted by flow cytometry from thymocytes treated with dexamethasone alone (lanes 7 and 8) or in the presence of Zn²⁺ (lanes 9 and 10). Neither normal nor apoptotic thymocytes treated by dexamethasone in the presence of Zn²⁺ exhibited DNA laddering (lanes 9 and 10).
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4.2.2. Cells fractionated by Percoll gradients

Discontinuous Percoll gradients were used as an alternative way to fractionate apoptotic cells. An increase in the percentage of apoptotic fraction (F4) was also found in dexamethasone treated thymocytes in the presence of zinc compared to dexamethasone alone (Fig.4.3). When these F4 cells were subjected to gel electrophoresis, DNA laddering was found in the F4 from dexamethasone alone treated thymocytes (Fig.4.2, lane 5), while no DNA laddering was found in F4 from dexamethasone treated thymocytes in the presence of zinc (Fig.4.2, lane 6). This observation further supported that formation of apoptotic population could be dissociated from DNA laddering.

Fig.4.3 Zn\(^{2+}\) does not prevent the formation of apoptotic thymocytes as assessed by Percoll gradients. Thymocytes were incubated for 4 h either alone or with dexamethasone (0.1 \(\mu\)M) in the absence of presence of Zn\(^{2+}\) (1 mM). The cells were fractionated by discontinuous Percoll gradients into 4 fractions (F1 - F4), respectively. Results were mean ± SEM (n ≥ 3).

4.2.3. Morphological features of apoptotic thymocytes in the absence of DNA laddering

Apoptotic thymocytes induced by dexamethasone alone, either sorted by flow cytometry or fractionated by Percoll gradients, showed distinct morphological
Chapter 4: Early morphological changes

features of apoptosis as described previously, i.e. the characteristic chromatin condensation and cytoplasmic contraction (Wyllie et al, 1980, Kerr et al, 1987 and Arends et al, 1991). They exhibited a condensed cytoplasm containing apparently normal organelles, apart from dilated cisternae of smooth endoplasmic reticulum, many of which were fused with the cell membrane. The nuclei were also shrunken, and although the euchromatin retained its normal appearance, the heterochromatin was condensed and usually coalesced against one pole of the nuclear membrane (Fig.4.4.a).

Remarkably different results were observed in the apoptotic thymocytes obtained from both flow cytometric sorting and Percoll fractionation following incubation with dexamethasone in the presence of zinc. Although no evidence of DNA laddering was observed, these thymocytes were also shrunken, with some dilation of the smooth endoplasmic reticulum. The heterochromatin in these cells was condensed and arranged in several sharply defined clumps that abutted against the nuclear membrane (Fig.4.4.b). A further clump was present in the centre of many these nuclei. The cell profiles were mostly regular but the nuclear membrane, although usually intact, was often convoluted. The euchromatin retained its normal density but often included one or more clusters of intensely stained nucleolar remnants. Similar characteristics have been described, in various cell types, as the earliest signs of apoptosis (Kerr et al, 1987 and Walker et al, 1988). Thus in the presence of zinc, thymocytes appeared to have been halted at a very early stage of apoptosis, possibly prior to the internucleosomal cleavage of DNA by Ca$^{2+}$/Mg$^{2+}$-dependent endonuclease due to the effects of zinc on this enzyme, although other possible actions of zinc cannot be excluded.
Fig. 4.4 Apoptotic thymocytes induced by dexamethasone in the presence of Zn$^{2+}$ show the earliest morphological changes of apoptosis. Apoptotic thymocytes (F4) were obtained by Percoll fractionation of thymocytes treated for 4 h with dexamethasone (0.1 μM) alone (a), which demonstrate the characteristic features of apoptosis, or those treated with dexamethasone in the presence of Zn$^{2+}$ (1 mM) (b), which show only the earliest signs of apoptosis.

Bars = 1 μm.
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4.2.4. Discussion

In the presence of zinc, dexamethasone induced the formation of a cell population that was apoptotic based on a range of criteria, including increased cell density revealed by Percoll gradients, decreased cell size and changes in cell membrane permeability to H33342 revealed by flow cytometry and morphological features, but which were devoid of DNA laddering. A clear dissociation has been observed between some morphological features of apoptosis and DNA laddering.

The morphology of the nucleus of apoptotic cells is obviously similar in different cell types. Ultrastructurally, the earliest recognised nuclear changes are condensation and margination of the chromatin to form dense masses (clumps) that abut on the nuclear membrane. The edges of the chromatin masses are characteristically sharply defined. At the later stage, chromatin masses further condense into crescent caps at one end, in most cases, of the nuclear periphery and the nucleus then breaks up into discrete fragments in which the characteristic segregation of chromatin is maintained (Kerr et al, 1987, Walker et al, 1988 and Arends and Wyllie, 1991). The selective activation of an endogenous endonuclease and consequential internucleosomal cleavage of DNA has been associated with the major nuclear morphological changes (Arends et al, 1990) and therefore with the manifestation of death (Wyllie, 1980, Cohen and Duke, 1984, Waring et al, 1990 and Schwartzman and Cidlowski, 1993). Recent studies have shown that internucleosomal cleavage of DNA does not seem to be an obligatory feature of apoptosis. Absence of internucleosomal cleavage of DNA has been observed in some cases of programmed cell death, for example in insect metamorphosis and normal limb development (Lochshin and Zakeri, 1991). In C. elegans, the functional mutation of nuc-1, a gene responsible for the digestion of nuclear DNA, does not stop cells dying (Ellis et al, 1991). In addition, chromatin condensation characteristic of apoptosis has been observed in oligodendrocytes (Barres et al, 1992), hepatocytes (Oberhammer et al, 1993a) and in murine embryonic fibroblasts (Tomei et al, 1993) in the absence of internucleosomal
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cleavage of DNA. It has also been suggested that endonuclease activation in apoptosis is neither necessary nor sufficient to induce chromatin condensation, and that DNA fragmentation and chromatin condensation may be triggered through separate pathways during apoptosis (Miller et al, 1993 and Sun et al, 1994a). Internucleosomal cleavage of DNA may thus be limited to a clean-up function rather than initiating the death program (Peitsch et al, 1994).

Zinc has also been reported to prevent apoptosis presumably by its inhibitory effect on a Ca^{2+}/Mg^{2+}-dependent endonuclease (Cohen and Duke, 1984 and Waring et al, 1990). It was therefore surprising that zinc did not decrease the formation of apoptotic cells under conditions where zinc almost completely inhibited dexamethasone induced DNA degradation as assessed by Burton method and agarose gel electrophoresis. Apoptosis induced by dexamethasone in the presence of zinc was assessed by the flow cytometric method that is mainly based on membrane changes and as well by Percoll fractionation that indicates cell size and buoyant density changes. Ultrastructural examination of these cells following cell sorting by flow cytometry or Percoll fractionation showed that the thymocytes were shrunken, with dilation and 'bubbling' of smooth endoplasmic reticulum. The heterochromatin in these cells was condensed and arranged in several sharply defined clumps, which were contiguous with the nuclear membrane. Similar nuclear changes have been described, in various cell types, as the earliest signs of apoptosis (Kerr et al, 1987, Walker et al, 1988 and Arends and Wyllie, 1991). In the presence of zinc, many of the cytoplasmic changes characteristic of apoptosis were still evident. Thus the nuclear changes in the cells were probably arrested at an early stage of apoptosis prior to the effect of endonuclease. This result not only dissociates internucleosomal cleavage of DNA from nuclear morphological changes but also dissociates it from other cytoplasmic events, e.g. changes in membrane permeability, cell size and buoyant density, which further suggests that they are individual downstream changes in apoptosis. Similar results were observed in thymocytes treated with etoposide in the presence of zinc (Sun et al,
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1994b). These results suggest that critical changes occur during the induction of apoptosis, both in nuclear DNA and in the cytoplasm prior to endonuclease cleavage of DNA into oligonucleosomal fragments. Whilst our results are consistent with a role for zinc in inhibiting the endonuclease (Cohen and Duke, 1984 and Waring et al, 1990), they do not exclude other possibilities such as zinc binding to the DNA and thus changing DNA conformation, in this way endonuclease will not be able to access to the cutting regions. Zinc may also modify apoptosis by activating protein kinase C or by inhibiting phosphorylases associated with inositol phosphate metabolism (Waring, 1990).

It is suggested here that the induction of the earliest morphological changes of apoptosis involves enzymes or biochemical events other than the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease and internucleosomal cleavage of DNA that is involved at later stages when it is responsible for degradation of DNA in apoptotic cells. Originally apoptosis was defined morphologically but it has now begun to be characterised biochemically, in particular by measurement of DNA laddering. Our results indicated that assessment of apoptosis by biochemical assays alone should be interpreted with caution.

4.3. Summary

An early key morphological change of apoptotic thymocytes was described in this study that was experimentally dissociated from internucleosomal cleavage of DNA. Internucleosomal cleavage of DNA is thus likely to be one of the downstream events responsible for degradation of DNA in dying cells. It is also possible that this event is independent of other cellular changes in apoptosis process. The identification of early events prior to this internucleosomal cleavage of DNA may help us to understand the mechanism of cell death via apoptosis.
Chapter 5. Identification of a transitional prepapoptotic population of thymocytes
Chapter 5: Preapoptotic population

5.1. Introduction: intermediate population of thymocytes observed in discontinuous Percoll gradients

A single step-wise decrease in cell volume and increase in cell buoyant density has been regarded as one of the features of apoptosis in thymocytes (Cohen et al, 1992). Normal and apoptotic thymocytes have been separated by different means with no reported evidence of cells with an intermediate size or buoyant density (Wyllie and Morris, 1982 and Walker et al, 1991). For example, after incubation of thymocytes with methylprednisolone, two populations of cells were characterised, one with lower modal density as normal thymocytes and the other with higher modal density as apoptotic (Wyllie and Morris, 1982). Our studies, using dexamethasone and etoposide to induce apoptosis in thymocytes, were in good agreement with the above studies, while a discrete population of cells of intermediate size and density (fraction F3) was also observed from discontinuous Percoll gradients (Table 5.1). Similar intermediate population of thymocytes was also observed in rats following irradiation (Borisova et al, 1987).

Table 5.1 Separation of a population of cells of intermediary volume by Percoll gradients

<table>
<thead>
<tr>
<th>Percoll fractions</th>
<th>Control</th>
<th>Etoposide</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume (fl)</td>
<td>FLS</td>
<td>Volume (fl)</td>
</tr>
<tr>
<td>F1</td>
<td>100.9 ± 1.7</td>
<td>100</td>
<td>104.9 ± 1.0</td>
</tr>
<tr>
<td>F2</td>
<td>96.8 ± 1.0</td>
<td>85 ± 2</td>
<td>99.9 ± 1.0</td>
</tr>
<tr>
<td>F3</td>
<td>86.6 ± 2.0</td>
<td>78 ± 3</td>
<td>90.7 ± 1.0</td>
</tr>
<tr>
<td>F4</td>
<td>58.1 ± 0.1</td>
<td>35 ± 3</td>
<td>60.1 ± 1.0</td>
</tr>
</tbody>
</table>

Thymocytes were incubated for 4 h either in the presence of etoposide (10 μM) or dexamethasone (0.1 μM). The cells were then fractionated by Percoll gradients. The volume of the cells in the different fractions was measured using the Coulter counter. The forward light scatter (FLS) was measured on flow cytometry using a UV laser, and the results expressed as a percentage of the light scatter of control cell in F1. Results represent the mean ± SEM of at least three determinations.
Chapter 5: Preapoptotic population

5.2. Characterisation of cells from different fractions separated on Percoll gradients

5.2.1. Time-dependent changes in the distribution of cells in the different fractions

The kinetic study of cell populations was achieved by measuring the percentage of cells in the different Percoll fractions at the indicated times (Fig. 5.1). In the untreated control cells, a slight decrease in the percentage of cells in F1 and F2 was observed, which was accompanied by a corresponding increase in the percentage of cells in F3 and F4 (Fig. 5.1, a). In cells exposed to etoposide (10 μM) (Fig. 5.1, b) or dexamethasone (0.1 μM) (Fig. 5.1, c), a much greater decrease was observed in cells of F1 and F2, which was accompanied by a concomitant rise in cells in F3 and F4 (Fig. 5.1, b and c). The most marked rise in cells in F3 was observed at 1 h, whereas the percentage of cells in F4 increased throughout the 4-h incubation period.

![Graph showing percentage of cells in different Percoll fractions at different times of incubation.](image)

Fig. 5.1 Percentage of cells in the different Percoll fractions at different times of incubation. Thymocytes were incubated either alone (a) or in the presence of etoposide (10 μM) (b) or dexamethasone (0.1 μM) (c) for 0 to 4 h. At the indicated times, the cells were separated on Percoll gradients into the four fractions of increased density, F1 (-○-), F2 (-□-), F3 (-△-) or F4 (-♦-). The results are mean ± SEM, n = 3.

5.2.2. Flow cytometric profile

The fractionated populations of cells from the Percoll gradients were then examined by flow cytometry, after incubation with H33342. Cells in F1 and F2 exhibited high forward light scatter and low blue fluorescence (Fig. 5.2, a and b). Cells
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in F4 were predominantly of a smaller size and exhibited high fluorescence (Fig. 5.2, d). These cells have been characterised as normal (F1 and F2) and apoptotic thymocytes (F4) on the basis of a series of biochemical and morphological criteria (see previous chapter and following sections).

Fig. 5.2 Flow cytometric analysis of cells fractionated on Percoll gradients. Thymocytes were incubated for 4 h with etoposide (10 μM). The cells were then separated on Percoll gradients into the four fractions of increasing density. The cells from different fractions were further incubated with H33342 and examined by flow cytometry.

Cells from F3, at 4 h, appeared to be heterogeneous, containing both smaller cells with high blue fluorescence (region 3, Fig. 5.2, c) and apparently normal cells that exhibited low blue fluorescence but somewhat smaller (lower mean forward light scatter) than the corresponding cells from F1 and F2 (region 1, Fig. 5.2, c). A third group of cells, present within F3 (region 2, Fig. 5.2, c) had properties intermediate between the other two populations.
5.2.3. DNA profile

Agarose gel electrophoresis of the cells from each Percoll fraction, obtained following exposure to etoposide (10 μM, 4 h), showed that little or no DNA laddering was observed from F1 and F2, whereas extensive laddering was observed from the apoptotic cells in F4 (Fig. 5.3). DNA laddering was also observed in cells from F3, but its extent was much less than that observed from F4 (Fig. 5.3).

![Fig.5.3 DNA gel electrophoresis of cells fractionated on Percoll. Thymocytes were incubated with etoposide (10 μM) for 4 h and separated on Percoll gradients into four fractions (F1 - F4). Cells (1 x 10^6) were then examined by agarose gel electrophoresis. Lane 1 contains markers of 100 bp or multiples thereof.]

5.2.4. CD4 and CD8 expression

The large majority of unfractionated thymocytes were labelled with antibodies to both CD4 and CD8, representative of double positive (DP) population of immature thymocytes (results not shown). The populations with single positive (either CD4 or CD8, SP), double positive and double negative (DN) labelling were found in Percoll.
fractionated Fl cells (Fig. 5.4, a), which indicated that Fl was composed of mature T-lymphocytes (either CD4 or CD8 positive, less CD8+ cells were found in this study, Fig. 5.4, a), immature thymocytes (DP) and proliferative thymoblasts (DN). In contrast, F2 contained much less DN and SP cells. Less SP or DN cells was found in F3 and F4 which consist mainly of DP cells. But a decrease in surface expression of both CD4 and CD8 was found in F3 and F4, compared to F1 and F2 (Fig. 5.4).

**Fig. 5.4 Expression of CD4 and CD8 in cells from Percoll gradients.** Thymocytes were incubated for 4 h with etoposide (10 μM) and separated into four fractions (Fl- F4) by Percoll gradients. The cells from different fractions were labelled with anti-CD4-FITC and anti-CD8-phycoerythrin (PE) and analysed by flow cytometry. Cells in A, B, C and D represent the cells in fractions F1, F2, F3 and F4, respectively.

### 5.2.5. Ultrastructural features of cells from different fractions

Thymocytes were incubated for 4 h in the presence of etoposide (10 μM) and fractionated on Percoll gradients in to four fractions. The fractionated cells were then fixed and examined by electron microscopy. The morphology of the majority (85%) of the cells in F1 and F2 (Fig. 5.5 a) was indistinguishable from that of most thymocytes.
Chapter 5: Preapoptotic population

isolated from untreated rats. The diameter of most cells in F1 was larger than that of cells in F2. The nuclei in both fractions were rounded and characterised by relatively condensed perinuclear heterochromatin that lined the nuclear membrane, except for small regions around the nuclear pore. This heterochromatin extended as several ill-defined projections into the euchromatin. The cytoplasm formed a thin rim around the nucleus containing numerous free ribosomes together with a few spherical mitochondria and profiles of endoplasmic reticulum. Some short cytoplasmic projections were present on cells in both fractions. Macrophages and thymic epithelial cells were present in these preparations, particularly in F1, together with a small proportion (<5%) of thymocytes with pyknotic nuclei.

Over 95% of the profiles in sections of F4 were pyknotic nuclear remnants. About 10% were identified as apoptotic bodies. The remainder were entire cells (Fig. 5.5.b), most of which had accumulations of condensed chromatin forming dense apical caps within the nucleus. The nuclear membrane overlying these caps was closely juxtaposed against the membrane of the cell and, in these regions, the continuity of the two membranes was evident, and aggregations of cytoskeletal filaments were present within the cytoplasm of these cells. Nucleolar remnants, particularly the dense fibrillar components, were also prominent in the cytoplasm. The cisternae of the endoplasmic vacuoles fused with the cell membrane, usually towards one pole of the cell.

Unlike the other three fractions, F3 was strikingly heterogeneous at 4 h (Fig. 5.5.c). It contained a few apoptotic bodies (<5%) and many profiles (40% to 50%) exhibited the features of cells in F4. The remaining profiles were cells with a distinctly different morphology. These cells had a rather dense, granular cytoplasm containing normal organelles, except for a few small vacuoles or dilated cisternae of the endoplasmic reticulum. The most striking feature of these cells was the compaction of the perinuclear heterochromatin into dense, sharply defined clumps abutting onto the nuclear membrane, the morphology of early apoptosis described in previous chapter. Unusually large regions of this membrane were devoid of condensed
Fig. 5.5 Ultrastructural characteristics of a population of preapoptotic thymocytes. Thymocytes were incubated for 4 h in the presence of etoposide (10 μM) and separated into four fractions (F1 - F4) on Percoll gradients. Cells in F2 (a) appeared normal, whereas most of those in F4 (b) were apoptotic.

At least two types of cell were evident in F3 (c). This fraction was subsequently sorted by flow cytometry according to their fluorescence intensity with H33342. Cells with high blue fluorescence (region 3, Fig. 5.2c) were apoptotic, similar to those in F4. The preapoptotic population (d), with low blue fluorescence (region 1, Fig. 5.2c) showed aggregation of heterochromatin into discrete clumps that were sharply delineated from the remaining nucleoplasm. Bars = 1 μm
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chromatin and the membrane itself was often convoluted. The euchromatin and fibrillar centres of these cells were apparently normal.

5.3. Evidence that F3 is a population of preapoptotic thymocytes

The flow cytometric, Coulter counter and electron microscopy data suggested that the cells in F3 were an intermediate population between the normal (F1 and F2) and apoptotic thymocytes (F4). In order to test this hypothesis, further experiments were carried out.

5.3.1. Distinctive chromatin condensation of cells in F3 prior to internucleosomal cleavage of DNA

After incubation for 4 h with etoposide (10 μM), thymocytes were separated on a Percoll gradient. The cells in F3 were further incubated with H33342 and sorted by flow cytometry into three subpopulations of cells with low, intermediate and high blue fluorescence (regions 1, 2 and 3 of Fig.5.2, c, respectively). On examination by agarose gel electrophoresis, no laddering was observed with the cells from region 1 (lanes 2 and 4, Fig.5.6), whereas significant laddering was observed with cells from regions 2 and 3 (lanes 3 and 5, Fig.5.6). Examination of the cells from region 1 by electron microscopy showed that this population was homogeneous (Fig.5.5, d) and characterised by distinctive perinuclear accumulations of condensed chromatin. These clumps of densely staining material were closely abutted against the nuclear membrane. The sharp demarcation between these clumps and the euchromatin was accentuated by the apparent loss of the perichromatin fibrils, which permeate this region in both untreated cells and in etoposide-treated cells collected in F1 and F2. In marked contrast, most of the profiles (>95%) in regions 2 and 3 showed the ultrastructural features associated with the apoptotic cells and apoptotic bodies, as was observed in the F4 fraction (Fig.5.5,b).
Fig. 5.6 Absence of internucleosomal cleavage of DNA in the preapoptotic population of thymocytes. Thymocytes were incubated for 4 h with etoposide (10 μM). Cells in F3 were collected from Percoll gradients and incubated with H33342. The cells were then kept on ice and sorted by flow cytometry into the regions indicated in Fig. 5.2c and examined by agarose gel electrophoresis. Lane 1 contains markers of 100 bp or multiples thereof. Lane 2 and 4 contain cells (0.91 and 0.86 × 10⁶, respectively) from region 1, lane 3 contains cells (0.7 × 10⁶) from regions 2 and 3, and lane 5 contains cells (0.47 × 10⁶) from region 2 alone. Lanes 4 and 5 were from a separated gel electrophoresis, but the markers for this have been omitted for clarity.

5.3.2. Cells in F3 develop an apoptotic morphology accompanied by internucleosomal cleavage of DNA

Shorter incubations, with etoposide, resulted in F3 fractions that were more homogeneous than after the 4-h incubation (Compare Fig. 5.7.a and b with Fig. 5.2.c). When F3 was obtained after 1- and 2-h incubation (and in some experiments at 3 h), it was homogeneous consisting primarily of a single population of cells. When examined by H33342 staining, they exhibited low blue fluorescence and a lower mean forward light scatter than those of F1 and F2. In some experiments at 3 h (Fig. 5.7.c) and in all studies at 4 h (Fig. 5.2.c), the population of cells in F3 was clearly no longer homogeneous, consisting of a population of cells exhibiting progressively increasing blue fluorescence of H33342.
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When the cells in F3 were examined by agarose gel electrophoresis, no laddering was observed at the earliest times, e.g. 1 to 2 h (Fig. 5.7.a and b) but at 3 h laddering was observed (Fig. 5.7.c), which coincided with the appearance of cells with higher fluorescence. There was good agreement between the above flow cytometric results and the ultrastructural studies. The cells in F3 at 1 and 2 h (and in some experiments at 3 h) were much more uniform. No apoptotic bodies were found, and very few intact cells were apoptotic. The majority of cells (>85%) contained a nucleus with peripheral accumulation of chromatin, in which, although rather less pronounced than those present in the same fraction after 4-h incubation, were distinct from those observed in F1 and F2 at same times.

![Fig. 5.7 Heterogeneity of Percoll fraction F3 develops with time. Thymocytes were incubated with etoposide (10 μM). At the indicated times (1 to 3 h), thymocytes were fractionated on Percoll gradients and the cells in F3 examined either by flow cytometry after incubation with H33342 or by agarose gel electrophoresis. DNA laddering accompanied the appearance of the cells with high blue fluorescence.](image)

Finally, when a homogeneous population of cells from F3 was isolated and then incubated for a further 2 h in the either presence or absence of etoposide, the cells with smaller size and high fluorescence were induced, and DNA ladders developed(Fig. 5.8).
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Fig. 5.8 Development of DNA laddering after incubation of homogeneous F3 for further 2 h. Thymocytes were incubated with etoposide (10 μM) for 2 h and separated on Percoll gradient. F3 cells (lane 2) were incubated without etoposide for further 2 h (lane 3) and examined by agarose gel electrophoresis. Lane 1 contains markers of 123 bp or multiples thereof.

5.4. Discussion

Cell shrinkage is a characteristic feature of apoptosis, especially in thymocytes (Kerr, 1971, Wyllie and Morris, 1982, Ohyama et al, 1981, and Thomas and Bell, 1981). The mechanism of this shrinkage is not known but has been suggested to be related to a Na⁺/K⁺/Cl⁻ cotransporter (Wilcock et al, 1988). It has been suggested that thymocytes undergo a single step-wise transition from normal to apoptotic in response to glucocorticoids on the basis of the changes in buoyant density or cell size (Wyllie and Morris, 1982 and Walker et al, 1991). Our results indicate the existence of a population of cells (F3) with intermediate size and buoyant density between normal (F1 and F2) and apoptotic (F4) thymocytes (Table 5.1). Cell volumes, in each of different Percoll fractions with corresponding buoyant density, were similar and independent of the agents used to induce apoptosis (Table 5.1). The volumes of normal (F1 and F2) and apoptotic (F4) cells were in good agreement with those in the literature (Thomas and Bell, 1981 and Walker et al, 1991). Cells in F1 had a slightly larger volume that those in F2 due to containing a higher percentage of the proliferatively competent subpopulation (Wyllie and Morris, 1981).
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An increase in the percentage of cells in F3 and F4 after incubation of thymocytes with either etoposide or dexamethasone (Fig. 5.1, b and c) is consistent with the known observation that normal thymocytes in F1 and F2 are converted to apoptotic in F4, as well as with the hypothesis that some of the normal cells may be converted initially to a transitional population (F3) prior to their conversion to apoptotic cells in F4, which are recognisable by the usual morphological and biochemical criteria. The kinetic changes of F3 cell did not correlate with that of normal thymocytes but did reflect the extent of apoptosis, i.e. increasing both with the time and severity of treatment. Some apparent differences were also observed in the relative sensitivities of normal cells in F1 and F2 to dexamethasone and etoposide. Cells in F2 appeared more sensitive to dexamethasone, whereas the proliferatively competent cells in F1 appeared more sensitive to etoposide.

A decreased expression of both accessory molecules, CD4 and CD8, was observed in apoptotic thymocytes (Swat et al, 1991). It was also noted that the loss of surface Ag was proportional to the reduction in surface area (Morris et al, 1984). Our results not only are in general agreement with these findings (Fig. 5.4), but also demonstrate that the cells in F3 have a CD4\textsuperscript{low}CD8\textsuperscript{low} phenotype, and that the decrease is greater than that can be attributed to the change in cell size (Table 5.1). This decreased expression of the accessory molecules is not like the downregulation of them during positive selection and maturation of thymocytes, in which only one molecule is downregulated in one type of cells (Von Boehmer et al, 1989). In this study, the decrease in both molecules appeared simultaneously, which suggests that alterations in surface expression of both CD4 and CD8 are early changes in the apoptotic process and appear to precede internucleosomal cleavage of DNA.

These results demonstrate that the subset of cells in F3 with low blue fluorescence on flow cytometry was a transitional preapoptotic population of thymocytes which had been artificially banded on F3 due to their higher buoyant density than normal thymocytes. This was further supported by flow cytometric analysis in conjunction with biochemical and morphological observations. Up to at
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least 2 h, F3 was apparently homogeneous, and no DNA ladders were observed (Fig.5.7, a and b) but at 4 h (Fig.5.2, c) or sometimes at 3 h (Fig.5.7, c). cells in F3 were clearly heterogeneous, and this was accompanied by the appearance of DNA ladders (Fig.5.3 and Fig.5.7, c). Cell sorting also suggested that DNA ladders coincided with the appearance of cells with high blue fluorescence (Fig.5.6), i.e. cells from region 1 (of Fig.5.2, c) without any detectable DNA laddering were moving to regions 2 and 3 (of Fig.5.2, c), which had significant DNA laddering(Fig.5.6). Again, if the increase in the fluorescence intensity is due to an increased cell membrane permeability according to the study in previous chapter, the loss of cell volume seems to precede the change in cell membrane permeability and DNA laddering. The cell volume change thus becomes a distinct stage in apoptosis process. All these suggest that the subset of cells in F3 is a transitional preapoptotic population of thymocytes. Interestingly, recent study showed that after \( \gamma \)- irradiation, rat thymocytes undergo apoptosis shrink in two stages (Klassen et al, 1993). Cell volume firstly decreased from original 99 \( \mu m^3 \) to 76 \( \mu m^3 \), followed by a gradual decrease to 57 \( \mu m^3 \) over the space of a few hours. The size of this intermediate population was remarkably similar to that observed in this study (Table 5.1). Moreover, when mature resting mouse spleen B cells progressed into apoptosis, a decrease both in cell size and membrane phospholipid packing was observed prior to internucleosomal cleavage of DNA (Mower et al, 1994). These results further support that there is a transitional change in cellular features, e.g. cell size and membrane permeability, during apoptosis process.
5.5. Summary

In this study, a transitional population of cells intermediate in size between normal and apoptotic thymocytes has been identified and characterised on the basis of a variety of physical, biochemical and morphological criteria. No internucleosomal cleavage of DNA was observed in these transitional cells until the changes of nuclear morphology characteristic of typical apoptosis were observed, which provides strong evidence for a causative link of these two events. The morphology of this population of cells was distinct from both normal and apoptotic thymocytes, but was identical to that described in previous chapter as early morphology of apoptotic thymocytes. These data support the existence of a transitional preapoptotic population of thymocytes.
Chapter 6. Formation of large fragments of DNA precedes internucleosomal cleavage of DNA during apoptosis
Chapter 6: Formation of large fragments of DNA

6.1. Introduction: organisation of DNA in eukaryotic nucleus

In eukaryotic cells, the nucleus packs a large amount of DNA (6×10⁹ base pairs per nucleus, 2 meters in length) into a three-dimensional organisation of only a few micrometers in diameter. This packaging is accomplished by arranging the DNA into high-order structures. Briefly, the 2-nm (fibre width) DNA helix wraps around a core of histone proteins to form the second-order structure of DNA, namely the nucleosome beads. The nucleosome beads then coil into the solenoid filament with 6 nucleosomes per turn, so called 30-nm filaments. The solenoid fibre forms loops of 10 to 180 kilobase pairs in length, which could represent well accepted functional loop domains of DNA in the interphase nucleus. The loop domains wrap radially around a central core to form minibands or radical arrays that then stack vertically to form the metaphase chromosome. The final result of DNA packaging is a set of metaphase chromosome of varying length, but with a markedly uniform chromatid width of approximately 800 nm (Table 6.1, see Pienta et al, 1991 for review).

The proteins of the eukaryotic nucleus play an important role in maintaining the high order structure of DNA and can be divided into three classes. One is defined as the non-chromosomal nuclear proteins (NCNPs) associated with the nuclear envelope and matrix. The other two are associated with chromatin and may be subdivided as histone and non-histone chromosomal proteins (NHCPs). The latter (NHCPs) is defined as all the other proteins isolated with chromatin except for the histones that represent a well-characterised group of proteins with quite unique compositions, sequence characteristics and functions. The NHCPs include enzymes associated with chromatin, transcription-regulation proteins, hormone receptors, high mobility group (HMG) proteins and chromosomal structure proteins (see van Holde, 1989 for review). The discussion of these proteins is beyond the scope of this thesis. Here I will only briefly discuss those
nuclear proteins that are more or less concerned with DNA organisation and morphology of the interphase nucleus.

Table 6.1. Higher order organisation of DNA in the nucleus

<table>
<thead>
<tr>
<th>Units of DNA order</th>
<th>Fibre width</th>
<th>Base pairs per turn</th>
<th>Packing ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA helix</td>
<td>2 nm</td>
<td>10 bp</td>
<td>1</td>
</tr>
<tr>
<td>Nucleosome</td>
<td>10 nm</td>
<td>80 bp (200 bp per nucleosome)</td>
<td>6 -7</td>
</tr>
<tr>
<td>Solenoid</td>
<td></td>
<td>1.2 kbp (6 nucleosomes)</td>
<td>40±</td>
</tr>
<tr>
<td>Loop filament</td>
<td>30 nm</td>
<td>10 - 180 (63±14) kbp</td>
<td>680</td>
</tr>
<tr>
<td><em>Chromatid coils</em></td>
<td>240 nm</td>
<td>(5 loops per radical array, 300 kbp)</td>
<td>1700</td>
</tr>
<tr>
<td><em>Minibands</em></td>
<td>0.8 μm</td>
<td>(18 loops per minibands, 1.1 ×10³ kbp)</td>
<td>1.2×10⁴</td>
</tr>
<tr>
<td>Nucleus</td>
<td>~ 10 μm</td>
<td>6×10⁶ kbp</td>
<td>2×10⁵</td>
</tr>
</tbody>
</table>

* Two major models have been proposed at this level of packaging.
  a. Radical coil model: loops are folded to form hemiloops and then arranged radically to form a radical array. These radical arrays are then turned sideways and coiled to form 240 nm coils that then stack to form a chromosome.
  b. Radical loop model: the loops are wound into the 18 radical loops that form a miniband unit. The minibands are continuously wound and stack along a central axis to form a chromosome.


**Histones and nucleosome.** In the early 1970's, Olinses and Woodcock separately proposed a 'beads-on-string' model as the basic structure of chromatin (Olins and Olins, 1973 and Woodcock, 1973, and see Van Holde, 1989 for review). These beads, or nucleosomes as named by Oudet later on, were found to consist of histone octamers with DNA, of approximately 200 bp in length, wound around each histone octamer core of two copies of histone H2A, H2B, H3 and H4. At this level of folding, histone H1 was considered as a more crucial factor than
core histones that determines the average periodicity of nucleosomes in chromatin. Recent studies have shown that replacement of H1 in the chromatin by its variants H5 does not alter the repeat length (Sun et al, 1990). Some other mechanisms may determine the length of DNA associated with each nucleosome, including core histone that may specify the repeat length, either by histone-histone or histone-DNA interactions (see Widom, 1991 for review). Besides genetic variants of histones that have a profound role in the regulation of gene expression, there are also a set of posttranslational covalent modifications of histones, including acetylation, methylation, phosphorylation, ADP-ribosylation, ubiquitination, glycosylation and covalent addition of peptides. Evidence has accumulated that such alterations of the histone may have important effects on chromatin structure and function, which may be involved in the regulation of genes (see van Holde, 1989, for review).

The nucleosomal structure was alternatively postulated by Hewish and Burgoyne without knowing the string of beads appearance of DNA (Hewish and Burgoyne, 1973). In their study, chromatin from rat liver nuclei was treated with endogenous Ca^{2+}/Mg^{2+}-dependent endonuclease. Instead of cutting the DNA randomly, the endonuclease attacked the DNA at remarkably regular intervals, which turned out to be at inter-nucleosomal regions, i.e. between beads. They then suggested that this region might be relatively less protected and be potentially accessible to endonuclease.

Given this hypothesis, the access of endonuclease to the internucleosomal regions could still be blocked by the highly compacted structure of chromatin. It is not clear yet if the core histones or linker histone H1 are directly involved in the regulation of chromatin conformation, which is related to the accessibility of endonuclease. *In vivo*, newly synthesised DNA is rapidly assembled into chromatin. Histones H3 and H4 are posttranslationally acetylated before assembly into chromatin but then progressively deacetylated following incorporation into a nucleosome (see Wolffe, 1991 for review). If deacetylation is inhibited, the
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Chromatin remains sensitive to nuclease. The protein kinase cdc2 phosphorylates the amino acid sequence Ser-Pro-Lys-Lys (SPKK), the tail of a typical somatic histone H1. Phosphorylation of H1 is highest at mitosis, suggesting that this modification is important for compaction of chromatin into chromosome (Lamb et al, 1990). Activation of cdc2 kinase has recently been suggested to have importance in turning on the machinery of the death program (Shi, et al, 1994).

Internucleosomal cleavage of DNA has also been considered as a predominant pattern of DNA degradation in the cell death via apoptosis (Wyllie, 1980 and see Arends et al, 1991, for review). The changes in chromatin conformation could enhance the sensitivity of chromatin to pre-existing nuclease, while the increase in the activity of Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease by an unknown mechanism could alternatively be responsible for the internucleosomal cleavage of DNA in apoptosis (Kyprianou et al, 1988). It is generally believed that proteolysis of nuclear histones is not involved in apoptosis, which implies that the integrity of histones is essential for internucleosomal cleavage of DNA in apoptosis.

Several candidates for endodeoxyribonuclease responsible for internucleosomal cleavage of DNA during apoptosis have been characterised from or detected in various tissues or cell lines. These include a Ca\(^{2+}\)-independent DNase from CEM-C7 cells (Alnemri and Litwack, 1990), NUC-18 (Gaido and Cidlowski, 1991), DNase I (Ucker et al, 1992) and DNase II (Barry and Eastman, 1993).

Nuclear matrix and loop domains. The proposed interphase structure of the DNA loop domain, of 10 to 180 kbp in length, consists of a 30-nm filament. In addition to histone H1 that is essential for this level of folding, nuclear matrix plays an important role in maintaining the loop domain in the interphase nucleus. The nuclear matrix is a residual framework scaffolding that forms the superstructure of the nucleus. It consists of peripheral lamins and pore complexes, an internal ribonucleic protein network, and residual nucleoli. The structural elements of the nuclear matrix give the nucleus its overall 3-dimensional...
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organisation and shape, which is accomplished by two major DNA-protein interactions, namely telomere regions to the peripheral lamina of the nuclear matrix and the base region of loop DNA to the internal matrix. The telomere regions on the end of the chromosome are attached to the peripheral lamina of the nuclear matrix, mainly lamins A and C (Shoeman and Traub, 1990). As the nucleus approaches metaphase, the nuclear lamina proteins are phosphorylated and diffuse as small vesicles into the cytoplasmic area as the nuclear envelope disintegrates (Gerace and Blobel, 1980 and Gerace et al, 1984). The chromosomes, now free of the lamina, collapse into condensed mitotic structures. At the end of telophase, the end of the chromosome serve as organising centres for the condensation of lamina proteins to reform the lamina of the nucleus (Benyajati and Worcel, 1976 and Jost and Johnson, 1981). DNA loop domains are attached to nuclear matrices at their bases and this organisation is maintained during both interphase and metaphase. Nuclear matrix shares some proteins with the chromosome scaffold including topoisomerase II, an enzyme that modulates DNA topology (Earnshaw and Heck, 1985, Berrios et al, 1985 and Halligan et al, 1984). The points of attachment of the loop DNA to the nuclear matrix are termed matrix-associated regions (MARs) or scaffold-attached regions (SARs). In general, MARs share several common attributes, including unique length of 200 bp, A-T rich regions, poly-A signal regions and high homology with topoisomerase II cleavage sequences. The significance of these homologous topoisomerase II cleavage sequences are still unknown. It may play a key role in maintaining a dynamic structure of eukaryotic cells. It is not known for certain that loops formed by 30-nm filaments exist as a periodic structure, like nucleosomes, at higher level packaging. One reason for this is that the great variations in loop sizes have been reported from 10 to 180 kbp, which argue that fragments of DNA of those sizes might be generated by randomly shearing of DNA. The great variations in loop size may come from the methods used in processing and extracting DNA or used to visualise fragments, e.g. different gel electrophoresis system used. If there was a periodic structure
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represented by loops, MARs or SARs might be the vulnerable regions like linker regions between nucleosomes. The sequences of the end of the loops would help us to understand if topoisomerase II is involved in this periodicity of higher order structure.

6.2. Formation of large fragments of DNA in the absence of DNA laddering.

Based on a number of criteria including cell density, size and various morphological features, it has been demonstrated that zinc did not inhibit dexamethasone-induced apoptosis, rather prevented some characteristic nuclear changes of apoptotic thymocytes in the absence of internucleosomal cleavage of DNA (chapter 4). The morphological features of the apoptotic thymocytes, obtained from thymocytes treated with dexamethasone in the presence of zinc, showed clumps of condensed heterochromatin abutting the nuclear membrane. It has also been demonstrated in a previous chapter that internucleosomal cleavage of DNA was also absent in the transitional preapoptotic population of thymocytes which had distinct morphology from normal and apoptotic thymocytes (chapter 5).

In this study, field inversion gel electrophoresis (FIGE) was used to biochemically characterise the thymocyte nuclear changes induced by dexamethasone in the presence of zinc and the changes in the preapoptotic population of thymocytes.

6.2.1. Dexamethasone-induced DNA degradation in the presence of zinc

Incubation of rat thymocytes for 4 h with dexamethasone (0.1 μM) resulted in the cleavage of DNA into nucleosomal fragments of 180-200 bp or multiples thereof (Fig.6.1, lane 3) with typical morphology of apoptotic thymocytes, in agreement with previous studies. This internucleosomal cleavage was prevented by zinc (1 mM) (Fig.6.1, lane 4). Field inversion gel electrophoresis (FIGE) was
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Fig. 6.1 No internucleosomal fragments were observed after treatment of thymocytes with dexamethasone in the presence of zinc. Thymocytes were incubated for 4 h either alone (lane 2) or with dexamethasone (0.1 μM) in the absence (lane 3) or presence of zinc (1 mM) (lane 4), or were incubated for 1 h with etoposide (10 μM) (lane 5). Lane 1 contains markers of 123 bp or multiples thereof. Whole thymocytes (1 x 10⁶) were examined by agarose gel electrophoresis.

Fig. 6.2 Zinc causes an accumulation in the large fragments of DNA after treatment of thymocytes with dexamethasone. Treated thymocytes were encapsulated in agarose plugs and then DNA fragments were separated by FIGE. Lanes 1a and 1b contain standards (STD) of S. Cerevisiae chromosomes (243 - 2200 kbp) and Pulse Markers (0.1 - 200 kbp), respectively. Thymocytes were incubated either for 1 h with etoposide (10 μM) (lane 2) or for 4 h either alone (lane 3) or with dexamethasone (0.1 μM) alone (lane 4) or in the presence of zinc (1 mM) (lane 5). Each agarose plug contained 0.3 - 0.5 μg of DNA. The brackets at right are size assignments of 30 - 50 or 200 - 250 kbp.
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further used to examine the possible existence of other forms of degradation of DNA in thymocytes exposed to dexamethasone in the presence of zinc. When thymocytes were incubated with etoposide (10 μM) for 1 h, although there were no oligonucleosomal fragments (Fig. 6.1, lane 5), an initial cleavage of DNA into a large fragment of kilobase pairs in length (Fig. 6.2, lane 1), which is approximately or beyond 700 kbp according to a previous study (Walker et al, 1991), was observed. Striking differences were found in the thymocytes treated with dexamethasone in the presence of zinc (Fig. 6.2, lane 5), compared to those treated with dexamethasone alone (Fig. 6.2, lane 4). In the former, particularly evident were the increased amounts of large fragments, which were resolved as two bands of approximately 30-50 kbp and 200-250 kbp in length as well as a large fragment similar in size to that observed with etoposide treatment for 1 h.

![STD Dex 2 3 MW (kbp)](image)

**Fig.6.3 Time course of the formation of the large fragments of DNA.** Thymocytes were incubated with dexamethasone (0.1 μM) alone or in the presence of zinc (1 mM) for 1, 2, 3 and 4 h (as indicated above each lane) and examined for large fragments of DNA by FIGE. Thymocytes were also incubated for 4 h with zinc (1 mM) alone. Standards (STD) used in this gel are same as Fig.6.2.

When thymocytes were then exposed to dexamethasone either alone or in the presence of zinc for 1-4 h, a time-dependent increase in the appearance of the different fragments was particularly evident in the first 3 h of incubation with
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dexamethasone in the presence of zinc (Fig. 6.3). It appeared that in the presence of zinc, the large fragments induced by dexamethasone treatment were stopped further being degraded into nucleosomal fragments.

6.2.2. Large fragments are present in preapoptotic population of thymocytes in the absence of nucleosomal fragments of DNA

It has been characterised in a previous study (chapter 5) that a subset of cells in Percoll F3 of thymocytes treated with dexamethasone or etoposide are a transitional preapoptotic population of thymocytes based on a series of criteria. DNA fragments in this population were examined by FIGE in this study in order to further establish the relation between the formation of large fragments and inter-nucleosomal cleavage of DNA.

When thymocytes were incubated with dexamethasone (0.1 µM) for 1 h and fractionated by discontinuous Percoll gradients, homogeneous F3 cells were obtained. These cells showed early morphological changes of apoptosis but no DNA ladders (Fig. 6.4, panel b, lane 4). When four fractions (F1 to F4) were examined by FIGE, no large fragments of DNA were observed in F1 and F2 (Fig. 6.4, panel a, lanes 3 and 4), while distinct large fragments, especially of 30-50 kbp in length, were found in both F3 and F4 (Fig. 6.4, panel a, lanes 5 and 6). The difference between F3 and F4 was that no DNA laddering was observed in F3 at this time (Fig. 6.4, panel b, lane 5), while in F4 there was a clear DNA laddering (Fig. 6.4, panel b, lane 6). When cells in F3 were incubated for further 2 or 4 h, DNA laddering was found (data not shown, ref. Fig. 5.8). These observations supported the hypothesis that the formation of large fragments of DNA preceded inter-nucleosomal cleavage of DNA.
6.2.3 Discussion

The cleavage of DNA in apoptotic thymocytes by etoposide has been proposed to have two components (Walker et al, 1991). Initially a cleavage into large fragments of approximately 700, 300 and 50 kbp in length, which was followed by an endonuclease cleavage into fragments of 180-200 bp or multiples thereof (Walker et al, 1991). This implied that the initial cleavage of DNA into kilobase pair fragments might be dissociated from further cleavage of DNA into nucleosomal fragments. In order to examine this hypothesis, FIGE was used to visualise the possible existence of large fragments of DNA in thymocytes exposed to dexamethasone in the presence of zinc. When thymocytes were incubated with dexamethasone in the presence of zinc, no oligonucleosomal fragments was found, while increased amounts of large fragments were observed. They mainly consisted
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of two bands of approximately 30-50 kbp and 200-250 kbp in length, and also a large fragment similar in size to that observed with etoposide treatment for 1 h (Fig. 6.2, lane 2). These results suggested that in thymocytes exposed to dexamethasone, there was an initial cleavage of DNA into large fragments which were rapidly cleaved into oligonucleosomal fragments. Zinc facilitated the visualisation of this first component of DNA cleavage in apoptosis, possibly due to its inhibitory effect on the further degradation of those large fragments into the oligo-nucleosomal fragments (Cohen and Duke, 1984). This is partially supported by the observation that zinc inhibits etoposide-induced internucleosomal cleavage of DNA in HL-60 cells without affecting the amount of DNA strand breakage (Shimizu et al, 1990). The size of two fragments, i.e. of 30-50 and 200-250 kbp in length, may represent cleavage of genomic DNA into loop domains or rosettes of a set of six loops as described by others (Filipski et al., 1990 and Solovyan et al, 1991). The accumulation of these large fragments was coincident with the ultrastructural changes previously described in chapter 4, i.e. condensation of heterochromatin and its arrangement in sharply defined clumps abutting the nuclear membrane, which have been described as some of the earliest morphological features of apoptosis (Kerr et al, 1987 and Walker et al, 1988).

The hypothesis that the formation of large fragments of DNA preceded internucleosomal cleavage of DNA during apoptosis was further supported by the fact that these large fragments existed in the transitional preapoptotic population of thymocytes which had no oligonucleosomal fragments (Fig. 6.4). After further incubation of this population of cells for 2 or 4 h, oligonucleosomal fragmentation developed.

Thus these studies demonstrate that in apoptosis, there is a two-step degradation of DNA, i.e. an initial cleavage of DNA into large fragments prior to subsequent degradation to nucleosomal fragments. To a certain extent, this hypothesis might reflect the different levels of DNA organisation in the nucleus. The first step of degradation of genomic DNA involves the cleavage of DNA into
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loop domains which were further cleaved into oligonucleosomal fragments at the second step. It is possible that the first degradation, or the formation of large fragments, is a precondition for the second step, because it can relax DNA and form a suitable conformation of DNA for subsequent degradation of DNA. The involvement and the effect of nuclear proteins (either NCNPs or NHCPs and histones) on the degradation of DNA during apoptosis remains unclear. It is important to establish the nature of these kilobase pair fragments and the biochemical events responsible for the formation of these fragments.

6.3. Formation of large fragments of DNA is a Mg$^{2+}$-dependent process

Cleavage of DNA into oligonucleosomal fragments, recognisable as a DNA ladder on agarose gel electrophoresis, is regarded as the biochemical hallmark of apoptosis. In our and other previous studies, it has been shown that this internucleosomal cleavage of DNA is preceded by the formation of large fragments of DNA. In this study, rat thymocyte nuclei were used to examine the mechanism by which DNA is cleaved into large fragments. The data further supported that the degradation of DNA in thymocytes during apoptosis occurs in at least two steps. The first step involves the formation of large fragments which is mediated by a Mg$^{2+}$-dependent process, whereas the second step is responsible for the internucleosomal cleavage of DNA which is mediated by a Ca$^{2+}$/ Mg$^{2+}$-dependent endonuclease.

6.3.1. Ionic dependence of the formation of the large fragments of DNA

Divalent (Ca$^{2+}$, Mg$^{2+}$) or polyvalent (i.e. the polyamines, spermine and spermidine) cations are commonly used in buffers for the isolation of nuclei in order to maintain nuclear membrane integrity and chromatin structure. In our preliminary studies, significant levels of DNA cleavage into large fragments of
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kilobase pairs in length were always found in nuclei isolated using buffers containing standard concentrations of Mg$^{2+}$ (5 -10 mM). Substitution of Mg$^{2+}$ with low concentrations of polyamines (0.15 mM spermine and 0.5 mM spermidine) resulted in the isolation of nuclei with intact DNA, as assessed by conventional gel electrophoresis (Kokileva, 1989). It was confirmed in this study that no DNA degradation was observed, as assessed by FIGE, after incubation of nuclei at 37°C for 1 h in the absence of any added Ca$^{2+}$ and Mg$^{2+}$ (Fig.6.5, lane marked 0). In this case, the DNA was assumed to be intact by virtue of it remaining in the well with no large fragments being observed.

**Formation of large fragments is Mg$^{2+}$- but not Ca$^{2+}$-dependent.** In the presence of Mg$^{2+}$ (0.5-5 mM), a concentration dependent formation of large fragments of DNA, of ≥460, 200-250 and 30-50 kbp in length was observed (Fig.6.5, panel a). In marked contrast, formation of large fragments was not observed using same concentrations of Ca$^{2+}$ (Fig.6.5, panel a).

![Fig.6.5 Formation of large fragments of DNA is Mg$^{2+}$ dependent.](image)

Thymocyte nuclei were isolated as described and incubated as follows: (a) for 60 min either alone (lane marked 0) or in the presence of the indicated concentrations of either Mg$^{2+}$ or Ca$^{2+}$ (0.5 - 5 mM, as indicated above lanes); (b) in the presence of Mg$^{2+}$ (5 mM) for 0 - 240 min as indicated above lanes. Agarose plugs were prepared and the fragments of DNA were separated by FIGE. The numbers in the centre of the figure represent the size of standards in kilobase pairs (kbp). The standards (STD) 1 and 2 were as described in Fig.6.2.
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The Mg\(^{2+}\)-dependent formation of large fragments was studied from 0 to 4 h at a fixed concentration of Mg\(^{2+}\) (5 mM) (Fig. 6.5, panel b). After 15 min incubation, large fragments of ≥460 kbp were observed followed at 30 and 60 min by the accumulation of fragments of 200-250 kbp. By 120 and 240 min, clear accumulations of fragments of 30-50 kbp were also observed (Fig. 6.5, panel b). These results clearly demonstrated that Mg\(^{2+}\) alone could mediate both the formation of large fragments of DNA of ≥460, 200-250 and 30-50 kbp as well as the further degradation of fragments of ≥460 and 200-250 kbp to 30-50 kbp. No oligonucleosomal fragments were observed in the presence of Mg\(^{2+}\) alone even when incubated for 4 h (data not shown) despite extensive accumulation of fragments of 30-50 kbp (Fig. 6.5, panel b). At 4 h, although no internucleosomal cleavage was observed a small amount of smearing of DNA was noted on conventional agarose gel electrophoresis, which may have been indicative of non-specific damage to the DNA due to the long incubation times. Thus in the presence of Mg\(^{2+}\) alone, large fragments of DNA were formed but these were not further degraded to the characteristic DNA ladders observed in apoptosis.

Mg\(^{2+}\)-dependent degradation of large fragments is facilitated by Ca\(^{2+}\). Nuclei were first incubated in the presence of Mg\(^{2+}\) (5 mM) for 1 h, pelleted, resuspended and washed once in buffer D (containing EDTA and EGTA, see chapter 2) in order to remove the Mg\(^{2+}\). The nuclei were then resuspended and washed once in buffer C, resuspended in buffer C with no added cations, or with either Ca\(^{2+}\) or Mg\(^{2+}\) alone, or both Ca\(^{2+}\) and Mg\(^{2+}\) together. The initial 1 h incubation with Mg\(^{2+}\) alone resulted in the formation of large fragments comparable to that shown in Fig. 6.5, panel a. Autodigestions were then carried out for a further 1 h. In the absence of any added cations or with Ca\(^{2+}\) alone, no further degradation of large fragments was observed (Fig. 6.6, panel a, lanes 1 and 3 respectively). In the presence of Ca\(^{2+}\) and Mg\(^{2+}\), the large fragments of ≥460 and 200-250 kbp in length were more rapidly and extensively degraded to fragments of
30-50 kbp (Fig. 6.6, panel a, lane 4) than in the presence of Mg$^{2+}$ alone (Fig. 6.6, panel a, lane 2). Oligonucleosomal fragments were only observed in the presence of both Ca$^{2+}$ and Mg$^{2+}$ (Fig. 6.6, panel b, lane 4) but not in the presence of either Mg$^{2+}$ alone or Ca$^{2+}$ alone (Fig. 6.6, panel b, lanes 2 and 3).

**Fig. 6.6 Further degradation of large fragments is facilitated by Ca$^{2+}$.** Nuclei were incubated initially for 60 min with Mg$^{2+}$ (5 mM) alone. They were then washed in buffer D (containing EDTA and EGTA, see chapter 2) to remove the Mg$^{2+}$ and further incubated for 60 min in buffer C (without EDTA and EGTA, also see chapter 2) either alone (lane 1), with Mg$^{2+}$ (5 mM) (lane 2), with Ca$^{2+}$ (5 mM) (lane 3) or with both Ca$^{2+}$ and Mg$^{2+}$ (both 5 mM) (lane 4). The DNA fragments were then examined either by (a) FIGE or (b) conventional gel electrophoresis. The size of standards is shown on the left of each panel.

### 6.3.2. Effect of cycloheximide on the formation of the large fragments

Previous work has shown that even in the presence of both Ca$^{2+}$ and Mg$^{2+}$, no internucleosomal cleavage of DNA was observed following autodigestion of thymocyte nuclei isolated from animals treated *in vivo* with the protein synthesis inhibitor cycloheximide (Nikonova et al, 1982). It was suggested that the
Ca^{2+}/Mg^{2+}-dependent endonuclease turned over at a high rate and therefore this offered an experimental model to investigate the possible formation of the large fragments in the absence of the Ca^{2+}/Mg^{2+}-dependent endonuclease. In this study, after 4 h, nuclei were isolated from control and cycloheximide-treated animals and incubated in the presence of either Mg^{2+} (5 mM) alone or Ca^{2+} (5 mM) and Mg^{2+} (5 mM). As expected, autodigestion of control nuclei in the presence of Ca^{2+} and Mg^{2+} resulted in marked internucleosomal cleavage of DNA (Fig. 6.7, panel b). This internucleosomal cleavage of DNA required the presence both Ca^{2+} and Mg^{2+} and did not occur in the presence of either Ca^{2+} (result not shown) or Mg^{2+} alone (Fig. 6.7, panel b). A dose dependent inhibition of the Ca^{2+}/Mg^{2+}-dependent internucleosomal cleavage was observed with nuclei from animals pre-treated with cycloheximide (Fig. 6.7, panel b). Internucleosomal cleavage was partially and completely inhibited at doses of cycloheximide of 1.5 and 3.0 mg/kg respectively (Fig. 6.7, panel b).

![Fig. 6.7 Pre-treatment of rats with cycloheximide inhibits both internucleosomal cleavage and Mg^{2+}-dependent formation of large fragments of DNA. Rats were dosed with normal saline or with cycloheximide (1.5 and 3.0 mg/kg), sacrificed 4 h later. Thymocyte nuclei were prepared and incubated with either Ca^{2+} (5 mM) alone, Mg^{2+} (5 mM) alone or with both Ca^{2+} and Mg^{2+} (both 5 mM) for 1 h. DNA fragments were examined either by (a) FIGE or (b) conventional gel electrophoresis. The numbers at the left hand side of each panel represent the size of the standards used.](image-url)
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Autodigestion of thymocyte nuclei isolated from control animals resulted in the formation of large fragments, particularly of ≥460 and 200-250 kbp, in the presence of Mg$^{2+}$ alone or Ca$^{2+}$ and Mg$^{2+}$ but not in the absence of both cations (Fig. 6.7, panel a). The distribution of the sizes of the fragments was somewhat different dependent on the presence of Mg$^{2+}$ alone or Ca$^{2+}$ and Mg$^{2+}$. In the presence of both cations, degradation of the large fragments to 30-50 kbp was facilitated. Autodigestion of nuclei isolated from cycloheximide pre-treated animals showed a dose dependent inhibition of both the Mg$^{2+}$-dependent and the Ca$^{2+}$/Mg$^{2+}$-dependent formation of large fragments (Fig. 6.7, panel a). This was most marked with the Mg$^{2+}$-dependent cleavage. The inhibitory effects of cycloheximide on the formation of large fragments and nucleosomal fragments were obviously different. At the dose completely inhibiting the formation of nucleosomal fragments, Mg$^{2+}$ and Ca$^{2+}$/Mg$^{2+}$-dependent formation of large fragments was still visible (compare Fig. 6.7, panel a and b).

6.3.3 Discussion

The data from this study further support the hypothesis that there are at least two steps involved in the degradation of DNA in apoptosis in thymocytes. The initial step involved a Mg$^{2+}$-dependent cleavage of DNA to large fragments of DNA, of ≥460, 200-250 and 30-50 kbp in length, which was not catalysed by Ca$^{2+}$ alone. However, the second step, i.e. the characteristic internucleosomal cleavage of DNA was not observed in the presence of either Mg$^{2+}$ or Ca$^{2+}$ alone but rather required both Ca$^{2+}$ and Mg$^{2+}$.

It is not clear whether these two steps in DNA degradation are mediated by different enzymes (endonucleases). Assuming that more than one endonuclease were involved in this two step degradation of DNA, the identity of the enzymes is unknown. To date, most work in this area has, by virtue of the assay used for measuring activity, concentrated on the enzymes responsible for internucleosomal
cleavage of DNA. The Ca\(^{2+}\)/Mg\(^{2+}\)-dependence of this enzyme was recognised in early studies (Wyllie, 1980 and Cohen and Duke, 1984), which has been confirmed in the present study (Sun and Cohen, 1994). Recent studies have suggested that this enzyme may be NUC-18 (Gaido and Cidlowski, 1991), DNase I (Peitsch, et al, 1993) or DNase II (Barry and Eastman, 1993). The identity of the Mg\(^{2+}\)-dependent enzyme, if there is one, responsible for the formation of the large fragments is not known. It appears to be distinct from both NUC-18 that is inactive in the absence of Ca\(^{2+}\) (Gaido and Cidlowski, 1991) and DNase I that also requires the presence of both Ca\(^{2+}\) and Mg\(^{2+}\) (Peitsch et al, 1993). Topoisomerase II is a major component of the nuclear scaffold and it has been suggested to play a role in the earliest stages of apoptosis (Arends and Wyllie, 1991, Walker et al, 1991 and Oberhammer et al, 1993). DNA topoisomerase II is a Mg\(^{2+}\)-dependent enzyme (Wang, 1985). However a role for topoisomerase II in the induction of apoptosis is far from clear. A recent study in mouse thymocytes suggested that both etoposide and teniposide, two topoisomerase II inhibitors, induced apoptosis by a mechanism independent of inhibition of topoisomerase activity (Ye et al, 1993). A Mg\(^{2+}\)-dependent endonuclease has been isolated from HeLa cells (Gottlieb and Muzyczka, 1990) and myeloid leukaemia cells (Kawabata et al, 1993). In the latter study, a Mg\(^{2+}\)-dependent and Ca\(^{2+}\)-independent endonuclease activity was found in the nuclei of myeloid cell lines including HL60, but this enzyme cleaved chromatin to give a characteristic DNA ladder pattern (Kawabata et al, 1993). It is also of interesting that in their early study, Hewish and Burgoyne found a fraction of endonuclease that was Mg\(^{2+}\)-dependnet (Hewish and Burgoyne, 1973). The relationship, if any, to the enzyme involved in the present study is nuclear.

Previous studies utilising cycloheximide both in vivo and in vitro had resulted in a loss of a Ca\(^{2+}\)/Mg\(^{2+}\) - catalysed internucleosomal cleavage, which was attributed either to the rapid turnover of the Ca\(^{2+}\)/Mg\(^{2+}\) - dependent endonuclease (Nikonova et al, 1982 and McConkey et al, 1990b) or its inhibition due to an activation of poly(ADP-ribosylation) (Nelipovich et al, 1988 and Sooki-Toth et al,
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1989). Our studies with the nuclei from cycloheximide treated animals confirmed the loss of the \( \text{Ca}^{2+}/\text{Mg}^{2+} \) - dependent internucleosomal cleavage of DNA, in particular at the high dose of cycloheximide. However both the \( \text{Mg}^{2+} \)- and the \( \text{Ca}^{2+}/\text{Mg}^{2+} \)-dependent formation of the large fragments also showed a dose-dependent inhibition with cycloheximide. These results suggested an alternative possibility for the loss of the \( \text{Ca}^{2+}/\text{Mg}^{2+} \)-internucleosomal cleavage in the nuclei from cycloheximide treated animals, i.e. a decrease in the formation of the large fragments which ultimately give rise to the oligonucleosomal fragments. Although the different ionic dependencies for the formation of large fragments and internucleosomal cleavage is suggestive of the involvement of more than one enzyme, I cannot state unequivocally whether more than one enzyme is involved in DNA degradation in apoptosis.

The role of \( \text{Ca}^{2+} \) in apoptosis has been well documented (Orrenius et al, 1992), while less attention has been paid to the possible role of intracellular free \( \text{Mg}^{2+} \). Recent reevaluations of the role of \( \text{Mg}^{2+} \) in cellular function suggest that intracellular free \( \text{Mg}^{2+} \) may also be a key physiological regulator of cell activity (Preston, 1990). The present study demonstrates a role for \( \text{Mg}^{2+} \) in the early degradation of DNA in apoptosis. Further studies on the regulation of intracellular and intranuclear concentrations of free \( \text{Mg}^{2+} \) will increase our understanding of mechanisms of apoptosis.
6.4. Summary

Different approaches have been used in this study in order to examine the steps of DNA degradation during apoptosis. We have established that the formation of large fragments of DNA, of ≥ 460, 200-300 and 30-50 kbp in length, precedes the internucleosomal cleavage of DNA. This first step of DNA degradation correlates both with the very early morphological features of apoptosis and with the formation of preapoptotic cells. Further studies showed that the initial degradation of DNA into kilobase pair fragments depended on Mg$^{2+}$ rather than Ca$^{2+}$, which was also inhibited by cycloheximide. The nature of these large fragments, the mechanisms by which DNA is cleaved into these large fragments and the relevance of its formation with death program remain to be established.
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7.1. Introduction

A variety of questions has always been asked by the people dealing with cell death phenomena. Is cell death solely a passive or degenerative process? Why do some cells die in apparently healthy tissues or in normal physiological processes? What is the significance of cell death both in physiological and pathological situations? Do the differences in the morphology of the dying cells mean that there exist more than one mode of cell death? Can cells die by a process other than a passive one, i.e. die actively? Are genetics involved in manifesting cell death? What are the biochemical mechanisms related to distinct changes of morphology in different modes of cell death? To what extent, can cell death be controlled.

More than one century ago, in 1858, the German pathologist Rudolf Virchow delivered a series of lectures concerning cellular pathology (Virchow, 1858). In one of those lectures, he demonstrated that a large number of passive processes, in which cells do not display particular activity, take place in the body. He called those changes in cellular elements as passive disturbances in which cells either immediately lose a portion of their functions, or are completely destroyed. In this way, degeneration, defined as a loss of substance and a diminution of the constituents of the body, is produced.

This idea dominated the understanding of cell death for nearly a century until early 1950's when Glucksmann first systematically described cell death in normal vertebrate ontogeny (Glucksmann, 1951). Actually, he was not the first person who noticed this phenomenon. At the beginning of this century, a few embryologists had already found it difficult to accept the idea that cells die during embryogenesis and particularly in actively growing regions. They therefore interpreted the 'granules' (i.e. pycnotic nuclei) in dying cells as 'mitotic metabolites'. It was a surprising fact that embryonic cells in actively growing regions die, and it was difficult to see what may cause the death of these cells. It appeared as an extravagant waste on the part of the organism to produce cells that
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were subsequently discarded. In his study, Glucksmann demonstrated that cell death occurred regularly at certain developmental stages of all vertebrate embryos. On the basis of the morphological changes, he divided the process of cell death into three stages, i.e. chromatopycnosis, hyperchromatosis of the nuclear membrane and chromatolysis. Interestingly, his description was so similar to what people used today to describe apoptosis. At the first stage, the morphology of dying cells consists in the separation of the chromatic from the nonchromatic material of the nucleus and the precipitation and coalescence of the former into large granules and finally into a single mass. The non-chromatic material seems to liquefy and to form confluent vacuoles. At the second stage, these nuclear changes result in the appearance of a single chromatic mass sitting as a cap on the vacuole formed by the non-chromatic material. At the third stage, after gradual shrinkage, a mere chromatic granule persists followed by the phagocytosis of the degenerating cell by a neighbour. He also observed that experimentally cells can be killed in various ways, resulting in different morphological changes. For example, radiation-induced changes concerned mainly the chromatic parts of the nuclei, while death due to interference with the blood supply caused a process that is characterised by the loss of staining power of the nucleus, i.e. predominantly a process of autolysis leading to karyolysis and cytolysis, or necrosis. Another point he made that is now important to the study of cell death was that cell death seems to play a significant role in embryonic processes, e.g. in the morphogenesis and histogenesis of tissues and organs, and the representation and regression of phylogenetic steps, i.e. cell death actively participates in embryonic development.

This principle was applied by Saunders in his study on death in embryonic systems (Saunders, 1966). The terminology he adopted in his study, e.g. phylogenetic death, histogenetic death and morphogenetic death, actually came from what Glucksmann used to describe the role of cell death in embryonic development. Saunders and his colleagues were the first to approach the question in an experimental manner. They identified a region on the chick posterior necrotic
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zone (PNZ) in which substantial cell loss helped to define the border of the wing bud. Cell death in the region was identified by nile-blue-sulfate uptake: that is, by recognition of phagocytosed cells in phagocytic vacuoles. They then explanted the tissue from that region as soon as it could be identified and grew it in a culture system *in vitro*. What they found was that at the appropriate age, numerous cells die in culture. In his studies, the control of cell death by tissue environment, hormone and even genetic factors was noticed.

At approximately the same time, Lockshin and Williams carried out a series of studies on insect and observed a similar phenomenon in insect metamorphosis (see Lockshin and Zakeri, 1991 for review). In Saturnine silk moths, the intersegmental muscles degenerated after the ecdysis of the moth. Although several biochemical changes coincident with death (e.g. an increase in lysosomal enzymes) could be identified, as could endocrine and neural timing events, death of the muscles could be blocked by any of several neuro-pharmacological agents. Thus, it appeared to these authors that there existed a program or sequence of events leading to involution and the term 'programmed cell death' was born. Their studies established an important concept that the cells actually carry within themself the program to commit suicide when required to by the organism, and that this property of cells is essential for an evolutionary imperative to nourish or otherwise maintain the organism. Two characteristics of programmed cell death were thus defined: (1) it is a developmental event; (2) it arises as a cellular response to an unknown stimulus (Lockshin and Zakeri, 1991). These notions led to a search for evidence for the existence and expression of the death program and genetic involvement in cell death, with early recognition that inhibition of protein synthesis can delay or prevent cell death (Tata, 1966, Lockshin, 1969, Lieberman et al, 1969 and Munck, 1971).

The concept of apoptosis arose at approximately the same time as that of programmed cell death. During the 1960's, there was an increasing recognition that spontaneous loss of cells is an important parameter in neoplastic growth (see Kerr
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et al, 1972, for review). Several groups discovered a distinctly different mode of cell death with ultrastructural features that were consistent with an active, inherently controlled phenomenon (Klion and Schaffner, 1966, Farbman, 1968, Kerr, 1969 and 1971). This mode of cell death seemed to play an important role in the regulation of cell numbers in a variety of tissues under both physiological and pathological conditions, including untreated malignant neoplasms, the regression of tumours following some forms of therapy, and even the tissues of healthy animals (Kerr et al, 1972). It was originally called 'shrinkage necrosis' (Kerr, 1971), which was found undesirable for the description of a phenomenon that occurs under physiological conditions. When Kerr, Wyllie and Currie realised that cell death with this morphology plays an opposite role to mitosis in regulating tissue size, the name 'apoptosis' was proposed (Kerr et al, 1972). The word, like mitosis, is derived from the Greek and means leaves falling off trees or petals falling off flowers, which highlights its kinetic significance and indicates that apoptosis may act as a counterbalance to mitosis.

Genetics provides an approach toward an understanding of the molecular mechanisms of cell death. During the 1980's, Horvitz's group in MIT first identified several genes that were related to the death of over 100 of 1090 somatic cells in C. elegans formed during development. Some of them were responsible for the onset of the program (so called death gene), e.g. ced-3 and ced-4, while some functioned as anti-death genes, e.g. ced-9 that protects cells from death (Horvitz et al, 1983, Ellis and Horvitz, 1986, Yuan and Horvitz, 1992 and Ellis et al, 1991). The homologues of some of these death related genes have been found in mammalian cells (Yuan et al, 1993, Hengartner and Horvitz, 1994 and for review see Raff, 1992 and Barinaga, 1994). It is hopeful that scientists may ultimately find ways to manipulate cell death, turning it on to destroy cancer cells or turning it off to prevent the loss of neurones in neurodegenerative disease (Barinaga, 1994).

From a toxicological point of view, it is important to understand the mechanism of cell death induced by exposure of cells or organisms to chemicals
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capable of causing cellular damage. It is becoming evident that cell death under a variety of conditions and environments is the product of a precise biochemical cascade. Therefore, we should not regard cell death only as a catastrophic failure of cell integrity caused by molecular damage, *per se*. In fact, molecular damage may be a start signal connecting a series of cellular events that immediately precede death in some cells that bear damage. It is these events that cause the catastrophic failure recognised as cell death rather than the original damage induced by toxic insult. The final molecular changes can occur following signal transduction that can be initiated either by damage itself or special receptor mediated pathways. Those final molecular changes can be defined as biochemical markers or morphologically recognisable changes. Identification of those molecular changes and corresponding morphological changes will help us to understand the mechanisms of cell death.

In my study, I started with the establishment of a flow cytometric method that can be used conveniently to separate and quantify apoptotic thymocytes. By using this method, I found that zinc did not inhibit the induction of apoptosis by dexamethasone. The internucleosomal cleavage of DNA was, for the first time, dissociated from early nuclear morphological changes, as well as from other cytoplasmic changes, e.g. membrane permeability, cell size and buoyant density, in apoptosis of thymocytes. Identification of a transitional preapoptotic population of thymocytes further confirmed this observation. In this population of cells, early nuclear morphological changes and other cytoplasmic changes were observed in the absence of internucleosomal cleavage of DNA. By using field inversion gel electrophoresis, I found that the formation of kilobase pair (kbp) fragments of DNA preceded internucleosomal cleavage of DNA. The formation of these large fragments of DNA was visualised when thymocytes were treated with dexamethasone in the presence of zinc. These large fragments of DNA were also present in the transitional preapoptotic population of thymocytes. Subsequent incubation of this population of cells generated oligonucleosomal fragments and a
nuclear morphology characteristic of apoptosis. Taken together, an early stage of apoptosis in thymocytes was identified (Fig. 7.1). Further studies with isolated thymocyte nuclei showed that the formation of these large fragments of DNA was a Mg$^{2+}$-dependent process that can be facilitated by Ca$^{2+}$ and inhibited by pretreatment of the animal with cycloheximide.

### 7.2. Separation and quantification of apoptotic cells

The death of a cell often means very different things to scientists in different disciplines. They utilise their own techniques to facilitate the identification of cell death or to detect dead cells as they understand. For example, to radiobiologists, cell death has a rather special functional meaning, i.e. the loss of reproductive ability that could be used as an indicator of cell death by them. To pathologists, atrophy or involution of a tissue or organ and regression of hyperplasia are indicative of cell death. They also tend to give a special morphological definition to cell death, in this way they could identify a cell as a dead or dying one on the basis of its morphology differing from that of neighbouring normal healthy cells. These morphological changes could be easily detected using microscopy. Biochemists like to detect the cessation of the properties that are associated with life, e.g. respiration of cells, or the appearance of degradative products, e.g. DNA fragments, as their marker of cell death. It is obvious that many methods used in cell death are still histological in nature. Although many histological studies are apparently based on the observations relating to selective uptake or exclusion of stains or dyes, which frequently reflect quite subtle changes in membrane permeability, their value should not be underestimated. The intravital dyes such as nile-blue-sulfate (Saunders, 1966) and acridine orange (McConkey et al, 1988) can still be used to advantage. This, in conjunction with other features of cell death, is the basis for some widely used flow cytometric methods that have been proved to be very efficient methods in the study of cell death.
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7.2.1. Flow cytometric methods in separation and quantification of apoptotic thymocytes - overall

Flow cytometry has been widely used to identify apoptotic cells, and seems to be the most reliable methodology to separate and quantify apoptotic cells on the basis of individual cells. This approach offers the advantage of both rapid, multiparameter, cell-by-cell analysis and the ability to sort purified subpopulation for further biochemical and morphological studies. Flow cytometric methods have been practically divided into two major classes, nuclear and cellular method (Elstein and Zucker, 1994). In the nuclear method, cells are usually either fixed and permeabilised, or lysed with non-ionic detergents to isolate nuclei. These procedures allow low-molecular weight DNA fragments that result from internucleosomal cleavage of DNA by endonucleases to diffuse out, leaving a nucleus with reduced DNA content. When stained with a nucleic acid fluorochrome (e.g. PI), the resulting nuclei exhibit less fluorescence than the diploid nuclei of viable cells (Nicoletti et al, 1991, Telford et al, 1992, Sun et al, 1992b, Afanasyev et al, 1993 and reviewed by Darzynkiewicz et al, 1992). Due to condensed chromatin in dying cells, it is still conceivable that both DNA loss and a decease of the accessibility of dye to DNA are responsible for the low fluorescence of apoptotic cells (Zamai et al, 1993). Another major problem associated with the nuclear method is that the method uses fixed or permeabilised cells, therefore no discrimination between viable and nonviable cells could be observed. In addition, the nuclear method consists in the discrimination between real apoptotic cells and debris. Since apoptosis results in the complete fragmentation of whole cells including the nucleus and cytoplasm, cell fragments recognised as debris are produced. Failure to discriminate these debris from apoptotic cells will bring inaccuracy to the analysis.

Cellular methods are usually based on the light scatter parameters or immunofluorescent and other fluorochrome staining to detect apoptotic cells (Swat et, 1991, Jones and Lafrenz, 1992, Ormerod et al, 1992 Dive et al, 1992 Sun et al,
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1992a, Elstein and Zucker, 1994). Apoptotic cells exhibit reduced forward light scatter as a result of gross changes in cell size and membrane refractive index, and increased side light scatter as a result of chromatin condensation. At earlier stage of apoptosis, altered membrane permeability can be detected by changes in dye retention due to altered membrane fluidity (Darzynkiewicz et al, 1992, Fadok et al, 1992, Ormerod et al, 1993 and Mower et al, 1994). Cellular methods normally utilise more than one feature of apoptosis, therefore are more reliable than nuclear methods. Since there are subtle changes between apoptotic and normal cells regarding cellular parameters, sample preparation, staining conditions (e.g. concentration of fluorochromes and incubation time), the setting of the instrument may influence the results. This leads to variability between studies carried out in different laboratories. In addition, since the changes of cellular parameters, e.g. cell size and membrane permeability, are not necessarily identical between cell types, the method validated by using one type of cell may not be suitable for other cell types. Finally, cellular methods are often dependent on changes in membrane permeability to certain types of fluorochromes and therefore can not be used in studies dealing with agents that change membrane integrity, \textit{per se}.

7.2.2. The flow cytometric method established in this study

The vital bisbenzimidazole dye Hoechst 33342 and the DNA intercalating dye propidium iodide were used in this study to separate viable cells from non viable cells. Immature rat thymocytes were incubated with dexamethasone for 2 - 6 h and then incubated with H33342 and PI. Two distinct populations of thymocytes were observed on the cytogram of blue versus red fluorescence (Fig.3.7, a and b). The population that fluoresced blue was viable due to uptake of H33342 and exclusion of PI. The population that fluoresced red was nonviable due to failure to exclude PI. When nonviable cells were gated out and viable cells were displayed on a cytogram of blue fluorescence intensity versus forward light scatter, two populations of cells were observed, one with low fluorescence intensity and high
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forward light scatter and the other with high fluorescence intensity and low forward light scatter (Fig.3.7, c and d). Forward light scatter is indicative of cell size, therefore one population of cells was larger than the other. Thymocytes from these two populations were further sorted by flow cytometry and examined by electron microscopy and agarose gel electrophoresis. The larger cells with low fluorescence showed normal morphology of thymocytes and the absence of any oligo-nucleosomal fragments of DNA, while the smaller cells showed the morphology characteristic of apoptosis and extensive internucleosomal cleavage of DNA into oligonucleosomal fragments (Fig.3.8 and 9). Apart from dexamethasone, other glucocorticoids, e.g. corticosterone and methylprednisolone (data not shown), and DNA topoisomerase I and II inhibitors (Fig.3.17) also increased the formation of the smaller cells with high fluorescence. The formation of this population of cells induced by those compounds mentioned above was prevented by inhibitors of macromolecular synthesis. The suggestion that this population of cells with smaller size and high fluorescence was apoptotic was further validated by the experiments using discontinuous Percoll gradient to isolate apoptotic thymocytes (Wyllie et al, 1982). The obtained apoptotic cells showed high blue fluorescence and low forward light scatter on the cytogram of blue fluorescence intensity versus forward light scatter (Fig.3.10). Finally, a flow cytometric method was established in this study, which offers a rapid and convenient way for separation and quantification of apoptotic thymocytes.

Further studies were carried out to establish the basis of this method. In several studies, apoptotic cells fluoresced more brightly on excitation of the H33342-DNA complex by UV light (Dive et al, 1992, Ormerod et al, 1992, Sun et al, 1992a and Elstein and Zucker, 1994). There are at least two possibilities responsible for the increase in the fluorescence intensity of H33342 in apoptotic thymocytes: (1) an alteration in DNA conformation due to DNA degradation, or (2) an increase in the intracellular concentration of the dye caused either by an increase rate of uptake or a deceased rate of efflux of the dye. Further experiments
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were designed to test these possibilities. Firstly, a time-dependent increase in the uptake of H33342 by either normal or apoptotic thymocytes was observed and the initial rate of increase in the fluorescence was higher in apoptotic population than in normal population (Fig.3.11). Verapamil, which blocks the p-glycoprotein pump and thus may be responsible for the decreased rate of efflux of dye from cell, had little effects on the fluorescence in either apoptotic or normal cell (Fig.3.12).

Secondly, when the nuclei of both apoptotic and normal thymocytes were incubated with H33342, a decrease in the fluorescence was observed in the nuclei of apoptotic thymocytes (Fig.3.14), which suggested that an altered DNA conformation due to DNA degradation could not be the reason for the increased fluorescence in apoptotic thymocytes. Finally, fluorescein diacetate (FDA) and its derivatives were used to investigate the changes in membrane permeability of apoptotic thymocytes. FDA, as well as its derivatives, diffuses into cells where it is hydrolysed by intracellular esterase to give a charged, fluorescent product, e.g. in the case of FDA, fluorescein. The rate of loss of fluorescent product from the cells is dependent on both the chemical structure of the analogues and the ability of the membrane to retain them inside the cell, i.e. membrane permeability (Kolber et al, 1989). After thymocytes were loaded with dyes for certain times, washed to get rid of remained dyes and resuspended in dye-free buffer, a measurable loss of the dye was only observed in apoptotic thymocytes (Fig.3.16). Thus, in this study, the possibilities that the alteration in apoptotic thymocytes in either DNA conformation or p-glycoprotein is responsible for the increase in the fluorescence intensity were basically excluded. The results suggest that there is a change in the membrane permeability of apoptotic thymocytes and it is this change that leads to a more rapid entrance of H33342 in apoptotic thymocytes and to an increased fluorescence in apoptotic thymocytes.

The nonviable population of thymocytes was also revealed by this method as PI-positive. It was suggested that this population of cells consisted of several types of cells that fail to exclude PI. Some of them were necrotic cells formed
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during isolation of thymocytes. In fact, a small percentage of freshly prepared thymocytes (approximately 4%) was trypan blue positive cells. In addition, at the later stage, the membrane integrity of apoptotic cells may be further damaged and thus apoptotic cells fail to exclude PI, which is usually regarded as secondary necrosis (Wyllie et al, 1980). In my studies, thymocytes were normally incubated for not more than 6 h either with or without the inducers of apoptosis. By this time, PI-positive cells in control cells were about 10% of total thymocyte preparation, while PI-positive cells in those treated with the inducers of apoptosis were slightly higher. To reduce the inaccuracy and specify the results, my data were always expressed as viable apoptotic thymocytes, i.e. the cells with high fluorescence intensity and low forward light scatter, in the total preparation of thymocytes.

It is also noteworthy that when this method was explored for the study of the induction of apoptosis by etoposide in vivo, some differences in the characteristics of apoptotic cells induced by etoposide between in vitro and in vivo studies were observed. One is the formation of the third population in vivo with both low fluorescence intensity and forward light scatter (Fig.3.22, region 3 of b, d and f). This population may represent apoptotic bodies according to their smaller size and exclusion of PI. A similar population was also noticed in dexamethasone-induced apoptosis in vivo as assessed by this method (data not shown). The other difference was that the increase in blue fluorescence in the apoptotic population in vivo was not as great as observed in vitro. It was also observed that a time-dependent decrease in viable cells was accompanied by an increase in nonviable PI-positive cells after in vivo exposure to etoposide (Fig.3.23). They could not be necrotic cells as judged by following observations. Examination of tissue sections at both light microscopic and ultrastructural levels did not provide any evidence for the presence of necrotic cells (Fig.3.19 and 20). Similarly, no evidence for an acute inflammatory reaction was observed. The reason for the existence of these PI-positive cells with apoptotic morphology may possibly be due to the fragility of
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apoptotic thymocytes and further loss of membrane integrity during processing. Another possibility that massive induction of apoptosis may exceed the capacity of physiological phagocytosis, resulting in apoptotic cells undergoing secondary necrosis, could also not be excluded. These results indicate some limitations to the extrapolation of *in vitro* results to *in vivo* studies.

7.3. The correlation of nuclear morphological changes and DNA degradation in apoptosis

It is generally believed that certain biochemical events are responsible for the apparently unique morphology characteristic of apoptosis, e.g. cell shrinkage and condensation of the nuclear chromatin into uniformly dense masses that abut on the nuclear membrane followed by the compaction of both the nucleus and the cytoplasm. Especially, it is suggested that chromatin condensation observed in thymocytes is the result of the internucleosomal cleavage of DNA by endonuclease (Arends et al, 1990). It is also proposed that internucleosomal cleavage of DNA is the cause of cell death based on several lines of evidence (Compton, 1992 and Schwartzman and Cidlowski, 1993). My study and many others do not support this as a universal hypothesis (Cohen et al, 1992, Barres et al, 1992, Barberi et al, 1992, Oberhammer et al, 1993a and b, Tomei et al, 1993 and Sun et al, 1994a). In these studies, morphological changes characteristic of apoptosis in different cell types were observed prior to or even in the absence of internucleosomal cleavage of DNA. It is also noticed that programmed cell death shares the unique morphology with apoptosis, e.g. chromatin condensation, but do not always have laddering pattern of DNA degradation (Lockshin and Zachary, 1991).

7.3.1. Internucleosomal cleavage of DNA

Cell death accompanied by internucleosomal cleavage of DNA was first found in mouse thymus following irradiation of animal (Skalka et al, 1976). In his landmark paper published in 1980, Wyllie, for the first time, associated this pattern
of DNA degradation with apoptosis induced by glucocorticoids in rat thymocytes (Wyllie, 1980). During apoptosis, DNA degradation occurred in a very specific pattern producing fragments of DNA that were multiples of 180 - 200 base pairs, which is the length of DNA wrapped around the histone octamer in a nucleosome, which indicates that the chromatin is being cleaved at the linker DNA between nucleosomes, producing oligonucleosomal fragments. It was the first and only, by that time, biochemical event that was characterised in apoptosis process. Since then, this process has been observed in almost all instances of apoptosis and has been regarded as a biochemical hallmark of apoptosis (see Schwartzman and Cidlowski, 1993 for review). It was not only associated with the morphological changes in apoptotic cells but also regarded as cause of cell death in those cells.

On the basis of this notion, a great deal of effort was devoted to identify the endonuclease(s) responsible for this process. To date, several candidate molecules for apoptosis related endonucleases have been characterised from or detected in various tissues or cell lines, including NUC-18 (Caron-Leslie et al, 1991 and Gaido and Cidlowski, 1991), DNase I (Ucker et al, 1992 and Peitsch et al, 1993) and DNase II (Barry and Eastman, 1993, also see Peitsch et al, 1994 for a review of the characteristics of these endonucleases). In the early 1970's, Hewish and Burgoyne described an endogenous nuclease in rat liver that required Ca^{2+} and Mg^{2+} for activity and had internucleosomal cleavage activity (Hewish and Burgoyne, 1973). This endonuclease was later believed to be responsible for the internucleosomal cleavage of DNA in apoptosis (Wyllie, 1980 and Cohen and Duke, 1984). It was observed that apoptosis and DNA degradation in several cell types are inhibited by the presence of zinc by its effects on Ca^{2+}/Mg^{2+}-dependent endonuclease (Cohen and Duke, 1984, Shimizu et al, 1990, Waring et al, 1990 and Martin et al, 1991, McCabe et al, 1993).
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7.3.2. Early morphological changes are dissociated from internucleosomal cleavage of DNA

When I validated the flow cytometric method, I also used zinc as one of the inhibitors of apoptosis induced by dexamethasone in rat thymocytes. It was unexpected that zinc did not inhibit the formation of the apoptotic population as assessed by flow cytometry (Fig. 4.1). The percentage of cells in the apoptotic fraction (F4 of Percoll gradient) was also increased (Fig. 4.4). However, cells treated with dexamethasone in the presence of zinc showed no DNA fragmentation as assessed by either agarose gel electrophoresis or the Burton method (Fig. 4.2 and Table 4.1). Similar observations were also obtained from the experiments using other glucocorticoids, e.g. corticosterone and methylprednisolone, and etoposide to induce apoptosis in the presence of zinc.

Further experiments were carried out to examine these 'apoptotic cells' induced by dexamethasone in the presence of zinc. Apoptotic cells were sorted by flow cytometry or fractionated by Percoll gradients and further subjected to electrophoretic and electron microscopic analysis. As expected, apoptotic cells from thymocytes treated with dexamethasone alone showed a typical laddering pattern of DNA degradation (Fig. 4.2, lanes 5 and 8 for apoptotic cells obtained by Percoll fractionation and flow cytometric sorting, respectively). While, 'apoptotic cells' from those treated with dexamethasone in the presence of zinc showed no DNA ladders (Fig. 3.2, lanes 6 and 10). Ultrastructurally, apoptotic cells from thymocytes treated with dexamethasone alone showed morphology characteristic of apoptosis as described previously (Fig. 4.4, a). Strikingly different morphology was observed in apoptotic cells from thymocytes treated with dexamethasone in the presence of zinc. These apoptotic cells were also shrunken, with some dilation of the smooth endoplasmic reticulum (SER). Instead of margination of uniformly condensed heterochromatin onto one end of the nuclear membrane, the heterochromatin of these cells was uniformly condensed but arranged in several sharply defined clumps that abutted against the different regions of the nuclear
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membrane (Fig.4.4, b). A further clump was present in the centre of many these nuclei. The euchromatin retained its normal density but often included one or more clusters of intensely stained nucleolar remnants. Similar ultrastructural characteristics have been described, in various cell types, as the earliest recognisable signs of apoptosis (Kerr et al, 1987 and Walker et al, 1988). In summary, in the presence of zinc, many of the cytoplasmic and nuclear changes, both gross and ultrastructural, were still evident. These include the changes in cell size, buoyant density and the membrane permeability to H33342 as assessed either by flow cytometry and Percoll gradients, as well as dilation of SER and condensation of heterochromatin and its abutting onto the nuclear membrane. These results suggested that critical changes occur during the induction of apoptosis in thymocytes, either in the cytoplasm or in the nucleus, prior to, or possibly independent of, endonuclease cleavage of DNA into oligonucleosomal fragments. In the nucleus, morphological changes appeared to have been halted at an early stage of apoptosis, possibly prior to the internucleosomal cleavage of DNA by a Ca^{2+}/Mg^{2+}-dependent endonuclease due to the effect of zinc on this enzyme.

The identification of a transitional preapoptotic population of thymocytes further supports the suggestion that morphological changes during apoptosis are not necessarily associated with internucleosomal cleavage of DNA. One of the characteristic feature of apoptosis, in most cell types, is cell shrinkage so that apoptosis was initially called 'shrinkage necrosis' (Kerr, 1971), although the mechanism of this shrinkage is still not known. It has been suggested that thymocytes undergo a single step-wise transition from normal to apoptotic in response to glucocorticoid on the basis of the changes in buoyant density or cell size (Wyllie and Morris, 1982 and Walker et al, 1991). My study indicated the existence of a population of cells with intermediate size and buoyant density between normal and apoptotic cells (F3, Table 5.1 and Fig.5.1). Besides cell size and buoyant density, there were other biochemical and morphological criteria that
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were used in this study to establish the nature of this transitional preapoptotic population of thymocytes. Firstly, a decreased expression of both accessory molecules, CD4 and CD8, was observed in apoptotic thymocytes (Swat et al., 1991). This study demonstrated that the cells in F3 had a CD4^{low}CD8^{low}-like phenotype (Fig. 5.4,c), and the decrease in expression of these molecules was greater than that could be attributed to the changes in cell size. It is suggested in this study that the alterations in the expression of surface markers appear to either be early changes in apoptosis or precede internucleosomal cleavage of DNA.

Secondly, these cells exhibited morphological characteristics (Fig. 5.5, d) with no evidence of DNA laddering (Fig. 5.6, lanes 2 and 4), which were similar to those from thymocytes treated with dexamethasone in the presence of zinc. When these cells were incubated for a further 2 h, DNA ladders developed (Fig. 5.8). Finally, as described previously, the increase in the fluorescence intensity is due to an increase in the cell membrane permeability to H33342. It was also demonstrated in this study that the loss of cell volume appeared to precede the change in cell membrane permeability and DNA laddering, which becomes a distinct stage in apoptosis process. All these suggest that the subset of cells in F3 is a transitional preapoptotic population of thymocytes.

In summary, although the morphology of apoptosis has been described in detail, little is known about the cellular machinery underlying this process. In addition, although chromatin condensation and internucleosomal cleavage of DNA are two remarkable features of apoptosis, whether they are triggered by the same pathway or represent two independent events remains controversial. This study and others have basically dissociated the link between these two features by different approaches. It is possible that internucleosomal cleavage of DNA is a later event responsible for the downstream degradation of DNA in dead cells. There may be other biochemical events prior to or even independent of it, which may also be responsible for the appearance of characteristic features of apoptosis.
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7.3.3. Formation of large fragments of DNA precedes internucleosomal cleavage of DNA

In the nucleus of eukaryotic cells, DNA is organised into high-order structure, from the double strand DNA helix to nucleosomes and then to 30-nm solenoid filaments (Pienta et al, 1991). The core structure for the topological organisation of chromatin in the interphase nucleus is the nuclear matrix. The protein composition of the nuclear matrix determines the basic morphological pattern of the nucleus (see Verheijen et al, 1988 for review). Nuclear histones may also play a role in high-order structure of DNA (van Holde, 1989). Except for endonucleases, these structural protein may also play a role in the degradation of DNA during apoptosis.

It was suggested that there may be a subsequent degradation of DNA in apoptosis (Walker et al, 1991). Initially a cleavage of DNA into large fragments of DNA, of approximately 700, 300 and 50 kbp in length, which may well represent DNA loop domains (Filipski et al, 1990). The subsequent cleavage of DNA into fragments of 180 - 200 bp or multiples thereof was suggested to be carried out by a Ca$^{2+}$/Mg$^{2+}$-dependent endonuclease (Wyllie, 1980 and Cohen and Duke, 1984), which was not necessarily associated with apoptosis in some types of cells (Barres et al, 1992, Oberhammer et al, 1993a and b, Tomei et al, 1993 and Miller et al, 1994). These results implied that the initial cleavage of DNA into kbp fragments might be dissociated from further cleavage of DNA into oligonucleosomal fragments. Field inversion gel electrophoresis was used in this study to examine the possible existence of large fragments of DNA in thymocytes treated with dexamethasone in the presence of zinc. As I have discussed previously, when thymocytes were incubated with dexamethasone in the presence of zinc, no oligonucleosomal fragments were found (Fig.6.1). However, an increase in amount of large fragments, mainly 30 - 50 and 200 -250 kbp in length, was observed (Fig.6.2), which suggested that in thymocytes undergoing apoptosis, there was an initial cleavage of DNA into large fragments that were rapidly cleaved into
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oligonucleosomal fragments. Zinc facilitated the visualisation of this first component of DNA cleavage in apoptosis, possibly due to its inhibitory effect on the further degradation of those large fragments into oligonucleosomal fragments (Cohen and Duke, 1984). The hypothesis that the formation of large fragments of DNA precedes inter-nucleosomal cleavage of DNA during apoptosis was further supported by the fact that these large fragments existed in the transitional preapoptotic population of thymocytes that had no oligonucleosomal fragments (Fig. 6.4). After further incubation of this population of cells for 2 or 4 h, oligonucleosomal fragments were observed. Thus, these studies demonstrate that in apoptosis, there is a two-step degradation of DNA, i.e. an initial cleavage of DNA into large fragments prior to subsequent degradation to nucleosomal fragments. It is suggested here that the first step of degradation of genomic DNA involves the cleavage of DNA into loop domains which are further cleaved into oligonucleosomal fragments at the second step. It is also suggested that the formation of large fragments is a precondition for the second cleavage, because it can relax DNA and create a appropriate conformation of DNA for subsequent cleavage of DNA by endonucleases.

Thymocyte nuclei were used in this study to further examine the mechanism by which DNA is cleaved into these large fragments. Preliminary results showed that the formation of these large fragments was a Mg\(^2+\) rather than Ca\(^2+\) dependent process (Fig. 6.5), while it can be facilitated by Ca\(^2+\) (Fig. 6.6). However, the second step, i.e. the characteristic internucleosomal cleavage of DNA, was not observed in the presence of either Mg\(^2+\) or Ca\(^2+\) alone but rather needed both Ca\(^2+\) and Mg\(^2+\) (Fig. 6.6, lane 4 of panel b). An inhibitory effect of either in vivo or in vitro exposure of cycloheximide has been observed on the internucleosomal cleavage of DNA by Ca\(^2+\)/Mg\(^2+\)-dependent endonuclease (Nikonova et al, 1982 and McConkey et al, 1990b). In my study, this effect was confirmed. However, both the Mg\(^2+\) and the Ca\(^2+\)/Mg\(^2+\)-dependent formation of the large fragments was also inhibited dose-dependently (Fig. 6.7). These results offered an alternative
possibility for the loss of the Ca$$^{2+}$/Mg$$^{2+}$$-internucleosomal cleavage in the nuclei from cycloheximide treatment, i.e. a decrease in the formation of the large fragments, resulting in a deficiency in the supply of the precursors for further cleavage by a Ca$$^{2+}$/Mg$$^{2+}$$-dependent endonuclease.

Except for certain types of unknown endonucleases being responsible for cleavage of DNA into these large fragments, there are also some other mechanisms by which large fragments are formed. It has been suggested that once the nuclear matrix-DNA interrelation is damaged by any reason, e.g. solubilisation of nuclear matrix protein, supercoiling organisation of DNA will be subsequently damaged (Miller et al, 1994). There are at least two outcomes for this damage. One is DNA cleavage into oligonucleosomal fragments if there exists an endonuclease and a machinery to activate it in special cell types, e.g. in thymocytes. Such a cell may have evolved mechanisms that give the destruction of its DNA a higher priority than other cells (Lockshin and Zakeri, 1991). Otherwise, the result will be the formation of large fragments of DNA due to the disorganisation of high order structure of DNA. DNA degradation occurs because of damage to structural elements that are responsible for a level of chromosomal organisation higher than that of the nucleosome. Therefore, these fragments may well represent DNA loop domains formed following the damage of the nuclear matrix proteins, which may represent an earlier biochemical event than cleavage of DNA into oligonucleosomal fragments. In my study, this hypothesis was further supported by using two experimental approaches. Firstly, when further cleavage of DNA into oligonucleosomal fragments was inhibited by zinc, the large fragments of loop domain size were visualised. Secondly, these large fragments were present in a transitional preapoptotic population of thymocytes that showed no oligonucleosomal fragments. To a certain extent, the formation of these large fragments was coincident with early nuclear morphological changes. We could not say for certain that it is the mechanism by which the changes of nuclear morphology are
induced, because the solubilisation of the nuclear matrix could also account for the morphological changes in the nucleus during apoptosis (Miller et al, 1994).

7.4. Early morphological and biochemical changes in thymocytes during apoptosis - a summary

In this study, by using different experimental approaches, an early stage of apoptosis was identified (Fig. 7.1). Several working hypotheses on the mechanism of apoptosis have recently been proposed (Carson and Ribeiro, 1993, Martin, 1993, Schwartzman and Cidlowski, 1993 and Jacobson et al, 1994). There are two major assumptions. One is that there is a cascade of events mediating a death program, with each event dependent on the previous one. Another is that the diverse events recognisable either biochemically or morphologically are activated in parallel by a central control system (Jacobson et al, 1994). There are numerous cellular changes in apoptosis, but it is still not clear which of them are directly associated with the death machinery or activation of death and then of greatest physiological importance, and which are associated with the effector of death machinery or only the degradation of dead cells. Similarly, I am not sure which and how these so called early cellular changes observed in this study fit in the death program.

7.4.1. Cell size

The early change in cell size was revealed by Percoll fractionation and further supported by Coulter counting, flow cytometry and electron microscopy. Following incubation of thymocytes for certain times with either dexamethasone and etoposide, thymocytes were subsequently fractionated on discontinuous Percoll gradients on the basis of cell size or buoyant density. A fraction of cells (F3) was found with intermediate size and buoyant density. The size was further confirmed by Coulter counting with intermediate volume, moreover assessed by
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flow cytometry with intermediate forward light scatter between normal and apoptotic thymocytes. EM examination supported this observation.

7.4.2. Cell surface markers

When the cells from this fraction (F3) were labelled with thymocyte surface markers, CD4 and CD8, a decreased expression of both molecules was observed. This decreased expression of cell surface markers may not be due to the decreased surface area of cell, and also does not resemble the downregulation of surface markers during positive selection in which only one of the markers is downregulated in one type of cells.

7.4.3. DNA degradation

At this stage, i.e. when cells shrunk and both surface markers were down-regulated, no oligonucleosomal fragments were found. Instead, large fragments of DNA, of 30 -50 and 200 -250 kbp in length, were observed. These fragments were also found in the thymocytes treated with dexamethasone in the presence of zinc, which implied that the formation of these large fragments preceded internucleosomal cleavage of DNA.

7.4.4. Nuclear morphology

The nuclear morphology of cells at this stage was different from that of both normal and apoptotic thymocytes. The heterochromatin of these cells condensed into sharply defined clumps and abutted onto the nuclear membrane. Large regions of this nuclear membrane were devoid of condensed chromatin and the membrane itself was convoluted. The euchromatin and fibrillar centres of these cells were apparently normal. Similar nuclear morphology was also observed in the thymocytes treated with dexamethasone in the presence of zinc. In addition, the cytoplasm of these cells was dense and granular and contained normal organelles. A few small vacuoles or dilated cisternae of the endoplasmic reticulum were also
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evident. These are very similar to the morphology of early apoptosis described previously (Kerr et al, 1987 and Walker et al, 1988).

**Fig.7.1 Summary of an early stage of apoptosis of thymocytes**

### 7.5. Future work

**7.5.1 Establishment of the nature of these large fragments**

It has been suggested that these large fragments of DNA represent the loop domains of high order structure of DNA (Filipski et al, 1990, Walker et al, 1991, Brown et al, 1993 and Oberhammer et al, 1993b). The real nature of these fragments remains unclear. Another related issue is that these fragments of DNA may well be generated artificially due to improper handling. There are two ways to deal with these issues. One is the identification of the end sequence of these large fragments, because the points of attachment of the loop DNA to the nuclear matrix, i.e. MARs or SARs, have several common attributes, e.g. unique length of 200 bp, A-T rich regions, poly-A signal regions and high homologous DNA topoisomerase II cleavage sequences (Pienta et al, 1991). If these large fragments
are loop domains, they should exhibit these consensus sequences. To avoid the problem of artefacts, the physiological conditions should be maintained through the whole experimental procedure, including the utilisation of physiological buffers to isolate the nuclei and chromatin components or the adaptation of the method by which DNA isolated can be maintained in natural condition (Jackson et al, 1988 and 1990).

7.5.2. Investigation of the mechanism involved in the formation of the large fragments and its relevance to death program

There are two possible mechanisms by which the large fragments of DNA are formed. One is endonuclease-dependent cleavage of DNA into these fragments. Another is the solubilisation of the nuclear matrix protein-induced damage in the high order structure, which causes the formation of these large fragments (Miller et al, 1994). For the first mechanism, identification of the endonuclease and understanding the mechanism related to the activation of the endonuclease will be essential. There will be some difficulties in identification of this endonuclease, because its natural substrate will be DNA which has maintained high order structure and the conformation of DNA will affect the activity of the enzyme. Routinely used single strand DNA might not be suitable in the assay system. DNA topoisomerase II may play a role in the formation of these large fragments (Filipski et al, 1990 and Walker et al, 1991), which needs to be further assessed. On the other hand, it is also recently proposed that telomeric association of chromosomes is involved in manifestation of programmed cell death (Pathak et al, 1994). The role of nuclear matrix protein, e.g. lamins, in DNA degradation and the nuclear morphological changes is also of interest to investigate. Finally, the place of the formation of these large fragments in the apoptotic process remains unclear and the correlation of this event with apoptosis is to be unequivocally established.
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