PROTEASE INHIBITORS ACT AT DIFFERENT STAGES OF THYMOCYTE APOPTOSIS

Howard Oliver Fearnhead (BSc. Lon)

Biochemical Toxicology Group

Medical Research Council Toxicology Unit

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Howard Oliver Fearnhead, Biochemical Toxicology Group, MRC Toxicology Unit, Hodgkin Building, University of Leicester, Leicester LE1 9HN.

ABSTRACT

Apoptosis is a form of cell death in which cells die in a controlled fashion. It is characterised by a distinct series of morphological changes and its incidence during embryogenesis, tissue homeostasis, T-cell maturation and following exposure to a number of toxins suggests that a pathway common to many diverse stimuli brings about the changes typical of apoptosis. The discovery that genes required for apoptosis in *Caenorhabditis elegans* are functionally conserved in man and the observation that some biochemical events recur in different examples of apoptosis supports the existence of a common pathway. In this thesis, rat thymocytes have been used to identify early events within a common apoptotic pathway.

Initially a previously identified early apoptotic population of thymocytes was further characterised and shown to be committed to progressing to a fully apoptotic phenotype. The hypothesis that proteins required for mitosis are also required for apoptosis and that apoptosis can be considered an aberrant mitosis was then tested. No correlation between cdc2 kinase activity and the incidence of mitosis was found showing that apoptosis cannot be considered an aberrant mitosis.

The effects of a number of protease inhibitors on different stages of thymocyte apoptosis were also investigated. Nα-tosyl-lysinyl chloromethyl ketone (TLCK), an inhibitor of trypsin-like proteases and benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethyl ketone, an inhibitor of interleukin-1β-converting enzyme-like proteases prevented apoptosis induced by diverse stimuli by all criteria used. The TLCK target was pre-existing and not synthesised in response to apoptotic stimuli. N-tosyl-phenylalaninyl chloromethyl ketone, a chymotrypsin-like protease inhibitor prevented only late changes of apoptosis. This work clearly that there are distinct stages within the apoptotic process and that a common apoptotic pathway contains a hierarchy of proteases.

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which commit thymocytes to apoptosis.

target is already present and is
not synthesised in response to
apoptotic stimuli.

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ABBREVIATIONS

ALLN	N-acetyl leucinyl-leucinyl-norleucinal
Aurin	Aurin tricarboxylic acid
bp	base pair
BrdU	5-Bromo-2'-deoxyuridine
CDK	cyclin dependent kinase
CHX	Cycloheximide
crmA	Cytokine response modifier gene
CTL	Cytotoxic T lymphocyte
DCI	3,4-dichloroisocoumarin
DEX	Dexamethasone
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene Glycol-bis-(β-aminoethyl ether) N,N,N',N',- tetraacetic acid
FBS	Foetal bovine serum
FIGE	field inversion gel electrophoresis
γ-IRR	γ-irradiation
HEPES	(N-[2-hydoxyethyl]piperazine-N'-[2-ethanesulfonic acid])
ICE	Interleukin- 1 β-converting enzyme
Ich-1	ICE and Ced-3 homolog gene
kbp	kilobase pair
kDa	kiloDalton
MDL-70,128	Benzyloxycarbonyl valinyl-phenylalaninyl-aldehyde
MEM	Minimum essential medium
NAD	Nicotinamide adenine dinucleotide
NDS	10mM Tris, 0.5M EDTA, 1% lauryl sarcosine
Nedd-2	Mouse neuronal precursor cell expressed developmentally down regulated gene
PAI-2	Plasminogen activator inhibitor
PBS	Phosphate buffered saline
PI	Propidium iodide
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl Fluoride
prICE	protease resembling interleukin- 1β-converting enzyme
SDS	Sodium dodecyl sulphate
SEM	standard error of the mean
STS	Staurosporine
TBE	Tris-Borate-EDTA
TCR	T-cell receptor
TE	Tris-EDTA
THAP	Thapsigargin
TIA	Cytotoxic granule protein
TIA-R	Related cytotoxic granule protein
TLCK	Nα-tosyl-lysinyl chloromethyl ketone
TNF	Tumour necrosis factor
TPCK	N-tosyl-phenylalaninyl chloromethyl ketone
Tris	tris (hydroxymethyl) amino methane
VP-16	Etoposide
YVAD.CHO	N-acetyl-tyrosinyl-valinyl-alaninyl-aspartyl aldehyde
YVAD.CMK	N-acetyl-tyrosinyl-valinyl-alaninyl-aspartyl chloromethyl ketone

YVAD.COCH₃   N-acetyl-tyrosinyl-valinyl-alaninyl-aspartyl methyl ether.

z-VAD.DCOMK   Benzyloxycarbonyl-valinyl-alaninyl-δethyl ester-aspartyl (2,6
-dichlorobenzylc methyl ketone

z-VAD.FMK   Benzyloxycarbonyl valinyl-alaninyl-aspartyl fluoromethyl ketone

CHAPTER 1
INTRODUCTION

The importance of apoptosis

Two different forms of cell death are recognised, necrosis and apoptosis. Necrosis occurs following massive cell injury that destroys cell homeostasis. It is characterised by a rapid cell swelling as the cell loses osmotic control, disruption of organelles and membranes and rapid cell lysis (Trump et al. 1981). During this form of death the cell is a passive target and is unable to regulate its internal environment. Apoptosis, a greek word which describes the falling of leaves from trees, is morphologically distinct from necrosis. Ultrastructurally the earliest changes are loss of cell junctions and other specialised plasma membrane structures such as microvilli and as a result the cell detaches from its surroundings and becomes more rounded. At the same time the cell shrinks and the chromatin condenses into one or more large masses at the nuclear membrane. Nuclear pores are not visible adjacent to the condensed chromatin but are observed and appear normal in the nuclear membrane not in contact with condensed chromatin. Another early nuclear change is the disintegration of the nucleolus into coarse osmiophilic granules. Following these changes there is continued condensation of the chromatin, distortion of the nuclear membrane and often disintegration of the nucleus into membrane bound fragments within the cytoplasm. In tandem with these nuclear changes is the dilation of the endoplasmic reticulum which often fuses with the plasma membrane and cell shrinkage causes involution of the plasma membrane. Other organelles such as the mitochondria appear normal without any evidence of high amplitude swelling or appearance of floculent densities within the mitochondrial matrix associated with necrosis (Arends and Wyllie 1991). In vivo cells at this stage are usually removed by either professional phagocytes such as macrophages or by neighbouring cells. Once ingested, the cell is broken down in the phagosome (Kerr and Harmon 1991). In vitro, perhaps because of the absence of professional phagocytes and the limited phagocytic ability of other cells types, cells at this stage of the process undergoes further degeneration and breaks up into membrane bound fragments called apoptotic bodies (Galili et al. 1982; Robertson et al. 1978). The formation of apoptotic bodies may be a function of cell size, large cells giving rise to multiple bodies but smaller cells such as thymocytes remaining as highly condensed but single particles. In the absence of phagocytosis these apoptotic bodies under go changes usually associated with necrosis, loss of membrane integrity and a progressive degradation of the remaining organelles, and called secondary necrosis because these changes are preceded

by apoptosis. It is important to stress that during apoptosis *in vivo* membrane integrity is maintained and therefore death is not associated with an inflammatory response.
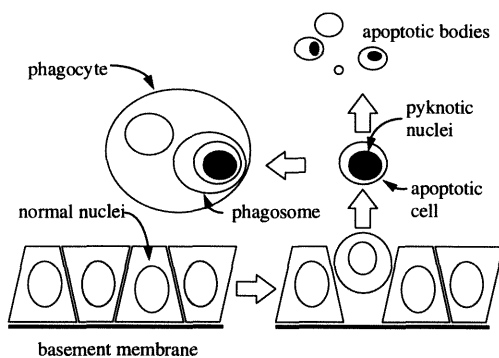
Figure 1. The sequence of events that occurs during apoptosis. The dying cell detaches from its neighbours and the basement membrane, shrinks, its chromatin condenses (pyknotic nuclei) and it is either engulfed by a phagocyte and degraded in the phagosome or undergoes further degenerative changes and fragments into apoptotic bodies. This may be associated with secondary necrosis.

Apoptosis occurs during normal tissue turnover, embryogenesis, metamorphosis and endocrine dependent tissue atrophy (Kerr et al. 1972; Saunders 1966; Kerr et al. 1987). It is also seen during cytotoxic T cell killing (Russel et al. 1972), in response to tumour necrosis factor (Gerschenson and Rotello 1992), during tumour regression (Kerr et al. 1972) and in response to cancer chemotherapeutic agents (Hickman et al. 1992). Thus apoptosis is a mechanism by which the death of a single cell can be brought about without damaging the surrounding and otherwise healthy tissue. Thus it is of vital importance for the development and homeostasis of multicellular organisms (Arends and Wyllie 1991; Williams et al. 1992) as well as being a defence mechanism allowing removal of self reactive T cells (Von Boemher et al. 1989; Smith et al. 1989; MacDonald and Lees 1990) and cells damaged by irradiation whose further survival is a risk to the organism as a whole (Lane 1992a; Lane 1992b). A loss of regulation of apoptosis will upset the balance of death and proliferation and may therefore contribute to tumourigensis and inappropriate apoptosis may be equally dangerous being responsible for neurodegenerative diseases and the loss of lymphocytes seen during AIDS (reviewed by Thompson 1995).

The removal of self reactive T cells is of critical importance in the development and maintenance of an effective immune response. During our life time we are exposed to a multitude of different immunological threats and in order to respond effectively the immune

system must have the ability to recognise a wide range of antigens. The development of the antigen receptor repertoire on progenitor T cells occurs in the thymus during the maturation (Ritter and Crispe 1992). Progenitor cells enter the thymus without any functional antigen specificity and exit with either α/β or γ/δ receptors. The function of cells bearing γ/δ receptors is unclear but those bearing α/β receptors possess defined biological properties. They recognise exogenous antigens associated with class I and class II molecules of the major histocompatibilty complex (Kronenberg et al. 1986; Samelson et al. 1985). As a thymocyte progresses towards maturity it undergoes a series of changes in its surface phenotype and these changes have been used to define stages of thymocyte development (Petrie et al. 1990; Shortman et al. 1991; Ritter and Crispe 1992)(Fig. 2).
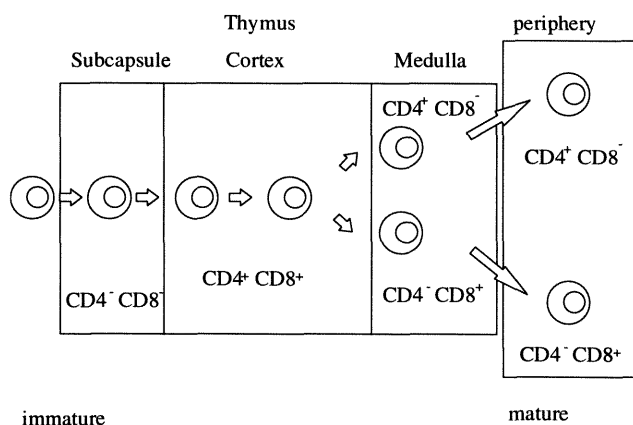
Figure 2. A very simplified diagram of α/β T cell development. Progenitor T cells from the bone marrow enter the thymus and undergo a series of changes. Rearrangement of their T cell receptor genes, believed to occur within the CD4+CD8+ fraction is followed by positive and negative selection. It is thought that the majority of apoptosis that occurs physiologically in the thymus occurs in this double positive population of cells.

During this process thymocytes in the cortex engage in a process of gene random re-arrangement that generates a diverse range of antigen receptors capable of recognising both self and foreign proteins (Snodgrass et al. 1985; Williams et al. 1986). Those clones which recognise foreign proteins will clearly be useful as they will trigger an appropriate immune response if such a protein is encountered. However, those clones which recognise self are potentially harmful as their activation results in an inappropriate and autoimmune response with drastic consequences. To build a competent immune system, the heterogeneous population of useful and dangerous cells is subjected to both positive selection for those

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clones which are useful, and negative selection to remove those which are self-reactive T-cells (Blackman et al. 1990) and apoptosis is the mechanism by which dangerous cells are removed in the thymus (Smith et al. 1989; Shi et al. 1989).

Changes associated with thymocyte apoptosis and techniques used to detect them

Thymocytes readily undergo apoptosis in-vitro in response to many agents, are easily obtained in large numbers and are therefore suited to an investigation of the biochemical changes that accompany apoptosis. In addition to these practical advantages, understanding thymocyte apoptosis is relevant to both understanding normal T-cell development and disorders of the immune system.

Glucocorticoid-induction of apoptosis in thymocytes is one of the best documented model systems for the study of apoptosis (Wyllie 1980; Cohen and Duke 1984; Arends and Wyllie 1991) and is characterised morphologically by condensation of nuclear chromatin, cell shrinkage, dilation of the endoplasmic reticulum and blebbing of the plasma membrane as previously discribed. Glucocorticoid induced apoptosis is dependent on new RNA and protein synthesis (Cohen and Duke 1984; Wyllie et al. 1984). A number of dramatic biochemical changes occurs in apoptotic cells, the best characterised being the fragmentation of chromatin into units of single or multiple nucleosomes (each nucleosome being 180 - 200 base pairs) following activation of an endogenous Ca^{2+}/Mg^{2+}- dependent endonuclease, which is inhibited by zinc (Cohen and Duke 1984). Endonuclease activation and the resultant internucleosomal cleavage is detected by agarose gel electrophoresis which reveals a "DNA ladder". (Wyllie 1980; Arends et al. 1990; Cohen et al. 1992a). The precise nature of the endonuclease(s) involved in apoptosis is unclear and has been the subject of some controversy . An 18-kDa calcium - dependent endonuclease responsible for internucleosomal degradation has been isolated and is inhibited by zinc and aurintricarboxylic acid (Compton and Cidlowski 1987). It was that suggested that this nuclease activity was associated with histone H2B (Alnemri and Litwack 1989) but the nuclease activity and the histone were subsequently dissociated (Gaido and Cidlowski 1991). Deoxyribonuclease I and II have been implicated in thymocyte apoptosis (Peitsch et al. 1993) and apoptosis in Chinese hamster ovary cells (Barry and Eastman 1992) respectively. The DNA ladder accompanies the typical apoptotic morphology of chromatin fully condensed at one pole of

the nucleus and a marked decrease in cell volume and so laddering is often considered as the biochemical hallmark of apoptosis (Wyllie 1980; Cohen et al. 1992a). However agarose gel electrophoresis is not quantitative. Quantitation of DNA laddering is possible (Wyllie 1980; Arends et al. 1990) following lysis of cells small fragments of DNA can be separated from large fragments of DNA by centrifugation. Following separation, the amount of DNA in each fraction, fragmented and intact, can be assessed by a colorimetric assay (Burton 1956) and so the level of DNA fragmentation in a population of cells can be determined. Like agarose gel electrophoresis, only the amount of DNA cleaved to nucleosomal fragments less than approximately 1 kilobase pairs in size (Arends et al. 1990) is detected and the amount of DNA present as larger fragments can not be determined. As a consequence the formation of 700, 200 and 10-50 kilobase pair sized fragments of DNA which also occurs during apoptosis (Walker et al. 1991; Brown et al. 1993; Oberhammer et al. 1993) was not recognised until alternative techniques (including field inversion gel electrophoresis, FIGE) were employed. Importantly, Oberhammer et al. (1993) recognised that apoptotic morphology was not always accompanied by DNA laddering but was associated with the formation of large DNA fragments, suggesting that DNA laddering occurs as a consequence of apoptosis but is not required for apoptosis. Thus the absence of laddering may not mean an absence of apoptosis, this is true even in thymocytes (Cohen et al. 1992b), the cells used to demonstrate DNA laddering (Wyllie 1980) and further support comes from the observations that the nucleus and DNA fragmentation are not required for apoptosis (Jacobson et al. 1994; Schulze-Osthoff et al. 1994). This high-lights the danger of using any single criterion in assessing the incidence of apoptosis unless that change can be shown to be causal.

Flow cytometry allows rapid quantitation of large numbers of cells and in contrast to traditional biochemical techniques which assess changes in a population of cells, flow cytometry allows changes in single cells to be measured. As a result flow cytometry has proved a very powerful tool in studying apoptosis (Darzynkiewicz et al. 1992; Ormerod 1993). Fluorescence activated cell sorting can also be used to separate distinct populations of viable cells for further study (Sun et al. 1992). A number of flow cytometric assays have been devised, some rely on detecting the changes described above (such as the loss of DNA content as a result of DNA cleavage and forward light scatter used to measure cell

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shrinkage) and others have revealed additional differences between normal and apoptotic cells.

The most widely used flow cytometric technique detects the loss of DNA from fixed cells that occurs as a result of DNA laddering. Using a method initially used to assign cells to different phases of the cell cycle (Gray et al. 1990), cells are fixed and stained with a fluorescent dye which binds nucleic acids such as propidium iodide and a DNA histogram recorded. In many instances apoptosis gives rise the appearance of an additional population of cells with less DNA than those of $G_{0/1}$ (Fig. 3). These cells are termed as a sub-G_1 population or as being hypodiploid and appear following degradation and loss of nucleosomal fragments of DNA (Darzynkiewicz et al. 1992) and rapid quantitation of the incidence of apoptosis is possible using the appropriate software (e.g. Lysis II, Becton Dickinson).
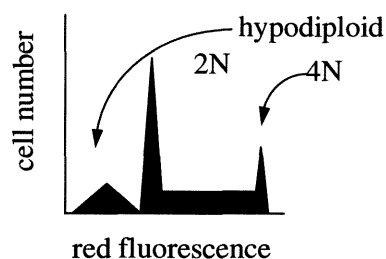
Figure 3. The appearance of hypodiploid cells is associated with apoptosis. Following fixation and staining with propidium iodide cells are assigned to phases of the cell cycle G1/0 (2N), S and G2/M (4N). Apoptosis is often accompanied by the appearance of a population of cells with hypodiploid DNA content (arrowed).

DNA fragmentation associated with single cells can be detected and apoptosis quantitated using in situ end labelling. This technique relies on the addition of digoxigenin-11-2'-deoxyuridine-5'-triphosphate to free ends of DNA by terminal deoxynucleotidyl transferase. Labelling can be detected with a fluorescent dye conjugated to an antibody against digoxigenin. Terminal deoxynucleotidyl transferase adds more than one digoxigenin-11-2'-deoxyuridine-5'-triphosphate to each break and therefore provides an amplification that makes the technique very sensitive (Gorczyca et al. 1993). Recent reports have shown that not only does this technique detect the DNA strand breaks produced by laddering but it is also able to detect the breaks associated with the formation of large fragments (Cain et al. 1995).

In addition to detecting nuclear changes of apoptosis, flow cytometry has been used to reveal and quantify membrane changes of apoptosis in viable thymocytes. The uptake of the DNA binding dye Hoechst 33342 has been used to detect changes in membrane permeability in chinese hamster ovary cells (Lalande 1981) and two populations of unfixed thymocytes are observed following a short incubation with Hoechst 33342 (Sun et al. 1992). Cells which have a higher blue fluorescence (containing more Hoechst 33342) and a low forward light scatter (indicative of a reduced cell volume) were sorted and shown to possess both apoptotic morphology and DNA ladders. In contrast cells which have lower blue fluorescence (containing less Hoechst 33342) and higher forward scatter were sorted and shown to be both biochemically and morphologically normal. Both these populations are defined as viable as they both exclude the dye propidium iodide and therefore 3 populations were identified; normal cells (low Hoechst 33342 fluorescence), apoptotic cells (high Hoechst 33342 fluorescence) and "dead" cells (high propidium iodide fluorescence) (Sun et al. 1992)(Fig. 4).
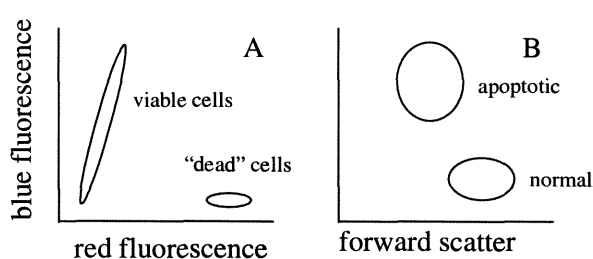
Figure 4. The Hoechst 33342/propidium iodide method. Thymocytes stained with Hoechst 33342 and propidium iodide (PI) are examined by flow cytomery and blue fluorescence (Hoechst) vs red fluorescence (PI) plotted (A). This allows the number of cells which have lost membrane integrity to be quantified. Cells with intact membranes (excuding PI) are plotted vs forward scatter (B) and then the number of viable but apoptotic cells can be quantified (Sun et al. 1992).

Three lines of evidence suggest that the difference in Hoechst staining is due to a change in plasma membrane permeability that accompanies apoptosis in thymocytes. First, nuclei isolated from both normal and apoptotic thymocytes stain to the same intensity with Hoechst 33342. Second, the difference between normal and apoptotic cells decreases if stained for longer periods of time and eventually both apoptotic and normal cells display the same blue fluorescence, suggesting that the difference in staining is due to different rates of uptake. Finally, apoptotic cells can be shown to be more permeable by their reduced ability to retain fluorescein derivatives (Ormerod et al. 1993).

Flow cytometry has also been used to detect the increased binding of the lipophilic dye, Merocyanin 540 to apoptotic cells (Ormerod et al. 1994; Mower et al. 1994). This may reflects altered lipid packing as a result exposure of phosphatidyl serine (Fadok et al. 1992) and the appearance of N-acetyl glucosamine (Savill et al. 1993) on the outer surface of the plasma membrane which may trigger the rapid recognition and subsequent phagocytosis of apoptotic cells (Del Buono et al. 1989; Savill et al. 1993).

A common pathway

The initiation of apoptosis is tightly regulated and many signals originating from both within the cell and from the external environment can influence the decision to live or die. These factors include the genes expressed by a particular cell type, cellular damage that may have been sustained as a result of physical or chemical insult, the presence or absence of extracellular survival factors, cell-cell interactions and hormones. These factors may either induce or protect from cell death and the same stimulus may have opposing effects in different cell types. Diverse signals are able to elicit apoptosis in thymocytes, these signals may be physiological; for example, glucocorticoids (Wyllie 1980), signalling via the T-cell receptor complex or via the Fas ligand, or non-physiological; for example, DNA damaging chemotheraputic agents (Hickman et al. 1992) and γ-irradiation (Sellins and Cohen 1987). In some instances, but not all, thymocyte apoptosis, requires the production of new proteins (Cohen and Duke 1984; Wyllie et al. 1984). In most tissues cell survival appears to depend on the constant supply of survival signals (from neighbouring cells and the extracellular matrix) and if cultured alone and in the absence of trophic factors these cells will undergo apoptosis (Raff 1992). In contrast to many instances of thymocyte apoptosis, these examples of apoptosis do not require gene-expression or synthesis of new proteins suggesting that the protein required to enact apoptosis are constitutively expressed. Therefore these cells are on the verge of suicide and will only continue to live if the appropriate signals are received from the environment. Extending this argument to thymocytes suggests the proteins synthesised in response to some apoptotic stimuli are required to modulate pre-existing apoptotic proteins in thymocytes, perhaps enabling the cell to overcome the inhibitory effect of antagonistic survival factors. Agents such as tributyl tin (Raffray and Cohen 1991), TPCK

and staurosporine induce thymocyte apoptosis in the absence of *de novo* protein synthesis, lending some support to the hypothesis that proteins synthesised during dexamethasone or etoposide induced thymocyte apoptosis are not absolute requirements for apoptosis.

A key question is how diverse signals bring about a controlled cell death that shares so many features in so many different cell types. It now appears that some proteins required for cell death are very similar whether apoptosis is occurring in the nematode or in man. Genetic studies of programmed cell death in the nematode *Caenorhabditis elegans* has identified a number of genes involved in apoptosis that are conserved in higher organisms. Genetic mosaic analysis shows that of these, *ced-3* which is required for cell death and *ced-9* which prevents cell death act within the dying cell and that the proteins encoded by theses genes are of direct relevance to the biochemical changes occurring in these cells (reviewed by Hengartner and Horvitz 1994). *Ced-3* encodes a protein with homology to a mammalian protease, interleukin-1β-converting enzyme (ICE)(Yuan et al. 1993). Further work has identified a family of ced-3/ICE-like proteases, encoded by *nedd-2* (Kumar et al. 1994), *ich-1* (Wang et al. 1994) and *CPP32* (Fernandes Alnemri et al. 1994) whose over expression induces apoptosis in mammalian cells. In addition, an ICE-like protease activity, prICE induces nuclear changes of apoptosis (Lazebnik et al. 1994) and a wide range of protease inhibitors prevent apoptosis in a wide range of cell types (Suffys et al. 1988; Bruno et al. 1992; Sarin et al. 1993; Squier et al. 1994) suggesting that proteolysis is a critical control point in apoptosis. As well as playing a role in the induction of apoptosis, a recent report shows that a member of the ced-3/ICE-like family (*ich-1$_S$*) can also prevent apoptosis and so members of this family are both positive and negative regulators of apoptosis. The gene ced-9, which prevents apoptosis (Hengartner et al. 1992) encodes a protein with both structural and functional homology to the mammalian protein Bcl-2 (Hengartner and Horvitz 1994). *Bcl-2* is an oncogene that was first discovered as a result of its translocation between chromosomes 14 and 18 and is present in human follicular lymphomas (Tsujimoto et al. 1985) and like *ced-9* in the nematode, *bcl-2* prevents apoptosis in mammalian cells induced by a wide array of stimuli (Sentman et al. 1991). Characterisation of the protein demonstarted that it possesses a hydrophilic membrane spanning C-terminal domain (Chen-Levy et al. 1989) and that it is associated with the membranes of mitochondria, the endoplasmic reticulum and the nucleus (Monaghan et al. 1992; Jacobson et al. 1993;

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Krajewski et al. 1993). The biochemical function of Bcl-2 is unknown although it may

modulate intracellular Ca^{2+} fluxes associated with the endoplasmic reticulum (Lam et al.

1994) and the subcellular localisation of p53 (Ryan et al. 1994). While bcl-2 plays an

important role there are examples of bcl-2 insensitive apoptosis, such as negative selection

in the thymus (Sentman et al. 1991; Milner et al. 1992; Reed 1994; Hawkins and Vaux

1994). It is now apparent that Bcl-2 is one of a family of related proteins, and like the ced-

3/ICE-like family, some members may induce apoptosis (Nunez and Clarke 1994). The

conservation of both structure and function in such different organisms, whether the protein

induces or prevents apoptosis, suggests that these proteins play a vital role in apoptosis and

are shared components of a pathway activated by many apoptotic stimuli. This data is

consistent with a model of apoptosis in which different stimuli act on private pathways that

converge on a series of biochemical changes common to apoptosis, irrespective of species

and that these changes produce the morphology typical of apoptosis (Fig. 5)(Cohen 1991).
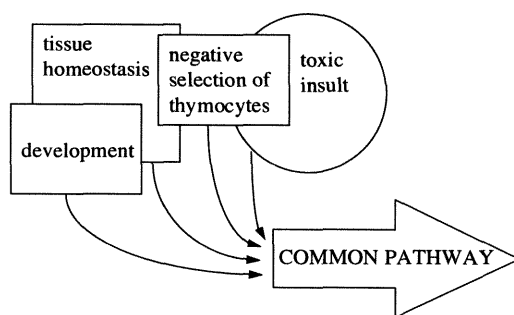
Figure 5. Convergence on a common pathway. Both physiological signals (boxes) and non-phsiological signals e.g. toxic insult (circle) can induce an identical response, that of apoptosis. The model shown suggests that different signals converge on a shared or common pathway in order to explain how such diverse signals may induce the same response.

The precise nature of the putative common pathway is unknown however , chromatin

condensation, changes in nuclear lamins are features similar in both apoptosis (Ucker et al.

1992; Lazebnik et al. 1993) and mitosis (Nurse 1989) and kinases usually associated with

mitosis are also activated during apoptosis (Shi et al. 1994). In addition apoptosis of prostate

epithelial cells following castration involves the re-entry of terminally differentiated cells into

an abortive cell cycle (Colombel et al. 1992) .This has given rise to the hypothesis that the

common apoptotic pathway utilises molecular machinery used in the cell cycle and that

apoptosis is an aberrant mitosis or "catastrophic"(Shi et al. 1994; Penninger and Mak 1994). This is addressed in more detail in Chapter 4.

The different stages of apoptosis

Apoptosis has been divided into distinct stages using morphological, genetic and biochemical criteria. A dying cell passes through a series of changes as it acquires an apoptotic phenotype; initially the cell undergoes shrinkage, the chromatin condenses, detaches from surrounding cells and the extracellular matrix and is rapidly phagocytosed by either its neighbours or professional phagocytes (Fig. 1) (Kerr and Harmon 1991). The rapidity of phagocytosis means that detection of apoptosis *in vivo* is difficult as traditional histological techniques supply only a snap shot in time of the changes occurring. As a result the incidence of apoptosis *in vivo* is probably underestimated. If phagocytosis does not occur the cell eventually fragments into membrane bound particles called apoptotic bodies (Galili et al. 1982; Robertson et al. 1978) a common fate of cultured cells undergoing apoptosis.

The majority of genes required for apoptosis have been identified using the nematode *Caenorhabditis elegans*. By mutating these genes and observing the effects on cell death the genes have been organised into a genetic pathway which is split into four stages. The first stage, the decision to die, involves the genes *ces-1*, *ces-2* and *egl-1* which were identified in the death of specific cells and cells committed to this stage cannot be distinguished morphologically from non-committed cells. The next stage, the execution of the death programme, involves the genes *ced-3*, *ced-4* and *ced-9* which are involved in all apoptotic deaths in nematodes and as a result of this stage the cell acquires the typical apoptotic phenotype. Cells then progress to the third stage, engulfment/phagocytosis, which requires the genes *ced-1*, *ced-2*, *ced-5*, *ced-6*, *ced-7*, and *ced-10* and are subsequently degraded (the fourth stage) which requires the gene *nuc-1* (Steller 1995 and references therein). *Nuc-1* encodes a nuclease responsible for DNA cleavage (Hedgecock et al. 1983), however *in vitro* studies with mammalian cells show that the activation of the endonuclease does not require engulfment although it is unclear whether internucleosomal cleavage of DNA precedes, accompanies or follows engulfment *in vivo*. The genetic pathway above describes epistatic relationships and therefore does not necessarily describe the sequence of biochemical events. For example, a gene which prevents apoptosis may be assigned a position near the apex of an

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assigned a position near the apex of an epistatic pathway. However, the protein encoded by this gene may exert its controlling effect either early or late within the biochemical pathway. Despite the danger of over extrapolation from genetic to biochemical pathways, the nematode model showed that the nuc-1 nuclease plays no role in the induction of apoptosis (Driscoll 1992). In mammalian systems endonucleases responsible for DNA laddering were considered causative (Arends et al. 1990) but, like the nematode have been relegated to a later and non-causal role (Cohen et al. 1992b; Oberhammer et al. 1993b).

Apoptosis has also been divided into stages using biochemical criteria. Cohen (1991) distinguishes both private and common pathways in apoptosis. This has parallels with the first two stages of the genetic pathway. Changes on private pathways, whether phosphorylation, dephosphorylation, proteolysis or translocation are not shared by all apoptotic stimuli but associated with a particular stimulus. These may be changes controlled by the genes of the "decision to die stage" of the genetic pathway in *C.elegans* and the genes expressed during dexamethasone induced apoptosis may be genes of this sort. As the morphology of apoptosis is similar irrespective of the system studied or the stimulus used the private pathways are believed to converge on a putative final common biochemical pathway and it is within this common pathway that the genes *ced-3*, *ced-4* and *ced-9* play a role. In thymocyte apoptosis there are a number of biochemical changes that are common, DNA degradation, cell shrinkage and changes in the plasma membrane. The temporal and casual relationships between these changes is poorly defined, but there is evidence that these changes occur at different stages within the common pathway. Zinc has been shown to arrest some of the nuclear changes in dexamethasone-induced apoptosis but it does not inhibit all nuclear changes. Ultrastructural examination of cells treated with dexamethasone plus zinc showed that they possess condensed chromatin abutting the nuclear membrane (Cohen et al. 1992b). Field inversion gel electrophoresis showed that these cells are characterised by the presence of large fragments of DNA, 200-250 and 30-50 kilobase pairs in length, in the absence of internucleosomal cleavage of DNA (Brown et al. 1993). Subsequent work, presented in part, in Chapter 2 has shown that the DNA ladder is derived from these large fragments and thus the cleavage of chromatin occurs in stages. The step wise degradation of DNA is associated with two distinct ultrastructures suggesting that chromatin condenses initially into discrete clumps at the nuclear membrane and then further condenses to one

initially into discrete clumps at the nuclear membrane and then further condenses to one electron dense mass at one pole of the nucleus more typical of apoptosis. However the relationship between these two step-wise nuclear changes may only be superficial and not causal as apoptotic chromatin condensation is not necessarily associated with DNA cleavage (Sun et al. 1994).

By identifying changes elicited by diverse apoptotic stimuli those which are part of a common apoptotic pathway can be identified. Once identified, the relationship between these changes can be established as either causal or consequential and a coherent picture of what is required for the apoptotic phenotype built up. While the later changes of apoptosis are clearly important for this phenotype, they are unlikely to control the process and the work that follows describes an attempt to trace the apoptotic process, starting with the terminal biochemical and morphological changes and working backwards towards the controlling events that occur early within the putative common apoptotic pathway. As a result, a population of thymocytes at an early stage of apoptosis were characterised (Chapter 2), evidence that normal thymocytes progress to apoptotic thymocytes via this early stage is shown (Chapter 3) and finally the role of proteases as mediators of both the early and late changes of apoptosis is discussed (Chapters 5 and 6).

CHAPTER 2

FORMATION OF LARGE MOLECULAR WEIGHT FRAGMENTS OF DNA IS A COMMITTED STEP OF APOPTOSIS IN THYMOCYTES

(work carried out in cooperation with Dr. M.MacFarlane and Dr. X.M.Sun)

INTRODUCTION

Glucocorticoid-induction of apoptosis in thymocytes is one of the best documented model systems for the study of apoptosis (Wyllie 1980; Cohen and Duke 1984; Arends and Wyllie 1991). In this system apoptosis is dependent on new RNA and protein synthesis (Cohen and Duke 1984; Wyllie et al. 1984). A number of dramatic biochemical changes occurs in apoptotic cells, the best characterized being the fragmentation of chromatin into units of single or multiple nucleosomes (each nucleosome being 180 - 200 base pairs) recognizable as a "DNA ladder" on agarose gel electrophoresis (Wyllie 1980; Arends et al 1990; Cohen et al. 1992a). This DNA ladder is often considered as the biochemical hallmark of apoptosis (Cohen et al. 1992a) and is thought to arise from the activation of an endogenous Ca^{2+}/Mg^{2+}- dependent endonuclease, which is inhibited by zinc (Cohen and Duke 1984). However, while zinc has been shown to arrest some of the nuclear changes in dexamethasone-induced apoptosis it does not inhibit all nuclear changes. Ultrastructural examination of cells treated with dexamethasone plus zinc showed that they possess condensed chromatin abutting the nuclear membrane (Cohen et al. 1992b). These cells are characterized by the presence of large fragments of DNA, 200-250 and 30-50 kilobase pairs in length, in the absence of internucleosomal cleavage of DNA (Brown et al. 1993). A transitional population of early apoptotic thymocytes (referred to in the text as F3 because of their separation on a Percoll gradient) has recently been identified , comprising cells intermediate in size and density between normal and apoptotic thymocytes and possessing sharply defined clumps of condensed chromatin, abutting the nuclear membrane (Cohen et al. 1993). F3 represents a population of cells at an early stage of apoptosis and were defined as such because they exhibited early biochemical and morphological changes associated with apoptosis without any internucleosomal cleavage of DNA (Cohen et al. 1993). In contrast, fully apoptotic cells are smaller and denser than those in F3. They exhibit extensive internucleosomal cleavage of DNA and characteristically shrunken nuclei, largely occupied by dense apical caps of condensed chromatin (Wyllie 1980; Wyllie et.al. 1980; Cohen et.al. 1993). This chapter describes the further characterisation of F3; large molecular weight fragments of DNA, 10-50 kilobase pairs in length were present in F3 cells. These fragments were absent in normal thymocytes and their formation was dependent upon protein synthesis (Walker et al. 1991). Their appearance is coincident with committment of these cells to apoptosis and is associated with the condensation of chromatin abutting the nuclear memebrane, which is recognised as one of the earliest ultrastrucutral changes of apoptosis. Subsequent cleavage of these large fragments gives rise to oligonucleosomal fragments and is independent of protein synthesis.

MATERIALS AND METHODS

Thymocyte incubations

Thymocytes were isolated from male Fischer 344 rats (4-5 weeks old). Thymuses were rapidly removed, adjoining lymph nodes and connective tissue dissected away and the thymus placed in ice cold Krebs-Henseleit buffer (pH 7.4). The thymuses were weighed and the ratio of thymus to body weight calculated in mg per 100g body weight. Thymuses with a ratio of less than 300 mg/100g body weight were rejected. Cell suspensions were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering, Gomshall, UK) followed by dissaggregation through a nylon mesh and filtering through nylon gauze. The cells were counted using a haemocytometer and the cell density was calculated. Viabililty was assessed by trypan blue exclusion. The cell suspension was then diluted with RPMI 1640 containing 10% fetal bovine serum to give a final suspension of $2x10^7$ cells/ml. Unless otherwise stated, incubations were carried out for 1h at $37^{\circ}C$ under an atmosphere of 95% air, 5% CO_2 .

Flow cytometry

After initial incubation with dexamethasone ($0.1\mu M$) or etoposide ($10\mu M$), thymocytes or cells from the Percoll fractions ($1 x10^6$ cells/ml) were further incubated with the bisbenzimidazole dye, Hoechst 33342 ($1.5\mu g$/ml) in RPMI 1640 containing 10% fetal bovine serum at $37^{\circ}C$ for 10 min. The cells were then cooled to $4^{\circ}C$ on ice for 3 min, centrifuged at 200g for 5 min, resuspended in phosphate-buffered saline containing propidium iodide (5mg/ml) and examined by flow cytometry. Cells were analysed on either an Ortho Cytofluorograf 50H (linked to a 2150 computer system) or a Becton Dickinson Vantage flow cytometer. Both were equipped with a coherent argon ion laser producing 200mW at 488nm and a coherent krypton laser producing 50 mW at 352 nm. Hoechst fluorescence (400-500 nm) and propidiium iodide fluorescence (>630 nm) were recorded. Normal thymocytes have a high forward light scatter (forward scatter is indicative of cell size) and exhibit a low blue fluorescence with Hoechst 33342. In contrast, apoptotic cells have a low forward light scatter and exhibit a high blue fluorescence. These cells have been shown to be apoptotic based on a number of criteria including ultrastructure, smaller size and the presence of DNA ladders (Sun et al. 1992).

Percoll gradients

Discontinuous Percoll gradients were used to separate populations of thymocytes. These populations were designated F1, F2, F3 and F4 in order of increasing bouyant density.

100% Percoll solution is defined as x10 MEM (105 mg/ml MEM powder, 250 mM HEPES pH7.4), solution diluted 1:10 with Percoll. 80%, 70% and 60% Percoll solutions were made up by diluting 100% Percoll solution with 1xMEM (10.5 mg/ml MEM powder, 25 mM HEPES pH 7.4) as shown below.

3ml	60%	F1
3ml	70%	F2
3ml	80%	F3
1ml	100%	F4
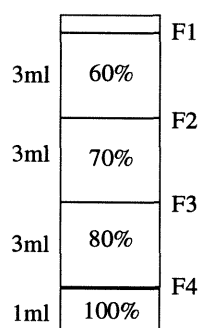
Thymocytes were washed once (200g, 5 min) and resuspended in ice cold PBS at $4x10^8$ cells/ml. $2x10^8$ cells were then loaded onto each 12 ml gradient and centrifuged at 2000g for 10 min. The density of the gradients was calibrated by density marker beads. The buoyant densities at the 0-60%, 60-70%, 70-80% and 80-100% interfaces were 1.063, 1.075, 1.099 and 1.119 g/ml respectively. After centrifugation cells were removed from these interfaces (fractions F1- F4 respectively) and mixed with five volumes of RPMI 1640, washed twice by centrifugation (200g, 10 min) and resuspended in RPMI 1640 with 10% FBS. (Wyllie and Morris 1982; Cohen et al. 1993).

Experimental plan

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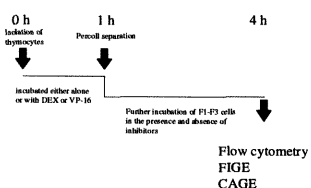
DNA analysis

Agarose gel electrophoresis was used to detect DNA laddering in whole cells (1 x10^6) (Sorenson et al. 1990) and the formation of kilobase pair sized fragments (Walker et.al. 1991; Brown et.al. 1993).

Conventional agarose gel electrophoresis.

Agarose gel electrophoresis was carried out to detect internucleosomal cleavage of DNA. of A 1.8% agarose gel in TBE buffer (89mM Tris, 89mMBoric acid and 2.5 mM EDTA pH 8.3) was prepared. To ensure that DNA from intact cells can enter the gel, a digestion gel (0.8% agarose, 2% SDS in TBE buffer) was made and 25 mg/ml Proteinase K added when the solution has cooled to just below 50°C. The digestion gel is quickly poured into a space above the wells previously cut in the resolving gel and allowed to set. 1-2x10^6 cells were washed (200g 5min) and resuspended in loading buffer (89mM Tris, 89mM Boric acid, 2.5 EDTA 50mg/ml RNAse A, 15% Ficoll, and 0.2% Bromophenol blue pH 8.3) and loaded immediately. The Proteinase K is introduced into the wells by electrophoresis at 25V for 1 h. The DNA fragments are subsequently resolved by electrophoresis at 100V for 3-4 h.
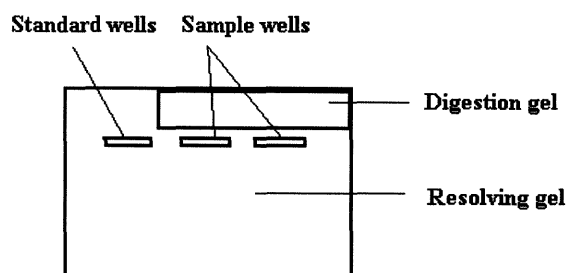
Field inversion gel electrophoresis (FIGE)

Field inversion gel electrophoresis was carried out to detect the formation of large (> 4 kilobase pairs) fragments of DNA.

1. Plug preparation. Agarose plugs containing 1x10^6 cells were prepared (after Anand and Southern, 1990); Briefly, 5x10^6 cells were resuspended in PBS at 2x10^7 cells/ml. Cell suspensions were then warmed to 50°C in a water bath for 3 min. After this time cell suspensions were diluted to 1x10^7 cells/ml with molton agarose L (1% in PBS; Pharmacia), transfered to 100μl wells and allowed to set . Once set plugs were transferred to NDS (10mM Tris, 0.5M EDTA, 1% lauryl sarcosine) and digested with Pronase (Boehringer, United Kingdom) (1mg/ml) for 48h at 50°C. The plugs were stored at 4°C until examined by FIGE as previously described (Brown et al.,1993).

2. Preparation of the gel. A 1% agarose gel (NA agarose, Pharmacia) (TBE x 0.5) was
poured into prewarmed (50°C) plates and the comb inserted (note; the comb was not placed
more than 1.5 cm into the agarose to allow easy removal of the comb). After the gel had set,
the comb was removed and the wells washed with TBE x 0.5.

3. Loading samples. Equal portions of plug were loaded into the wells and the wells then
sealed with 1% agarose (as above).

4. Running the gel. The gel was run in TBE x 0.5 under the following conditions using a
200V power supply and a Hoeffer PC750 pulse controller. The total run was 7 hours and 15
minutes. The first 15 minutes was at 200V constant current followed by 1 h at 200V with a
pulse rate of 2.4 seconds forward and 0.8 seconds reverse without ramping the current. The
gel was subsequently pulsed at 200V with a pulse rate of 2.4 seconds forward and 0.8
seconds reverse and ramp factor of 1.5.

Under the conditions used, DNA fragments ranging in size from 4.4 to 460 kilobase
pairs were resolved. *Saccharomyces cerevisiae* chromosomes, 243- 2200 kilobase
pairs (Clontech, Cambridge, United Kingdom) and 0.1 -200 kilobase pairs (Sigma
Chemical Co., Poole, United Kingdom) were used as standards.

Isolation of nuclei and DNA autodigestion

Thymocyte nuclei were isolated as previously described (Bellard et al. 1989) with
minor modifications as follows. Briefly, thymocytes were washed once in buffer A
(10 mM Tris HCl, 60mM KCl,15mM NaCl and 5 mM $MgCl_2$, pH 7.5),
resuspended in buffer B (10mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5), left on ice for 20
min., homogenized and centrifuged at 200 g for 5 min., resuspended and washed
twice in buffer B. The pellets were then resuspended in buffer A with the indicated
concentrations of calcium or inhibitors. DNA autodigestions were carried out at
37 °C.

Microscopy

Cell suspensions were fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer
(pH 7.2), pelleted and postfixed for 90 min., in 1% osmium tetroxide and 1.7%
potassium ferrocyanide. They were then stained en bloc for 1h with 2% aqueous
uranyl acetate, dehydrated and embedded in Araldite. Sections were stained with
toluidine blue for light and lead citrate for electron microscopy.

RESULTS

Induction of apoptosis in thymocytes is accompanied by the formation of large fragments of DNA

Incubation of thymocytes with dexamethasone (0.1 μM) or etoposide (10 μM) for 4h resulted in the formation of 22 ± 3.3 and 27 ± 1.6 % apoptotic cells respectively compared to 8.5 ± 1.8 % in control thymocytes (Mean ±S.E.M., n = 4) as assessed by flow cytometry. Thymocytes were examined by conventional agarose gel electrophoresis for internucleosomal cleavage of DNA and by field inversion gel electrophoresis (FIGE) for the presence of large fragments of DNA. Following incubation with dexamethasone for 4h, unfractionated thymocytes exhibited an increase in both internucleosomal cleavage of DNA and in the formation of large molecular weight fragments of DNA, particularly of 30-50 kilobase pairs, compared to thymocytes incubated for 4 h wothout additional treatment (Fig.1a & b, lanes 4 & 3 respectively). The thymocytes were also fractionated by discontinuous Percoll density gradients into four fractions of decreasing size and increasing density (fractions F1- F4). Based on both ultrastructural and biochemical criteria, cells in F1 and F2 are normal thymocytes, cells in F3 are preapoptotic, whilst those in F4 are apoptotic (Wyllie and Morris,1982; Cohen et al.,1993). After 4h, there was little or no internucleosomal cleavage or formation of large fragments of DNA in normal cells in F1 and F2 (Fig.1a & b, lanes 5 & 6 respectively). In marked contrast, at this time, both oligonucleosomes and large fragments of DNA, in particular of 30-50 kilobase pairs in length, were observed in the early apoptotic cells in F3 and the apoptotic cells in F4 (Fig.1a & b, lanes 7 & 8 respectively). In order to ascertain a possible inter-relationship between the large molecular weight fragments and internucleosomal cleavage of DNA, cells obtained from Percoll fractionation at earlier times were examined.

Formation of large fragments of DNA in preapoptotic thymocytes precedes internucleosomal DNA cleavage

Thymocytes were incubated with dexamethasone (0.1μM) for 1h, fractionated on Percoll and examined by electrophoresis. Cells in F1 and F2 contained primarily intact DNA (as defined by it not entering the gels), whereas cells in F3 contained large fragments of DNA, 30- 50 kilobase pairs in length (Fig.2a). Further incubation for 2 or 4 h of cells in F1 or F2 resulted in formation of similar large fragments (Fig.2a). After 1 h, no internucleosomal cleavage was observed in cells from F1,F2 or F3 (Fig.2b). Following a further incubation of 2 h, slight internucleosomal cleavage was observed with cells from F2 whilst

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Figure 1. Detection of large DNA fragments and DNA ladders in preapoptotic and apoptotic thymocytes after 4h exposure to dexamethasone.
Thymocytes from each treatment were examined as described in Experimental Procedures by a) field inversion gel electrophoresis (FIGE) and b) agarose gel electrophoresis. Thymocytes were incubated either alone (lane 3) or with dexamethasone (lane4) (0.1μM) for 4 h. Dexamethasone treated thymocytes were then separated on discontinuous Percoll gradients into 4 fractions (F1-F4) (lanes 5-8 respectively). Standardsfor FIGE in lanes 1 and 2 contain 0.1-200 kilobase pair (kbp) and *S.cerevisiae* chromosomes (243-2200 kbp) as size markers, respectively. The standard (lane 1) for the agarose gel electrophoresis contains markers of 123 base pairs or multiples thereof .
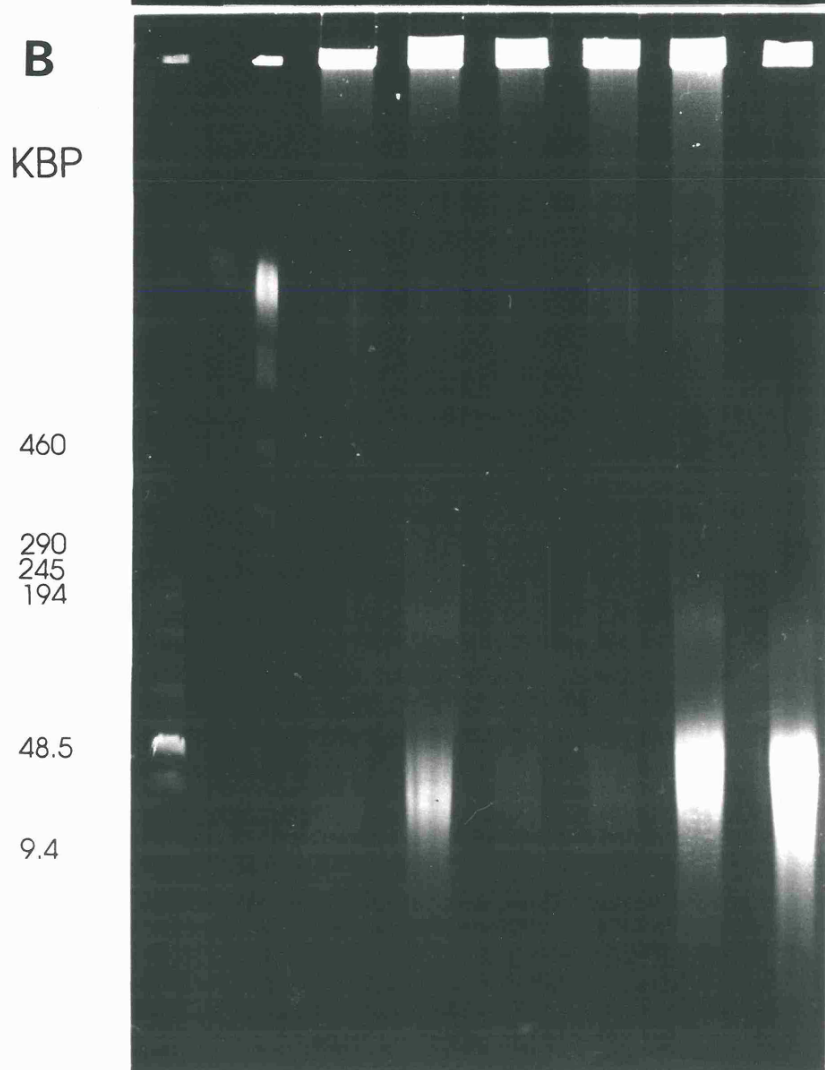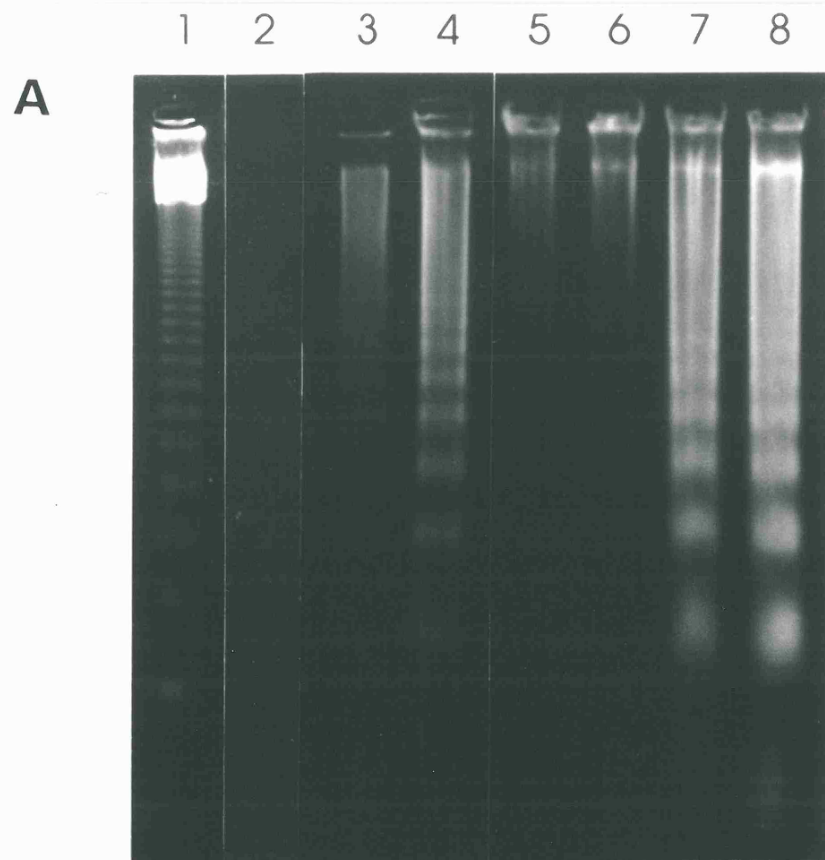
**Figure 2. Detection of large DNA fragments in preapoptotic thymocytes prior to
the appearance of DNA laddering following 1 h incubation with dexamethasone.**
Thymocytes were examined both by a) FIGE and b) agarose gel electrophoresis.
Thymocytes were incubated with dexamethasone (0.1μM) for 1 h and then separated on
discontinuous Percoll gradients and fractions F1-F3 examined either immediately or after a
further incubation of 2 or 4 h. The standards used were as described in the legend to figure
1 except for FIGE only the 0.1-200 kilobase pair standard was used.
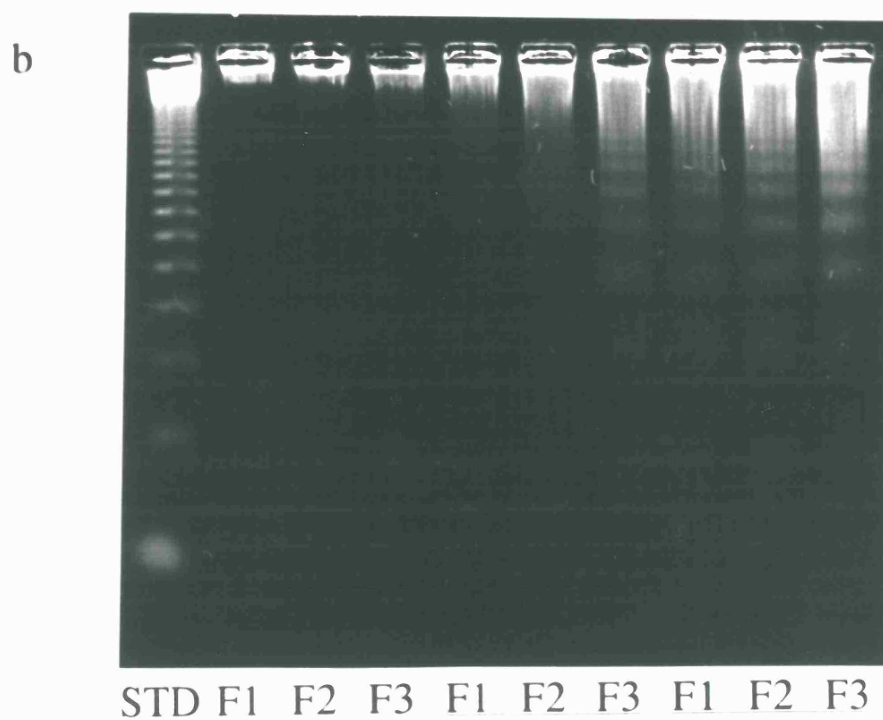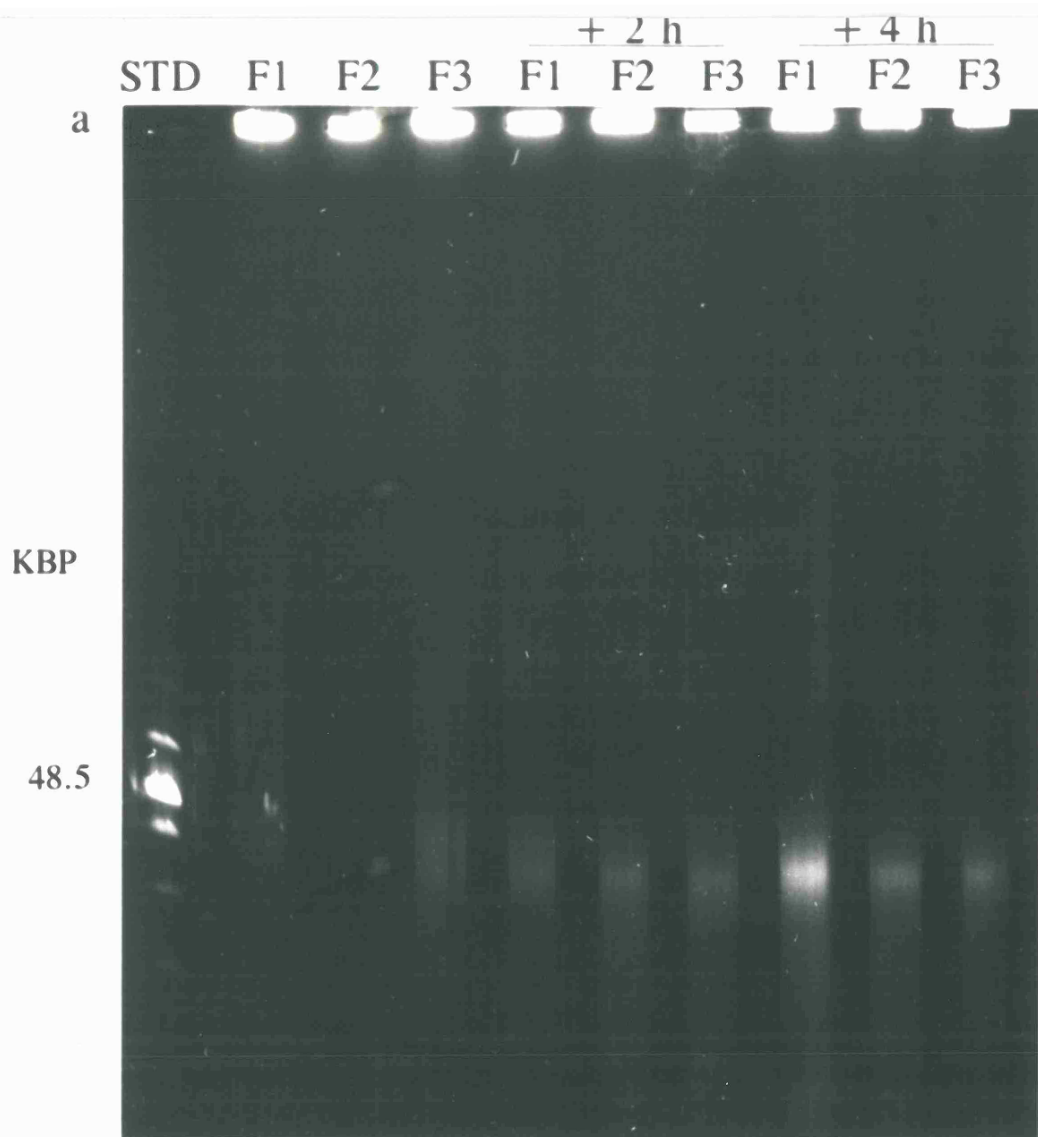
extensive cleavage was seen with cells from F3 (Fig.2b). By 4 h, internucleosomal cleavage was clearly observed in cells from F1-F3 (Fig.2b). Similar results were obtained following incubation of thymocytes with etoposide (10μM) (results not shown). Thus in the preapoptotic cells in F3, formation of large fragments of DNA preceded internucleosomal cleavage.

DNA ladders characteristic of apoptosis are derived from the large fragments of DNA
The above results suggested that the formation of large fragments of DNA preceded endonuclease cleavage of DNA into nucleosomal fragments. In order to examine the possible relationship between the large fragments and internucleosomal cleavage of DNA, the autodigestion of isolated thymocyte nuclei in the presence of Ca^{2+} and Mg^{2+} was investigated. A time-dependent internucleosomal cleavage of DNA, resulting in a DNA ladder pattern characteristic of apoptosis, was observed (Fig.3b). No internucleosomal cleavage was noted at times up to 30 min but after this it increased markedly with time (Fig.3b). When these samples were examined by FIGE, a progressive degradation of large fragments greater than 30 kilobasepairs was observed (Fig.3a). At the earliest times, fragments of > 460 kilobase pairs (beyond the resolving power of the gel under the conditions used) were also observed.

In order to further investigate the relationship between the large and oligonucleosomal fragments of DNA, several inhibitors of internucleosomal cleavage were used. A concentration dependent inhibition of the internucleosomal cleavage of DNA was observed in the presence of zinc (10, 25 & 50 mM) (Fig. 4b, lanes 6, 7 & 8 respectively) , aurin (50, 100 & 250 μM) (Fig. 4b, lanes 9, 10 & 11 respectively) and spermine (0.5, 1 & 5mM) (Fig. 4b, lanes 12, 13 & 14 respectively), almost complete inhibition being observed at the highest concentration of all three inhibitors (Fig.4b, lanes 8, 11 & 14). These inhibitors partially blocked the conversion of the >460 kilobase pair fragments to 190-240 and 30-50 kilobase pair fragments, the most marked effects being observed at the highest concentration of the inhibitors (Fig.4a, lanes 8, 11 & 14 respectively). The highest concentration of spermine (5mM) almost totally prevented formation of the 30-50 kilobase pair fragments (Fig.4a, lane 14).

Figure 3. Detection of large DNA fragments prior to internucleosomal cleavage of DNA in isolated nuclei.

Thymocyte nuclei were prepared and examined by a) FIGE for large DNA fragments and b) agarose gel electrophoresis for internucleosomal cleavage as described in Experimental Procedures. Nuclei were incubated at 37°C, pH 7.5 in the presence of 5 mM Ca^{2+} and 10 mM Mg^{2+} for 0, 15, 30, 60, 90 or 120 min as indicated in the figure. The standards used were as described in the legend to Figure 1.

a)

STD

| 1 | 2 | 0 | 15 | 30 | 60 | 90 | 120 |

KBP

460

245

48.5

b)

| 1 | 2 | 0 | 15 | 30 | 60 | 90 | 120 |

STD   0   15   30   60   90   120
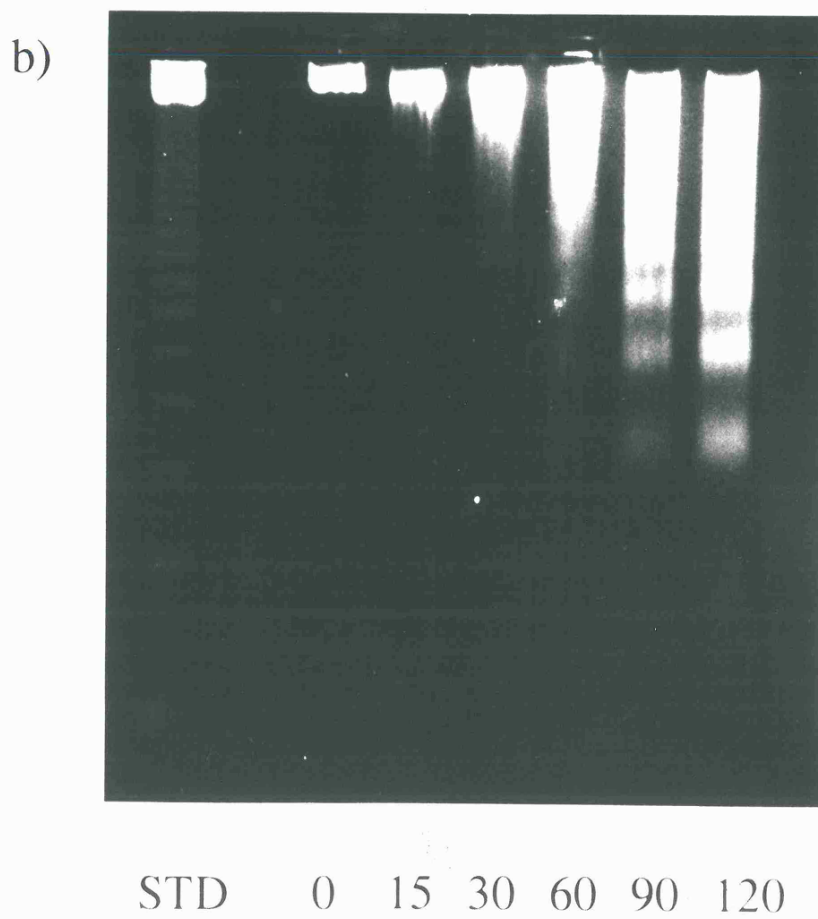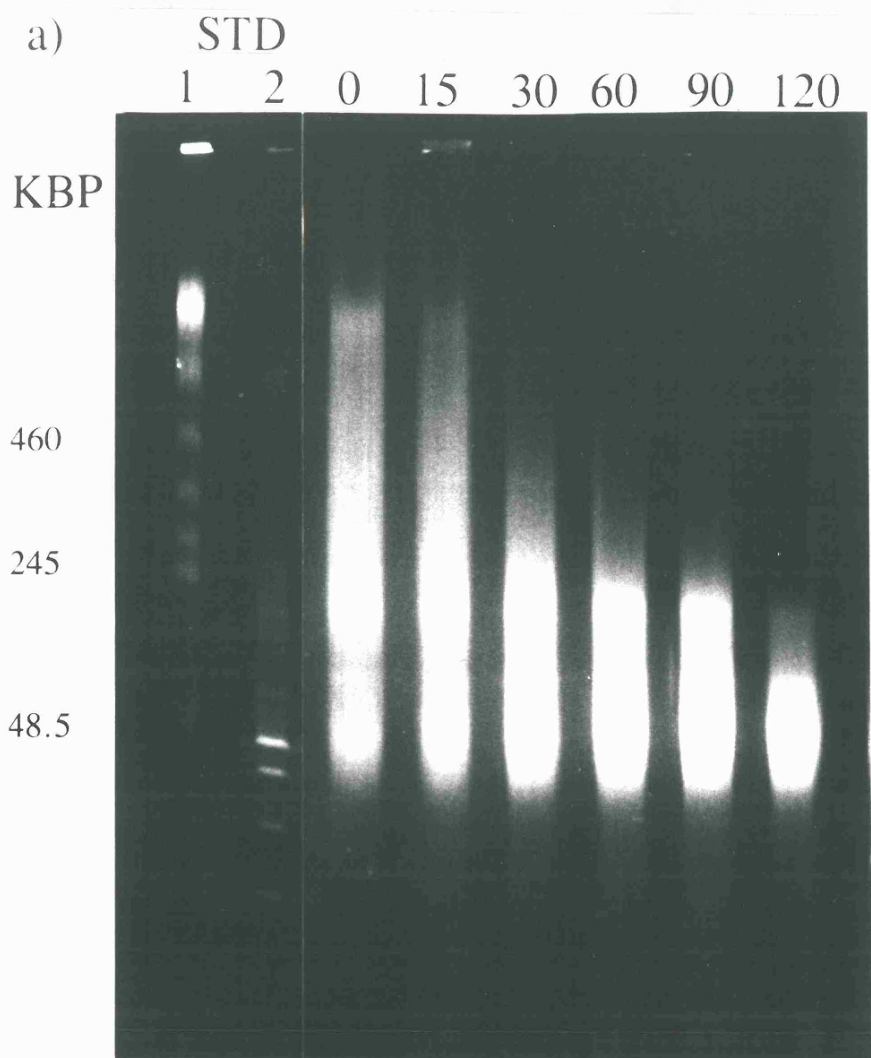
Figure 4. Inhibition of internucleosomal cleavage of DNA and simultaneous accumulation of large DNA fragments.

Thymocyte nuclei were prepared and examined by a) FIGE for large DNA fragments and b) agarose gel electrophoresis for internucleosomal cleavage. Nuclei were incubated for 90 min as described in the legend to Figure 3 either alone or in the presence of the indicated concentrations of Zn^{2+} (10, 25 & 50 µM) (lanes 6, 7 & 8), aurin (50, 100 & 250 µM) (lanes 9, 10 & 11) or spermine (0.5, 1 and 5 mM) (lanes 12, 13 & 14). The standards used were as described in the legend to Figure 1. To confrim the ionic dependence of nuclei auto-digestion nuclei were incubated in the absence of Ca^{2+} and Mg^{2+} (lane 3), with Mg^{2+} alone (lane4) or with Mg^{2+} plus Ca^{2+} (lane 5).
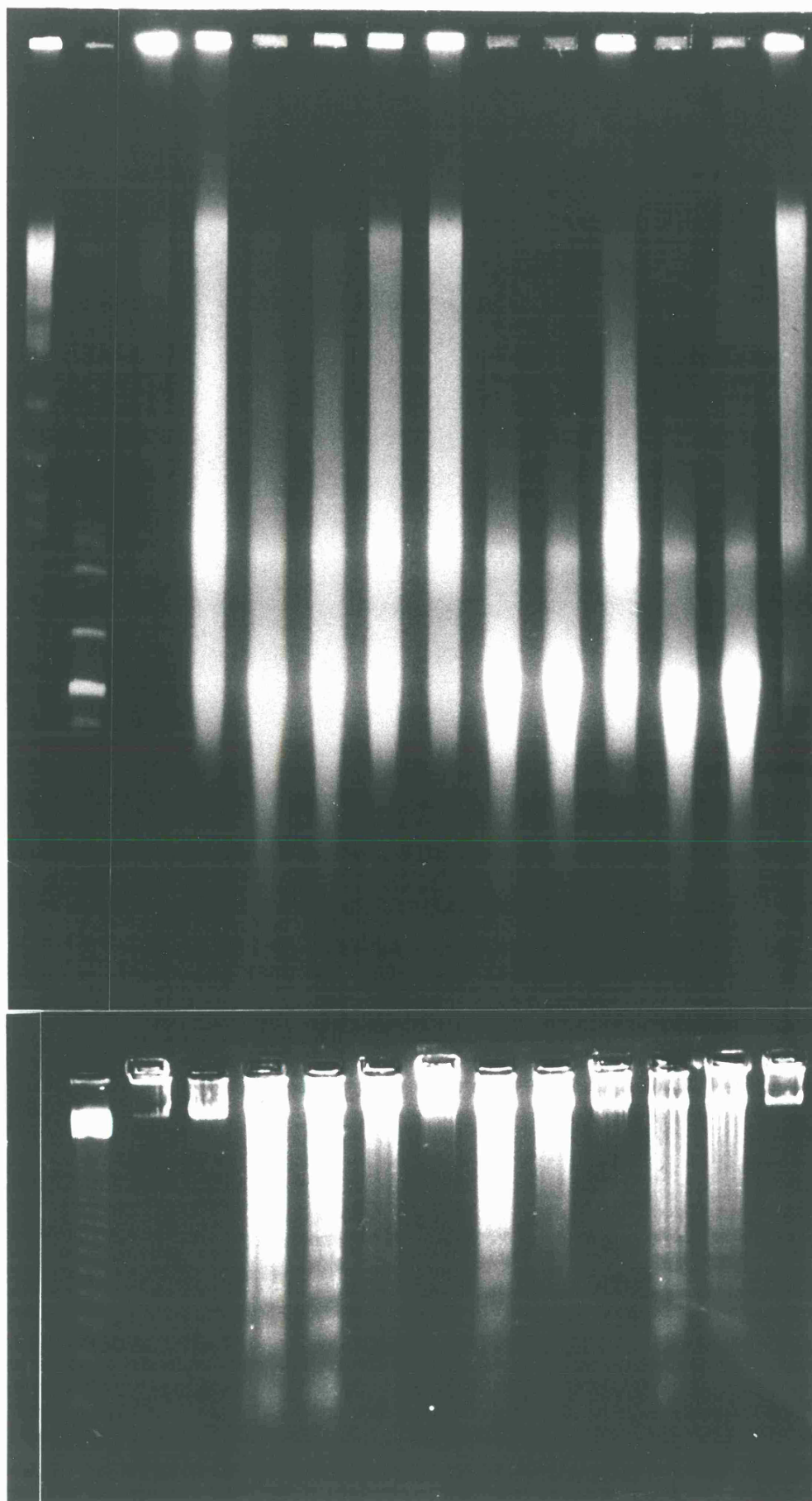
Protein synthesis is required for the formation of large fragments of DNA in normal thymocytes in F1 and F2.

Percoll fractions, obtained from thymocytes incubated for 1h with dexamethasone (0.1 µM) were further incubated for up to 4h in the presence or absence of cycloheximide (10µM) and examined by FIGE. Further incubation of cells in F1 or F2 for 2 or 4 h resulted in the formation of detectable amounts of large fragments of 30- 50 kilobase pairs in length, which was largely prevented by coincubation with cycloheximide (10µM) (Fig.5). Similar results were obtained with cells from F1 and F2 following initial treatment of the thymocytes with etoposide (10µM) (data not shown).

Protein synthesis is required for the progression of thymocytes in F1 & F2 but not the preapoptotic cells in F3 to fully apoptotic cells

Glucocorticoid- and etoposide- induced apoptosis in thymocytes are dependent on new protein and mRNA synthesis (Cohen and Duke,1984; Wyllie et al.,1984; Walker et al.,1991). In order to assess whether cells in F1, F2 or F3, obtained after 1h of incubation either alone or with dexamethasone or etoposide, were already committed to apoptosis, they were incubated for a further 2 or 4 h either alone or in the presence of cycloheximide (10 µM). They were then examined by agarose gel electrophoresis, flow cytometry and light and electron microscopy. Further incubation of cells in F1, F2 or F3 for 4h resulted in the formation of DNA ladders (Fig.2b) as well as a time-dependent increase in the percentage of apoptotic cells (Fig.6). Normal and apoptotic thymocytes can be readily separated and quantified by flow cytometry using Hoechst 33342; normal cells exhibit a low blue fluorescence and a high forward light scatter whereas apoptotic cells have a lower forward light scatter and a high blue fluorescence (Sun et al 1992). When cells from F1 and F2 were further incubated in the presence of cycloheximide, the time-dependent increase in the formation of the apoptotic cells was inhibited to control values (Fig.6a & b). Cells in F3 progressed to apoptotic cells more rapidly than cells in F1 and F2 (compare Fig.6c with Fig.6a & b) and this progression was not inhibited by cycloheximide (Fig.6c).

These observations were consistent with morphological changes evident by both light and electron microscopy in resin sections. Cells in both F1 and F2, fractionated after 1h, appeared normal, but when incubated for a further 2-4h, a small number showed apoptotic morphology. In contrast, most of the cells in F3, fractionated after 1h, exhibited perinuclear accumulations of dense sharply

Figure 5. Formation of large fragments of DNA in normal thymocytes (F1 and F2) is inhibited by cycloheximide. Thymocytes were incubated for 1h with dexamethasone (0.1µM) and cells in fractions F1 and F2 isolated by Percoll density gradient centrifugation. When these cells were examined for large fragments of DNA immediately after isolation, none were observed (Fig.2a). Cells were then incubated for a further 2 or 4 h either alone (-) or in the presence of cycloheximide (+) (10µM) and examined by FIGE. The standards used were as described in the legend to Figure 2.
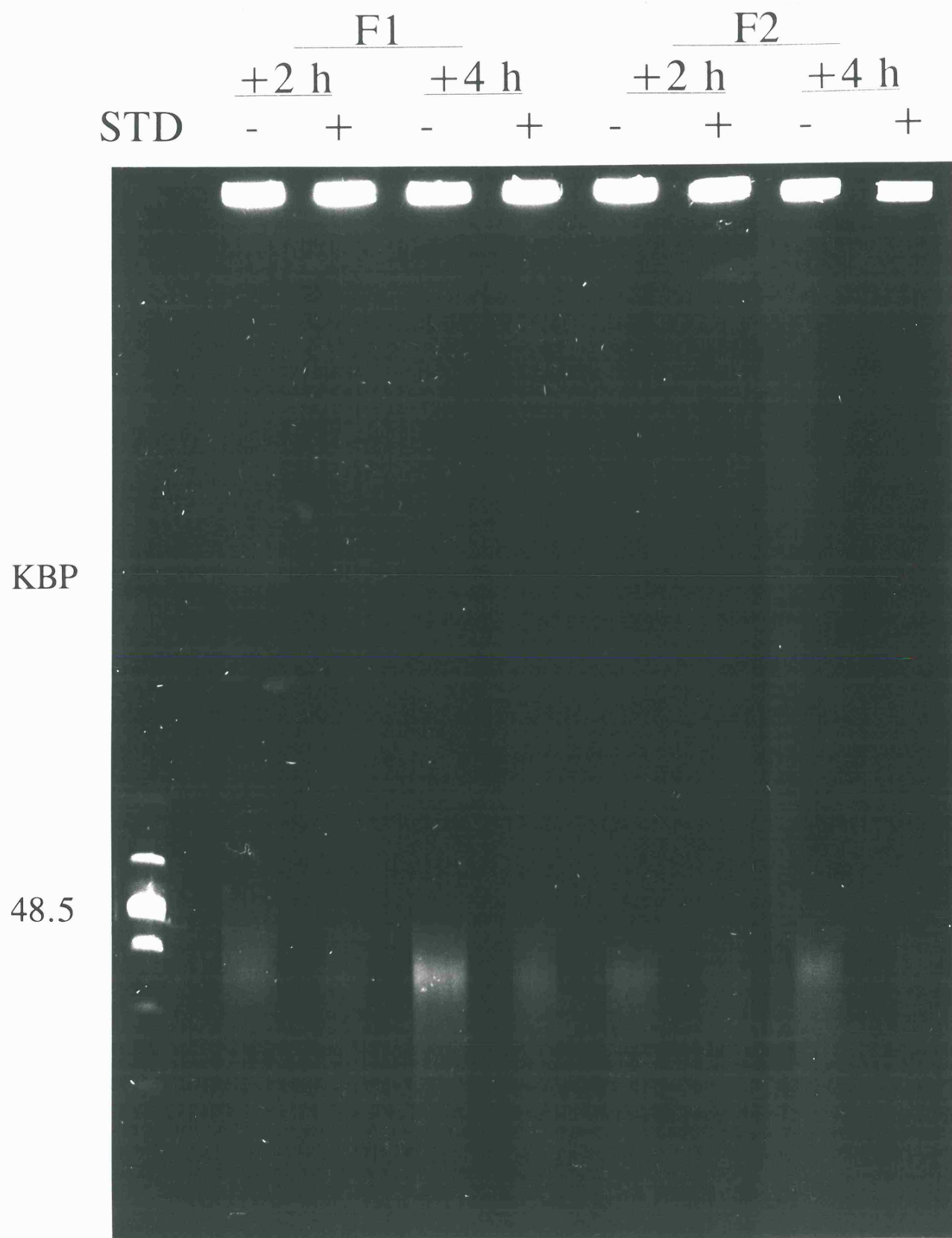
delineated chromatin (Fig. 7a), characteristic of this population of preapoptotic cells (Cohen et al., 1993) and cells with the characteristic morphology of the late stages of apoptosis were rarely observed (<3%). When cells in F3 were further incubated either alone or in the presence of cycloheximide for 4h, many cells were observed (>20%) possessing all the characteristic features of fully apoptotic thymocytes in particular the coalescence and condensation of chromatin (Fig.7b&c). No differences were observed in the proportion of these cells following incubation in the presence or absence of cycloheximide (results not shown). The remaining cells retained the features of cells normally found in F3, particularly the perinuclear and centrinuclear clumps of moderately condensed chromatin (Fig.7c). Thus the progression of cells in F3 to fully apoptotic cells was not inhibited by cycloheximide.

Figure 6. Cycloheximide inhibits the progression of normal thymocytes (F1 and F2) but not that of preapoptotic thymocytes (F3) to fully apoptotic cells.
Thymocytes were fractionated into normal (F1 and F2) and preapoptotic (F3) cells by discontinuous Percoll density gradient centrifugation after incubation of thymocytes for 1h either alone (□—□) or in the presence of dexamethasone (0.1 µM) (O—O) or etoposide (10µM) (Δ—Δ). Cells from F1, F2 or F3 (Fig.6a, b or c respectively) were then incubated for a further 2 or 4h either alone (solid lines) or in the presence of cycloheximide (10 µM) (dashed lines). The percentage of apoptotic cells was quantified by flow cytometry using Hoechst 33342 as described in Experimental Procedures. The results represent the means of at least four determinations.
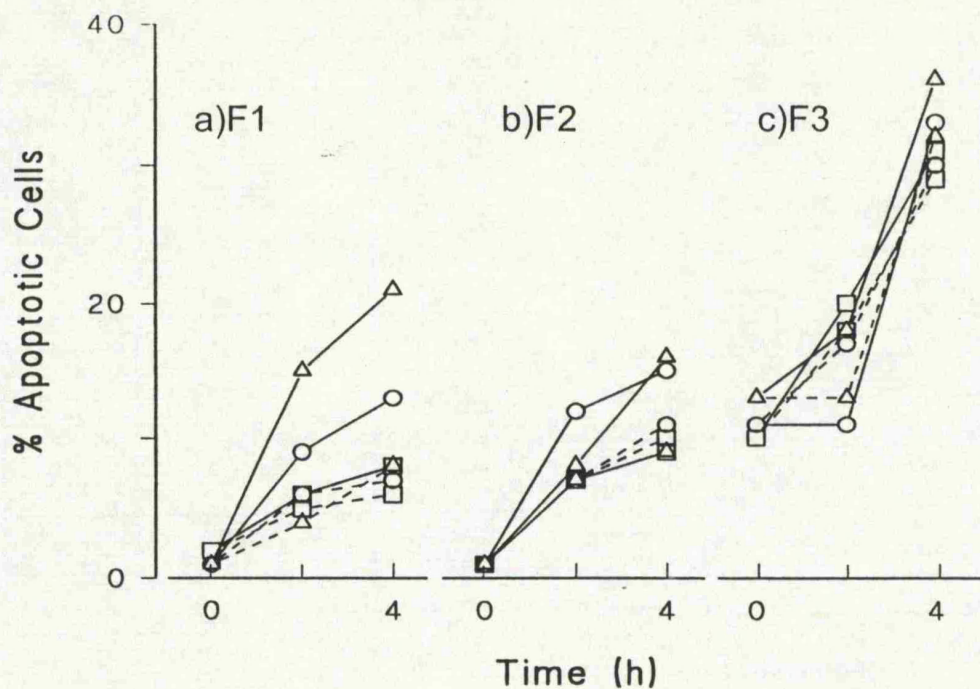
Figure 7. Progression of preapoptotic to apoptotic thmocytes is not inhibited by cycloheximide.
Thymocytes were incubated for 1h with dexamethasone (0.1 µM) and preapoptotic cells in F3 were
isolated by Percoll density gradient centrifugation. Immediately after fractionation, the majority of cells in F3 showed dense
sharply defined clumps of perinuclear chromatin associated with the nuclear membrane (Fig.7a). Only a few apoptotic cells
(arrowheads) were present in this fraction. Incubation of F3 for a further 4 h in the presence of cycloheximide (10µM)
resulted in the appearance of many apoptotic cells (a small proportion of these are indicated by arrowheads) (Fig.7b).
Ultrastructural examination of this sample (Fig.7c) confirmed the the condensation of chromatin (*) in the apoptotic cells.
The chromatin of the remaining cells was moderately condensed and concentrated in centrinuclear and perinuclear clumps
typical of cells in F3. Bars = 5µm.
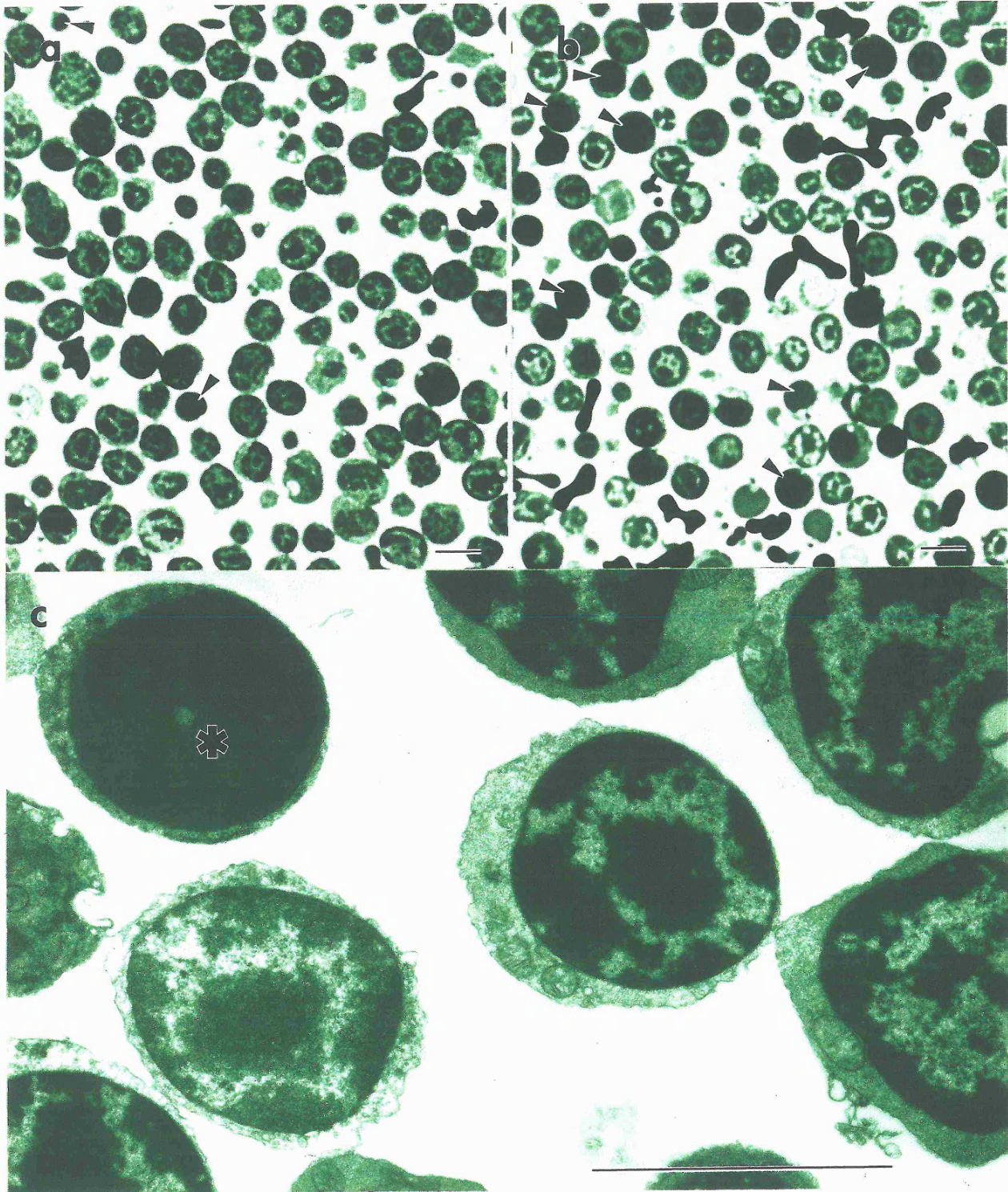
DISCUSSION

Large fragments of DNA are present in preapoptotic but not normal thymocytes
Percoll density gradients were utilized to separate incubated thymocytes into four
fractions (F1-F4) so as to determine in which fraction detectable changes in DNA
occurred. At 1h, large fragments of DNA, in particular of 30- 50 kilobase pairs in
length, were present in preapoptotic cells in F3 but not in normal thymocytes in F1
and F2 (Fig.2a). Further incubation of cells in F1 and F2 for 2 or 4h resulted in the
formation of large fragments of DNA, 30-50 kilobase pairs in length, particularly in
cells in F1 (Fig.2a) followed or accompanied by internucleosomal cleavage of
DNA (Fig.2b). The reason for the accumulation of large fragments in cells in F1 at
4h is not clear but may be due to less extensive internucleosomal cleavage in these
cells. In none of the studies was internucleosomal cleavage observed prior to
formation of large fragments.

The appearance of large fragments in cells in F3 also preceded internucleosomal
cleavage (compare Figs.2a & b). Following further incubation of cells in F3 for 2
or 4h, large DNA fragments were still present (Fig.2a) but were accompanied by
internucleosomal cleavage of DNA (Fig 2b). At 1h the majority of cells in F3
exhibited ultrastructural changes characteristic of cells at an early stage of apoptosis
(Fig.7a) (Cohen et al.,1993). At later times (2 or 4h) cells in F3 comprised cells
with ultrastructural characteristics typical of both early and late stages of apoptosis
(Fig 7b & c). Thus it is evident that cells in F3, which exhibited the earliest
ultrastructural changes of apoptosis, possessed large fragments of DNA prior to
internucleosomal cleavage.

DNA ladders characteristic of apoptosis are derived from the large fragments of DNA
Isolated thymocyte nuclei possess a Ca^{2+}/Mg^{2+}-dependent and zinc-sensitive
endonuclease, which results in the formation of DNA ladders (Cohen &
Duke,1984). In agreement with these studies, a similar Ca^{2+}/Mg^{2+}-dependent
internucleosomal cleavage of DNA (Fig.3b) was observed. Formation of large
fragments of DNA clearly preceded the appearance of DNA ladders (Fig.3) and
suggested that the ladders were derived from the large fragments. Incubation of
nuclei with zinc, aurin and spermine, inhibitors of internucleosomal cleavage
(Cohen & Duke,1984; McConkey et al.,1989; Brune et al.,1991), resulted in a
concentration dependent inhibition of internucleosomal cleavage (Fig.4b)
accompanied by a concomittant increase in large fragments of DNA (Fig.4a). This

provided very strong evidence to support the hypothesis that there was an initial formation of large fragments, which subsequently gave rise to the characteristic DNA ladders of apoptosis. This conclusion, in agreement with an earlier study (Brown et. al.,1993) and a recent report with human tumour cell lines (Oberhammer et al.,1993b), showed the formation of DNA fragments of 50 kilobase pairs prior to internucleosomal cleavage . Different DNA fragmentation pattterns have been found in some human tumour cells (Dusenbury et al.,1991; Canman et al.,1992; Oberhammer et al.,1993). In some, a discrete population of DNA fragments ranging from approximately 50-200 kilobase pairs was observed which did not give rise to DNA ladders (Canman et al., 1992; Oberhammer et al.,1993b). It is possible that in these cells as well as in thymocytes, the early DNA cleavage to large fragments is similar but only certain cells, such as thymocytes, possess the endonuclease responsible for the cleavage of the large fragments into classical DNA ladders.

Zinc, spermine and aurintricarboxylic acid inhibited the interconversion of the large fragments but only at the highest concentrations of inhibitors were signficant amounts of the very large fragment (>460 kilobase pairs) observed (Fig.4a). This is in contrast to the Ca^{2+}/Mg^{2+}-dependent endonuclease most frequently associated with internucleosomal cleavage of DNA, which is completely inhibited by zinc (Fig.4b and Cohen and Duke,1984). The present results together with previous observations are compatible with one or more of the following possibilities:- a) there are at least two Ca^{2+}/Mg^{2+}-dependent enzymes responsible for the cleavage of DNA, one of which is inhibitable by low concentrations of zinc,aurin and spermine; b) one Ca^{2+}/Mg^{2+}-dependent endonuclease is responsible for the cleavage but it has at least two active sites, one being more strongly inhibited by these three inhibitors than the other or c) the inhibitors may act to alter the conformation of the DNA so it is not accessible to internucleosomal cleavage. Some support has recently been provided for the last suggestion by the findings that spermine inhibits apoptosis by altering chromatin conformation (Brune et al.,1991). Aurintricarboxylic acid is a general inhibitor of nucleases and blocks glucocorticoid-induced chromatin cleavage and cell killing in thymocytes (McConkey et al.,1989). However, recently aurin has been shown to inhibit programmed death of neuronal cells independent of its ability to block nucleolytic DNA degradation (Mesner et al.,1992). The results suggest that the inhibition of internucleosomal cleavage of DNA by aurin may be due in part to inhibition of the breakdown of the large fragments of DNA (Fig.4). The significance of the sizes of the large fragments formed in both intact cells and isolated nuclei is not clear. Fragments of approximately 50 and 250 kilobase pairs

may represent cleavage of chromosomal domains such as supercoiled loops and rosettes respectively (Filipski et al., 1990; Walker et al.,1991; Solovyan et al.,1991).

Formation of the large fragments of DNA is dependent on protein synthesis

The requirements for new protein and mRNA synthesis in the induction of apoptosis in thymocytes by a wide variety of diverse agents including glucocorticoids (Cohen and Duke,1984; Wyllie et al.,1984), Ca^{2+} ionophores (Wyllie et al.,1984), γ- irradiation (Sellins and Cohen,1987) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (McConkey et al.,1988) are well documented. In contrast there are only a few examples of induction of apoptosis in immature thymocytes, including hyperthermia (Sellins and Cohen, 1991), gliotoxin (Waring,1990) and tributyltin oxide (Raffray et.al.,1993), which appear to be independent of protein synthesis. In cells other than thymocytes, the requirement of protein synthesis in the induction of apoptosis is more poorly defined and inhibitors of protein synthesis often induce apoptosis (Martin et al,1990; Martin,1993). The precise roles of the newly synthesized molecules in the induction of apoptosis in thymocytes are not known although there have been several suggestions including synthesis of either the Ca^{2+}/Mg^{2+} -dependent endonuclease (Compton and Cidlowski,1987), synthesis of a protein required to transport Ca^{2+} into the nucleus (Cohen and Duke,1984) or that macromolecular synthesis blocks apoptosis by depleting nuclei of an endonuclease which is undergoing rapid turnover (McConkey et al.,1990).

Further incubation of cells from F1 and F2 resulted in the formation of large fragments of DNA, which was inhibited by cycloheximide (Fig.5). These results suggested, that in both dexamethasone- and etoposide-induced apoptosis, protein synthesis was required for the formation of large fragments of DNA. However, once these were formed, as in cells in F3, no further protein synthesis was required in order for apoptosis to proceed (Fig.6c). Thus the well documented requirement for protein synthesis in the induction of apoptosis in thymocytes (Cohen and Duke,1984; Wyllie et al.,1984) is apparently essential for the formation of the large fragments of DNA.

Cells in F3 but not in F1 or F2 are committed to apoptosis

Further incubation of cells from F1, F2 or F3, fractionated after 1h of treatment with dexamethasone or etoposide, resulted in the formation of apoptotic cells as

determined by the appearance of high blue fluorescent cells with Hoechst 33342
(Fig.6), morphological criteria (Fig.7) and DNA ladders (Fig.2b). In the case of F1
and F2 but not F3, these changes were inhibited by cycloheximide (compare Figs.6a
& b with Fig.6c). These results suggested that normal thymocytes in F1 and F2
were not yet committed to apoptosis and still required protein synthesis, whereas
cells in F3 were already committed and no longer required new protein synthesis.
As protein synthesis was required for the formation of the large fragments (Fig.5),
the results support the hypothesis that this represents a key committed step in
apoptosis of thymocytes.

The fate of cells in F3 isolated after 1h incubation appeared independent of the
previous treatment of the thymocytes (Fig.6c), which suggested that most of the
preapoptotic cells at this early time point may have arisen by spontaneous apoptosis
of cells already primed to die in vivo. These results support an earlier suggestion
that cells in F3 are a transient preapoptotic population of thymocytes (Cohen et
al.,1993). Further evidence for the preapoptotic nature of these cells was provided
by their rapid transition to fully apoptotic thymocytes as assessed both by flow
cytometry and morphology (Figs.6c,7b & 7c). A greater variability was observed
in the percentage of apoptotic cells formed in cells from F3 upon further incubation
when compared to data obtained with cells from F1 or F2. The precise time that
cells in F3 are isolated appears critical, which is compatible with these cells being a
transient population of preapoptotic thymocytes at an early committed stage in the
apoptotic process, when there is a wide range of rapid biochemical and
ultrastructural changes occurring.

Recent studies from our laboratory as well as a number of others have suggested that the
morphological features of apoptosis may be dissociated from internucleosomal cleavage of
DNA in thymocytes as well as a number of other cell types (Cohen et al.,1992b; Barres et
al.,1992; Oberhammer et al.,1993 a & b; Tomei et al.,1993).These studies and others
(Filipski et al.,1990) together with the present data enable us to propose a model to explain
the relationship between DNA degradation, chromatin structure and changes in nuclear
morphology (Fig.8). It is proposed that normal thymocytes, in fractions F1 and F2, are
converted to preapoptotic cells in F3 and this coincides with the formation of the large
fragments of DNA.The data suggest that this step is dependent on protein synthesis. These
large fragments, possibly associated with the areas of condensed chromatin abutting the
nuclear membrane in cells in F3, are then rapidly cleaved by a zinc-inhibitable endonuclease

Figure 8. A proposed sequence of events which commit thymocytes to apoptosis. Intact DNA of normal thymocytes (F1 and F2) is cleaved initially into large fragments present in the preapoptotic population (F3) of cells containing condensed chromatin abutting the nuclear membrane. These cells are then rapidly converted to cells with the classical ultrastructure and biochemistry (i.e. DNA ladders) of apoptotic thymocytes (F4).

Normal

F1 & F2

Intact DNA

CHX

Preapoptotic

F3

190-240 kbp

Zn

30-50 kbp

Zn

Apoptotic

F4

~180 bp
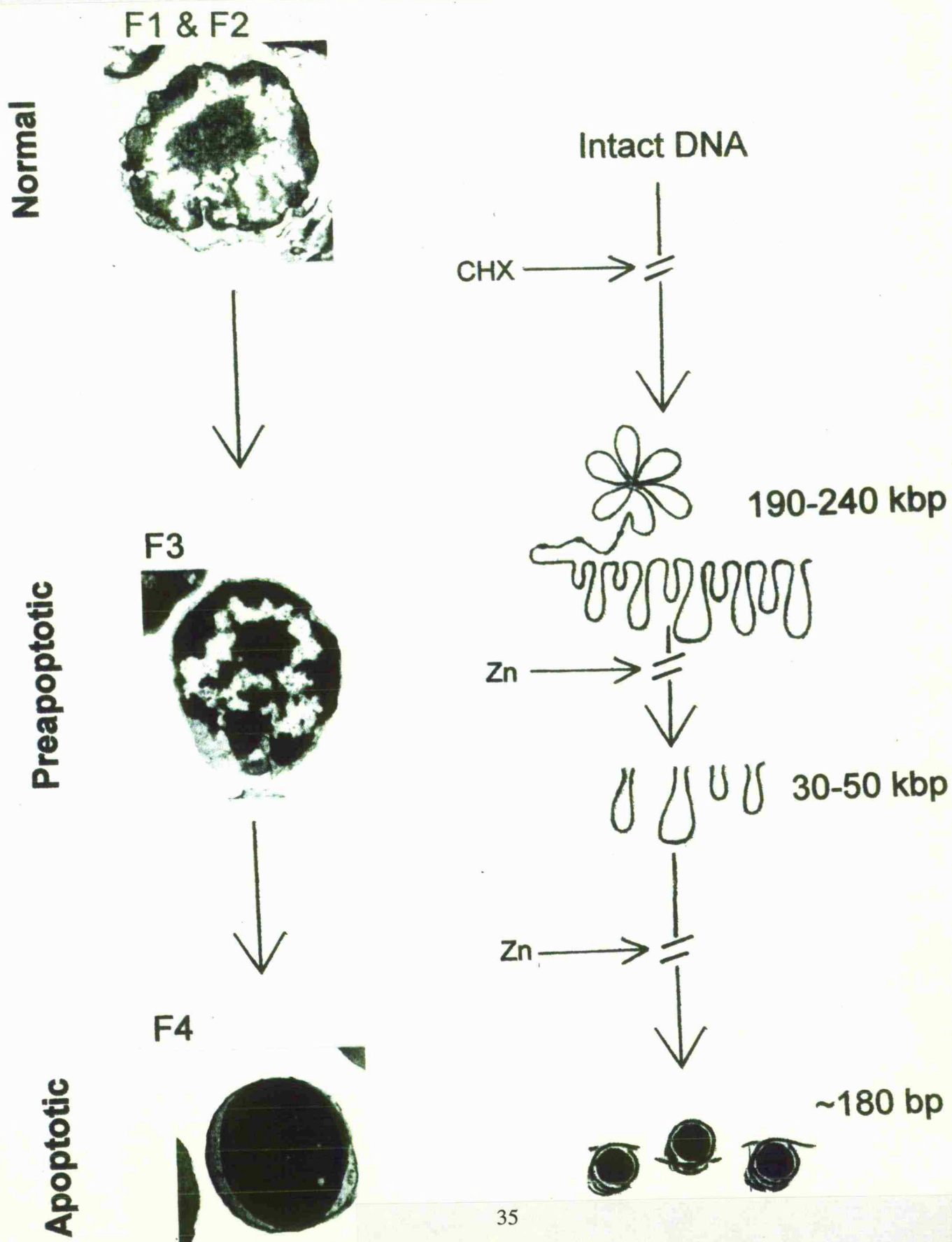
(Walker et al.,1991;Brown et al.,1993) to give rise to characteristic apoptotic cells (F4) possessing DNA ladders. It is posssible that the conformational changes in the chromatin of cells in F3 facilitate access of the endonuclease to DNA so enabling its rapid breakdown to fully apoptotic cells. Thus internucleosomal DNA cleavage is dependent upon an early degradation of higher order chromatin structure.

The present study demonstrates the formation of large fragments of DNA in preapoptotic thymocytes at an early but committed stage of apoptosis. Their formation clearly precedes internucleosomal cleavage of DNA and it is from these large fragments that the classical DNA ladders of apoptosis are derived. The well documented dependence of glucocorticoid-induced apoptosis on de novo protein synthesis may be related to the formation of these large fragments, whose appearance accompanies the early morphological changes of apoptosis. It is likely that the initial steps in the induction of apoptosis may differ in different cell types or even in the same cell depending upon the initiating stimulus and there is convergence to a small number of common pathways only in the later stages of apoptosis.

CHAPTER 3

DEXAMETHASONE AND ETOPOSIDE INDUCE APOPTOSIS IN RAT THYMOCYTES FROM DIFFERENT PHASES OF THE CELL CYCLE.

INTRODUCTION

In the developing thymus, during the process of negative selection >90% of immature thymocytes die by apoptosis (Cohen et al. 1992). Diverse stimuli, such as antibodies to the T-cell receptor (Smith et al. 1989), ionising radiation (Sellins and Cohen 1987), glucocorticoids (e.g. dexamethasone) (Wyllie 1980) and DNA topoisomerase II inhibitors (e.g. etoposide) (Walker et al. 1991; Sun et al. 1992) induce apoptosis in thymocytes. Although the majority of cells in the thymocyte are quiescent (Cohen 1991), there is also a population of proliferating cells. Whether these populations showed a differential sensitivity to the induction of apoptosis by dexamethasone and etoposide was investigated.

Thymocytes separated on a discontinuous Percoll gradient (Wyllie and Morris 1982) yield four fractions (F1 to F4)(Wyllie and Morris 1982; Cohen et.al. 1993; Sun et.al. 1994). F1 contains proliferative cells; the majority of cells in F2 are quiescent immature T-lymphocytes; F3 contains preapoptotic cells i.e. cells at an early stage of apoptosis. These cells are defined as early apoptotic because they are smaller in size and of higher density than the normal cells in F1 and F2. They exhibit early ultrastructural alterations of apoptosis i.e. the condensation of chromatin abutting the nuclear membane but they do not yet exhibit internucleosomal cleavage of DNA. In contrast cells in F4 contain fully apoptotic cells based on characteristic ultrastructure of apoptotic thymocytes and also internucleosomal cleavage of DNA (Cohen et al. 1993; Sun et al. 1994).

Thymocytes were pulse labelled with 5'Bromo-2'-deoxyuridine (BrdU) prior to drug treatment. At different times after pulse labelling cells were fixed. BrdU incorporation and DNA content were measured by flow cytometry to study the effect of the drugs on progression through the cell cycle. Cells were also fractionated on Percoll gradients and flow cytometry used to reveal the phase of the cell cycle from which the cells became apoptotic.

The data demonstrated that dexamethasone and etoposide induced apoptosis in cells from different populations. Etoposide activated apoptosis in proliferating cells whereas dexamethasone induced apoptosis only in quiescent cells.

MATERIALS AND METHODS

Materials

BrdU and goat anti-rat fluorescein conjugate were from Sigma Chemical Company (Poole, England). Anti-BrdU antibody (supernatant, ICR2 available commercially from Sera Labs Ltd., Crawley Down, England) was a gift from Dr. C.J. Dean, Institute of Cancer Research, Sutton, England.

Thymocyte incubations

Thymocytes were isolated from male F344 rats and incubations of unfixed cells were carried out as described in chapter 2. Thymocytes (2 x 10^7 cells/ml) were pulse labelled with BrdU (10μM) for 30 min and then washed twice (200g for 10 min). The cells were resuspended at 2 x 10^7 cells/ml and incubated for up to 8 hours either alone (control), or with dexamethasone (0.1μM) or etoposide (10μM). In some experiments, cells were treated as described above and at 3 hours separated into four populations by centrifugation on a discontinuous Percoll gradient as previously described (Chapter 2). The number of cells in each fraction was determined using a Coulter Counter (Coulter Electronics, Luton U.K.).

Flow cytometry

Cells were analysed on an Ortho Cytofluorograf 50H equipped with an Ortho 2150 computer system and a Coherent argon-ion laser producing 500 mW at 488 nm. Data were transferred to an IBM-PC computer and the results analysed and displayed using software written by Mike Ormerod of the Institute of Cancer Research, Sutton, England.

Cell cycle progression

Following treatment, cells (5 x 10^6) were fixed in ice cold 70% ethanol and stained to label the incorporated BrdU and the DNA (McNally and Wilson 1990). Briefly cells were treated with 2M HCl for 27 min at 37°C to produce single stranded DNA, washed and resuspended in 200 μl labelling buffer (PBS with 0.5% Tween-20 and 10% FBS). Rat anti-BrdU monoclonal antibody (10μl) was added and the cells incubated at 4°C for 1 hour. The cells were washed at least twice and then incubated in labelling buffer (200 μl) with goat anti-rat IgG fluorescein conjugate (10 μl) at 4°C for 1 hour. Finally, the cells were washed twice and resuspended in ice-cold PBS containing propidium iodide (PI) (20 μg/ml).

In the flow cytometer, fluorochromes were excited at 488 nm and red (PI-DNA - >630 nm) and green (fluorescein-BrdU - 520 nm) fluorescence recorded. After having gated on a bivariate histogram (cytogram) of the peak versus integrated area of the red fluorescence signal to remove debris and clumped nuclei from the analysis (Ormerod 1990), a cytogram of

green versus red fluorescence was displayed. The labelling index was expressed as the percentage of thymocytes which had taken up BrdU and was estimated from this cytogram.

RESULTS

Induction of apoptosis

Both dexamethasone (0.1μM) and etoposide (10μM) induced apoptosis in thymocytes as assessed by agarose gel electrophoresis and flow cytometry in agreement with previous studies (Wyllie 1980; Walker et al. 1991; Sun et al. 1992) (data not shown).

Cell cycle progression in unfractionated cells

Freshly isolated cells were incubated with BrdU (10 μM) for 30 min, washed and then incubated in normal medium with or without dexamethasone (0.1 μM) or etoposide (10 μM). Cells were harvested at different times, fixed and labelled with anti-BrdU and PI for flow cytometry as described in Materials and Methods. Immediately after incubation with BrdU, labelled cells were distributed throughout S phase with a labelling index of approximately 20% (Fig.1a). Controls in the absence of antibody to BrdU (Fig.1b) or in the absence of BrdU (Fig.1c) showed no non-specific labelling of the cells. In control cultures after 3 h many of the labelled cells had progressed through to late S and G_2/M phases (compare Figs 1a and 2a). After 8 h, most of the labelled thymocytes had divided and were now in G_1 (Fig. 2d).

The addition of dexamethasone, immediately after labelling the cells with BrdU, had little effect on cell cycle progression (Fig. 2b and 2e). In contrast, the presence of etoposide (10 μM) prevented the movement of labelled cells through S phase (Fig. 2c and 2f).

In all the cytograms, cells were observed with a DNA content less than those in G_0/G_1 (arrowed in Fig. 2). Such cells were most evident at later times and after drug treatment (Fig. 2). These cells are probably apoptotic as they are equivalent to the cells in the hypodiploid peak in other commonly used methods to assess apoptosis (reveiwed in Darzynkiewicz et al. 1992)

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Figure 1. Thymocytes were pulse-labelled with BrdU, incubated at 37°C, fixed and incubated with rat anti-BrdU followed by FITC-anti-rat Ig; PI was added to label DNA. Cytograms of green (BrdU) versus red (PI) fluorescence were produced as described in Materials and Methods. a: Untreated cells at time 0. b: BrdU-labelled cells stained with the second antibody alone. c: cells not labelled with BrdU, but stained with both primary and secondary antibodies.
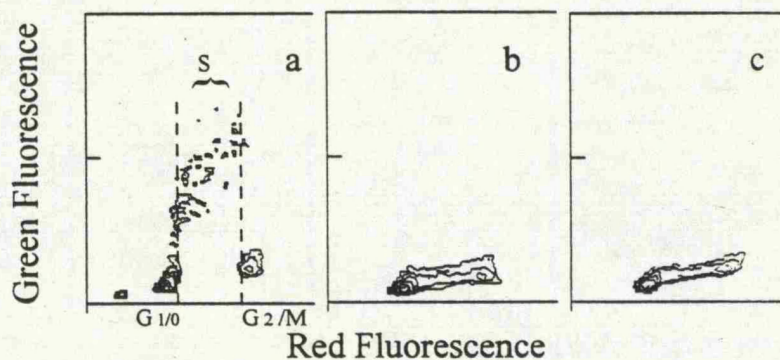
Figure 2. Effects of dexamethasone and etoposide on cell cycle progression. Thymocytes were pulse labelled with BrdU for 30 minutes and then incubated for a further 3 (Figs. a,b and c) or 8 hours (Figs. d,e and f) either alone (Figs. a & d) or with dexamethasone (0.1μM) (Figs. b & e) or etoposide (10μM) (Figs. c & f).
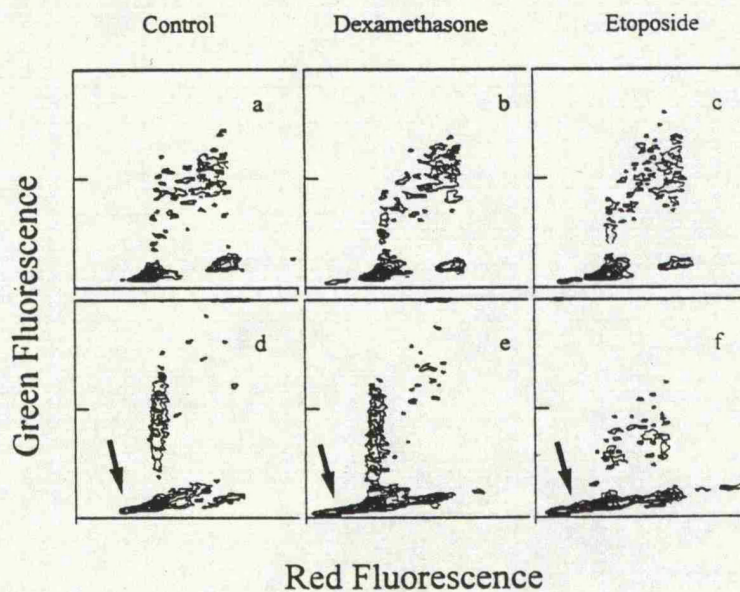
Normal and apoptotic thymocytes fractionated on discontinuous Percoll gradients.

Thymocytes were incubated for 3 h either alone, with dexamethasone (0.1μM) or with etoposide (10μM) and then separated on discontinuous Percoll gradients. After etoposide treatment, the percentage of cells in F1 was significantly less than in control cells. In contrast, the percentage of cells in F2 was decreased following dexamethasone treatment but not following treatment with etoposide (Table 1). These data suggested that, whilst both treatments induced apoptosis, quiescent cells from F2 were more sensitive to dexamethasone whereas the proliferative cells from F1 were more sensitive to etoposide. The percentage of cells in F3 (pre-apoptotic) and F4 (apoptotic) following either dexamethasone or etoposide treatment was greater than in control cells.

Treatment	F1	F2	F3	F4
Control	25±2	63±3	6±2	5±1
Dexamethasone	19±3	49±3*	15±3*	16±1*
Etoposide	14±2*	62±2	13±2*	11±1*

TABLE 1. **Percentage of cells in fractions F1 to F4 following 3 hours incubation and fractionation on Percoll gradients** Thymocytes were incubated either alone or with dexamethasone (0.1μM) or etoposide (10μM) for 3 hours and then fractionated on Percoll gradients as described in Materials and Methods. The number of cells in each fraction was found using a Coulter Counter and expressed as a percentage of the total number of cells from each treatment. The results are expressed as the mean±SEM of 6 experiments. The results were compared to their corresponding controls using a paired t-test and determined to be statistically different if p≤0.05 ( ).

In further experiments, thymocytes were pulse labelled with BrdU and incubated for 3 hours without drug or with either dexamethasone or etoposide and then separated on Percoll gradients. The flow cytometric data from control cells showed that the cells actively synthesising DNA (in S phase) were in the least dense fraction, F1. The cells in this fraction had a labelling index of approximately 50% compared to an index of approximately 15% for those in F2 (Table 2 and Fig. 3a and 3b). There was an absence of unlabelled cells in G_2/M showing that cells initially in G_2/M at the time of labelling 3 h earlier had since divided and moved into G_1. There was an accumulation of labelled cells in G_2/M and some labelled cells had moved into G_1 (Fig. 3a). The majority of the cells in F2 were unlabelled in G_0/G_1 of the cell cycle (Fig. 3b). Cells in F3 and F4 were predominantly unlabelled and had arisen from cells in the G_1 and G_2 phases (Fig. 3c and 3d and Table 2). These results suggested that it was quiescent cells which underwent spontaneous apoptosis.

Figure 3. The effect of dexamethasone and etoposide on the pattern of BrdU labelling in different subpopulations of thymocytes. Thymocytes were pulse labelled with BrdU as described in Materials and Methods and incubated for 3 hours, either alone (a-d) , with dexamethasone ($0.1\mu M$)(e-h) or with etoposide ($10\mu M$)(i-l). The cells were then separated on Percoll gradients into 4 fractions of increasing density, F1(Figs. a,e & i), F2(Figs. b,f & j), F3(Figs. c,g & k) and F4(Figs. d,h & l) The four fractions were fixed and incubated with rat anti-BrdU followed by FITC-anti-rat Ig; PI added to label DNA. Cytograms are of green (BrdU) versus red (PI) fluorescence. In this figure, low numbers of labelled cells may not be revealed by the contour plot.

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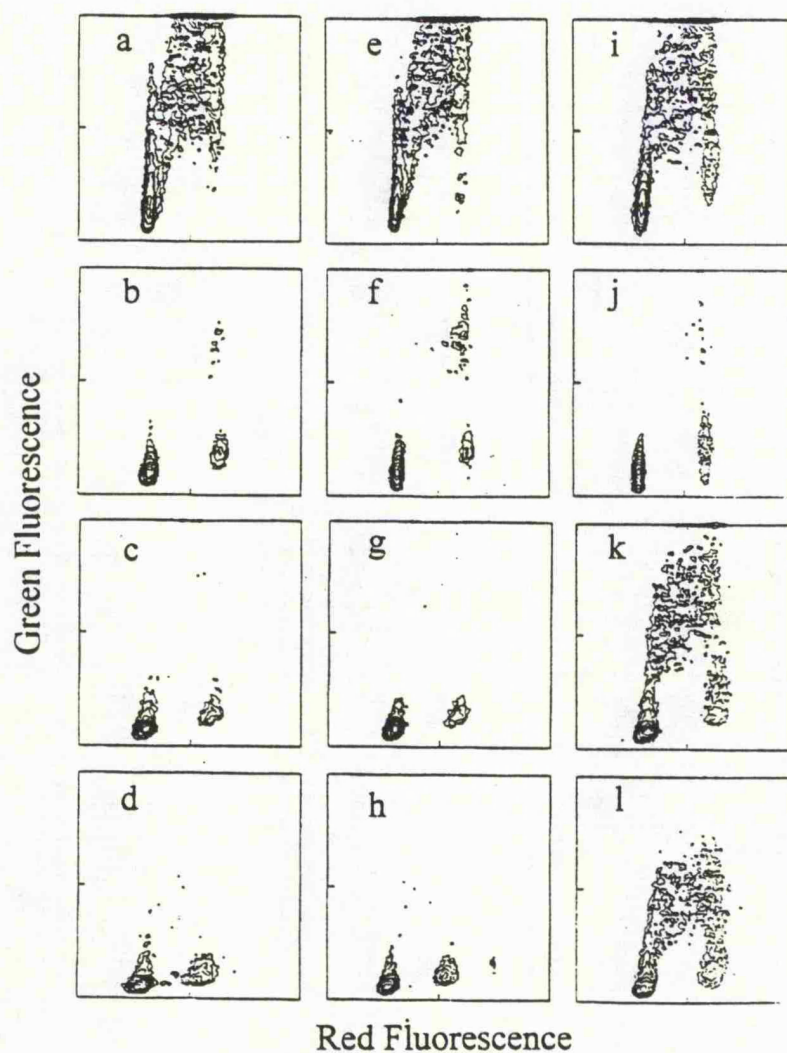
Treatment	unfractionated	F1	F2	F3	F4
Control	25±3	52±7	15±5	5±1	4±1
Dexamethasone	19±1	53±3	16±5	8±1	5±1
Etoposide	20±3	39±9*	17±7	29±3*	25±2*

TABLE 2. Labelling indices of thymocytes incubated for three hours and then fractionated on Percoll gradients. Thymocytes were incubated either alone or with dexamethasone ($0.1\mu M$) or etoposide ($10\mu M$) for 3 hours and fractionated on Percoll gradients. The labelling indices of cells in the different fractions were determined as described in Materials and Methods. The results are expressed as the mean±SEM of 3 experiments. The results were compared using a paired t-test and determined to be statistically different if $p \leq 0.05$ (*).

Cells treated with dexamethasone (Fig. 3e-h) showed a pattern of labelling similar to that of control cells. The cells in F1 had continued to cycle (Fig. 3e) and the majority of cells in F2 were unlabelled in G_0/G_1 (Table 2 and Fig. 3f). The cells in F3 (preapoptotic) and F4 (apoptotic) had also arisen from G_0/G_1 and G_2 (Fig. 3g, 3h and Table 2).

Marked differences were observed following treatment with etoposide. The drug stopped the cells in F1 cycling - there was no movement of the labelled cells through the cycle and the unlabelled cells in G_2/M remained in that phase of the cycle (Figure 3i). Of particular interest was the observation that in cells from both F3 and F4, there were labelled cells in addition to cells from G_1 and G_2 demonstrating that apoptosis had occurred from all phases of the cell cycle (Fig. 3k, 3l and Table 2).

Comparison of the intensity of green (BrdU) fluorescence of etoposide treated cells in F1, F3 and F4 (Fig. 3i, 3k and 3l) revealed a progressive decrease through these fractions.

DISCUSSION

Previous work carried out in this laboratory has shown that both dexamethasone and etoposide induce a time dependent increase in apoptosis (Sun et.al. 1994, Cohen et.al. 1993). These two drugs have different modes of action, and experiments with p53 knockout mice suggestred that the mesechanism of induction of apoptosis was different (Clarke et al. 1993, Lowe et al. 1993). Experiments on untreated thymocytes demonstrated that cells in the proliferative fraction continued to cycle over a period of 8 hours and that cells became apoptotic predominantly from G_0/G_1 and G_2 phases of the cell cycle, not from S phase (Fig. 3a-d). These data indicate that the quiescent, not the proliferating, cells undergo spontaneous apoptosis in culture. This behaviour reflects the fate of quiescent immature (CD4$^+$/CD8$^+$) thymocytes *in vivo*, the majority of which are removed to ensure the development of a competent immune system (Smith et al. 1989, Shi et al. 1989)

Dexamethasone did not affect the cell cycle.
Treatment with dexamethasone did not affect the movement of the proliferative cells through the cell cycle (Fig. 2). The pattern of apoptosis also followed that of the untreated cells (Fig. 3). That apoptosis occurred from the quiescent population was supported by the observation that the number of cells in F2 had decreased over 3 hours (Table 1). The physiological role of glucocorticoids in the survival of thymocytes could explain why dexamethasone appeared to mimic the pattern of spontaneous apoptosis seen in control cultures. Recently much attention has been given to the hypothesis that apoptosis is a result of an aberrant mitosis (Rubin et al. 1993; Shi et al. 1994; Meikrantz et al. 1994) and in one instance cells enter S phase prior to apoptosis (Colombel et al. 1992). If these observations reflect changes within a common pathway of apoptosis, cells in F3 and F4 should display a DNA content greater than that of G_1/G_0. No such increase was observed and therefore entry into an abortive cell cycle (Colombel et al. 1992; Rubin et al. 1993) is unlikely to be a common feature of apoptosis. This issue is dealt with in more detail in the next chapter.

Etoposide induced apoptosis from all phases of the cell cycle.
Etoposide prevented proliferating cells from cycling (Fig. 2f, and Fig. 3i). This observation is consistent with other reports; etoposide and other topoisomerase II inhibitors have been shown to slow movement through S phase and to induce a G_2/M block in cell lines (Kalwalinsky et al. 1983; Zucker and Elstein et al. 1991).
It is clear that etoposide induced apoptosis in proliferating cells (note the reduction in fraction F1 and the increase in F4 after incubation with etoposide, Table 1) and from all phases of the cell cycle including S phase (Fig. 3l). Other topoisomerase II inhibitors (teniposide, fostriecin and m-AMSA) also caused apoptosis from S phase in HL60 cells

(Hotz et al. 1992, Del Bino et al. 1992, Del Bino et al. 1991). The sensitivity of proliferating cells to topoisomerase II inhibitors may be explained both by the importance of this enzyme in DNA replication (Earnshaw and Heck 1990), chromatin condensation (Heck et al. 1988, Hsiang et al. 1988), chromosomal segregation (Earnshaw and Heck 1990). The levels of topoisomerase II are also greater in proliferating than in quiescent cells (Earnshaw and Heck 1990).

The data presented in this chapter apparently contradict the data of Bruno et al. (Bruno et al.1992) who reported that prednisolone (a glucocorticoid), camptothecin (a topoisomerase I inhibitor) and teniposide all triggered apoptosis in rat thymocytes selectively in cells in G_0 of the cell cycle. However, Bruno et al. used unfractionated thymocytes and an acridine orange stain to detect the decrease in DNA content seen in apoptotic cells. The loss of DNA from some of these cells may have been more difficult to detect in their system since the proliferating thymocytes only constitute a minor proportion of the total population (about 25%, see Table 1).

The experiments described here did not unequivocally detect apoptosis of quiescent cells after treatment with etoposide. However, that etoposide induces apoptosis in this population is supported by three lines of evidence. First the progression experiments show etoposide induced apoptosis in F2 cells after 4 h exposure (see previous chapter). Second, after 4 hours incubation with etoposide, the percentage of apoptotic cells in F4 is greater then the labelling index of unfractionated thymocytes (Table 2). Thirdly, at higher concentrations and longer times of incubation, etoposide induced a level apoptosis which exceeds the percentage of cells found in the proliferating compartment at the start of the incubation (Sun et al. 1992, Clarke et al. 1993). Whether etoposide triggers apoptosis in quiescent cells by reacting with low levels of topoisomerase present (presumably topoisomerase IIβ) or whether it acts on another target remains to be determined.

p53 expression is not required for apoptotic death in either untreated thymocytes in culture (Clarke et al. 1993) or for the induction of apoptosis by dexamethasone (Clarke et al. 1993, Lowe et al. 1993). In contrast the absence of p53 prevented apoptosis in irradiated cells or in etoposide treated cells (Clarke et al. 1993, Lowe et al. 1993). While the mechanism by which p53 participates in the induction of apoptosis is unknown (Lane, 1992) it does have an important role in the regulation of the cell cycle (Kastan et al. 1992, Lane 1992). The data in this chapter may explain, in part, the results observed with the p53 "knockout" mice.

Progression from F3 to F4.

We have recently suggested that cells in F3 represent a population of thymocytes at an early stage of apoptosis. F3 cells can be distinguished from those in both F2 and F4 by a number of criteria including morphology, pattern of DNA fragmentation and size (Cohen et al. 1994). The present study showed that, after treatment with etoposide, a significant number

of cells in both F3 and F4 were labelled with BrdU (Fig. 3k and 3l). Thus these cells have arisen directly from the proliferative cells in F1 (scheme A, see below). The absence of labelled cells in F2 following etoposide treatment shows that F1 cells do not pass through F2 (scheme B). The similarity of the labelling patterns in F3 and F4 supports the suggestion that cells in F3 represent an intermediate population of thymocytes at an early stage of apoptosis.

A.   B.   C.   D.

F 1   F 1   F 1   F 2
                                                       F 2
        F 2   F 2   F 3
                                            F 3
   F 3   F 3   F 4
                                            F 4
  F 4   F 4
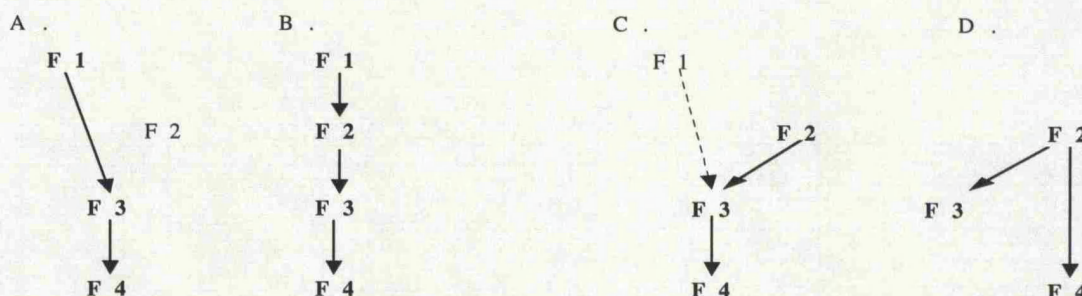
In contrast, after treatment with dexamethasone, the cells in F3 and F4 were unlabelled. The percentages of cells in F3 following dexamethasone treatment support the progression of F2-F3 (Table 1, scheme C). Further, data from the previous chapter showing the progression of DNA fragmentation in F3 cells supports a progression from F2 to F4 via F3 following dexamethasone treatment. However, in the absence of a marker (anaologous to BrdU) that would allow selective labelling of F2, the possiblity that cells from F2 enter F3 but do not susequently progress further and reach F4 directly cannot be ruled out (scheme D). In summary, it has been demonstrated that different populations of thymocytes show differential sensitivity to the induction of apoptosis by dexamethasone and etoposide. In particular, cells in S phase were sensitive to etoposide but not to dexamethasone. This study also provided further support for the existence of a preapoptotic population of thymocytes (F3), which was derived, at least in part, from the proliferating thymocytes (F1).

CHAPTER 4

CDC2 ACTIVATION IS NOT PART OF A COMMON APOPTOTIC PATHWAY.

(work carried out in collaboration with Dr. C. Norbury)

INTRODUCTION

Apoptosis and mitosis have common features. During mitosis there is chromatin condensation which is also one of the most obvious manifestations of apoptotic cell death (Wyllie et al. 1980). However, during apoptosis individual chromosomes are not seen, unlike mitosis. Recent descriptions of lamina disassembly in apoptosis (Ucker et al. 1992; Lazebnik et al. 1993) support the notion that apoptosis might share a common biochemical event with mitosis (Eastman 1990, Rubin et al. 1993). The observation that apoptotic death can follow arrest in the G2 phase of the cell cycle prompted Eastman (Eastman 1990) to propose an involvement of Cdc2 in apoptotic events. In addition Cdc2 activity is induced by a protease which induces apoptosis (Shi et al. 1994) and cyclin-A dependent kinases are activated during apoptosis in HeLa cells (Meikrantz et al. 1994). Apoptosis also occurs in response to chemotherapeutic agents which perturb the cell cycle (previous chapter, Sun et al. 1992, Evans et al. 1993). This has given rise to the hypothesis that apoptosis requires the mitotic "machinery" to acheive its characteristic morphology and that therefore apoptosis may be considered as an aberrant form of mitosis (Ucker 1992). Further support for the hypothesis comes from the observation that during apoptosis in prostate cells there is re-entry into a defective cell cycle (Colombel et al. 1992). If correct, apoptotic death of quiescent cells must also utilise the mitotic "machinery".

The highly conserved cyclin-dependent protein kinase p34^{cdc2} (Cdc2) is responsible for bringing about the onset of mitosis in eukaryotic cells (Nurse 1990). Substrates of Cdc2 include the nuclear lamins and histone H1 (Norbury and Nurse 1992). Phosphorylation of these proteins is thought to be responsible, in part at least, for the initiation of the mitotic processes of nuclear lamina disassembly and chromatin condensation, respectively (Gerace and Blobel 1980, Marsden and Laemmli 1979). Primary rat thymocytes provide both one of the best characterised model systems for studying apoptosis as well as a convenient system in which to examine apoptotic death from G_0. As previously described (Wyllie et al. 1982, previous chapter) F1 and F2 comprise normal proliferating cells and quiescent thymocytes respectively. It would be surprising if the universal mitotic regulator Cdc2 was involved in death from a quiescent (G_0) state, as the expression of Cdc2 is generally restricted to proliferating cells (Lee et al. 1988, Furukawa et al. 1990). In addition etoposide inhibits cdc2 activation in chinese hamster ovary cells (Lock and Ross 1990a; Lock and Ross 1990b), an effect hard to reconcile with its induction of thymocyte apoptosis if mitotic machinery is involved. The following work attempted to substantiate or invalidate the aberrant mitosis hypothesis. To acheive this Cdc2 activity in primary rat thymocytes was examined following treatment with dexamethasone and etoposide. The effect of a known

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inhibitor of thymocyte apoptosis, Nα-tosyl-lysinyl chloromethyl ketone (TLCK) on Cdc2 activity was also assessed.

MATERIALS AND METHODS

Thymocyte incubations and Fractionation

Thymocytes were prepared and incubated as described in Chapter 2. In initial experiments, thymocytes were incubated for 1 h at 20 x 10^6 cells/ml in RPMI 1640 containing 10% foetal calf serum (Gibco Ltd., Paisley, Scotland) either alone or with etoposide (10μM). They were then separated on a discontinuous Percoll gradient into four fractions (F1 - F4) (13) and assayed for Cdc2 kinase activity. F1 and F2 mainly comprise normal proliferatively competent and quiescent thymocytes respectively; cells in F4 are apoptotic and those in F3 represent a transient population of preapoptotic cells, at an early stage of the apoptotic process. In subsequent experiments, thymocytes were isolated and immediately separated into four subpopulations (F1-F4). Quiescent thymocytes (F2) were then further incubated for 4 h either alone, with etoposide (10μM) or with dexamethasone (0.1μM). In some experiments, the serine protease inhibitor Nα-tosyl-L-lysinylchloromethyl ketone (TLCK) (50μM) (Boehringer Mannheim, Mannheim, Germany) was included in the incubation.

Cdc2 Kinase Activity (carried out by Dr C. Norbury)

Cdc2 protein (histone H1) kinase activity was measured after immunoprecipitation of thymocyte lysates using polyclonal antiserum raised against the C-terminus of mammalian Cdc2 as described (Norbury et al. 1991). The thymocyte lysates were normalized for total protein content before precipitation, using the BCA method (Pierce). Quantitation was performed by densitometric scanning of autoradiographs or by direct scintillation counting of [^{32}P] histone H1.

Quantitation of apoptotic cells by flow cytometry

Apoptosis was assessed by a flow cytometric method, which separates and quantifies viable normal and apoptotic thymocytes (Sun et al. 1992). The cells exhibiting high blue fluorescence with Hoechst 33342 have been shown to be apoptotic based on a number of criteria including ultrastructure and that the DNA shows extensive internucleosomal cleavage (Sun et al. 1992)

Percentage of cells in S and G_2/M phases of the cell cycle and the etoposide induced appearance of hypodiploid cells

Thymocytes (2 x 10^6) were fixed (ice cold 70% EtOH, 60 min), washed (600g, 5 min) and resupended in ice cold PBS. The cells were then stained with propidium iodide (5μg/ml) for 48 h after which time they were analysed on a Becton Dickinson FACScan flow cytometer. Cells were assigned to phases of the cell cycle as previously described (Chapter 3).

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to detect DNA laddering in whole cells (2 x 10^6 cells) as previously described (Chapter 2).

RESULTS

Cdc2 activity is not increased in apoptotic populations of thymocytes.

Isolated thymocytes were incubated for 1 h either alone or with etoposide ($10\mu M$) and then separated using Percoll gradient centrifugation into four fractions (F1-F4) (Cohen et al. 1993). Cdc2 kinase activity was measured in these fractions (Fig. 1).
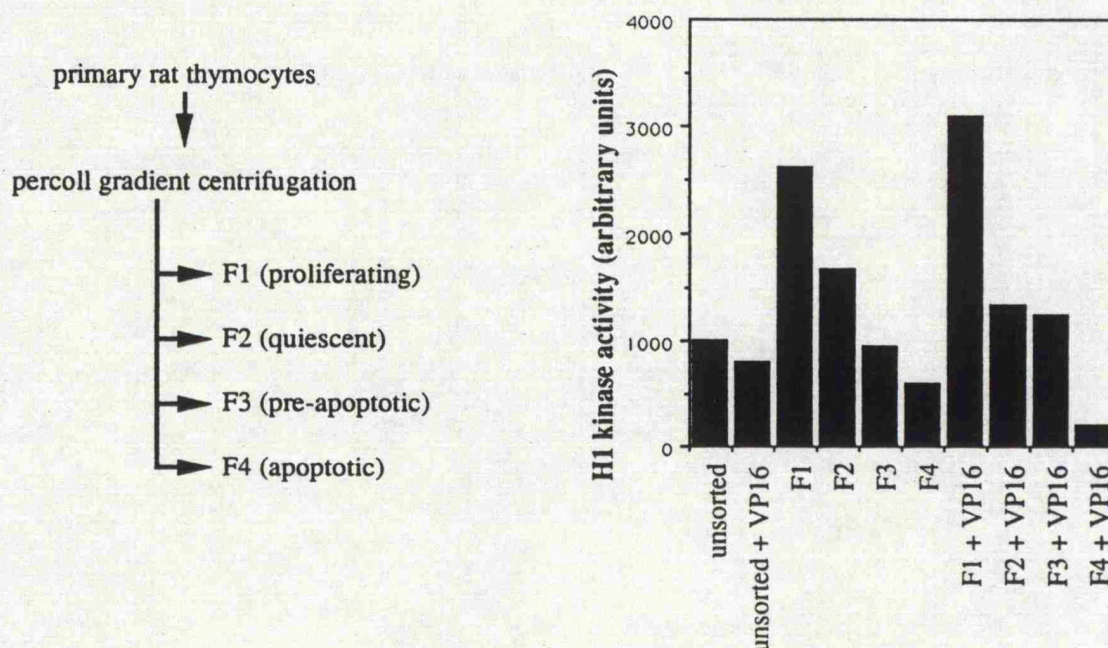
Figure 1. Cdc2 kinase activity in normal, preapoptotic and apoptotic thymocytes.
Thymocytes were isolated, and incubated for 1 h either alone or with etoposide ($10\mu M$) before separation on discontinuous Percoll gradients into four fractions (F1-F4) as shown. The Cdc2 kinase activity of immunoprecipitates prepared from the various populations was assessed as described in Materials and Methods. Cdc2 kinase activity in unfractionated cells was not significantly altered by 1 h etoposide (VP-16) treatment. Subsequent separation of thymocytes from both control and etoposide treated cultures into four subpopulations on discontinuous Percoll gradients showed that the greatest kinase activity was in F1 (proliferating cells) and that there was decreased kinase activity in F4 (apoptotic cells)

F2 cells contained reduced Cdc2 protein kinase activity compared with F1, in line with previous findings in T-lymphocytes, postmitotic neurons and fibroblasts, where Cdc2 activity and gene expression is reduced to very low or undetectable levels as cells become quiescent (Lee et al. 1988, Furukawa et al. 1990, Freeman et al. 1994). Cdc2 kinase levels were further reduced in F3 and F4. This progressive decrease in Cdc2 kinase activity from F1 to F4 was also seen when thymocytes were incubated for 1 hour before fractionation, with the DNA topoisomerase II inhibitor etoposide (VP-16) (Fig. 1), a treatment that on further incubation increases the proportion of apoptotic cells. Thus thymocyte populations enriched for cells engaged in the active phase of apoptosis do not contain elevated levels of Cdc2 activity.

Cdc2 activity in quiescent cells was not increased following exposure to dexamethasone or etoposide

To minimize the contribution of Cdc2 from proliferating cells, quiescent (F2) thymocytes were isolated and incubated either alone, or with etoposide or dexamethasone, agents commonly used to induce apoptosis in thymocytes (Wyllie 1980, Walker et al. 1991). Apoptotic cells were clearly detected by 2 hours (Fig. 2). Both agents caused a time-dependent induction of apoptosis in comparison with control cells (Fig. 2).
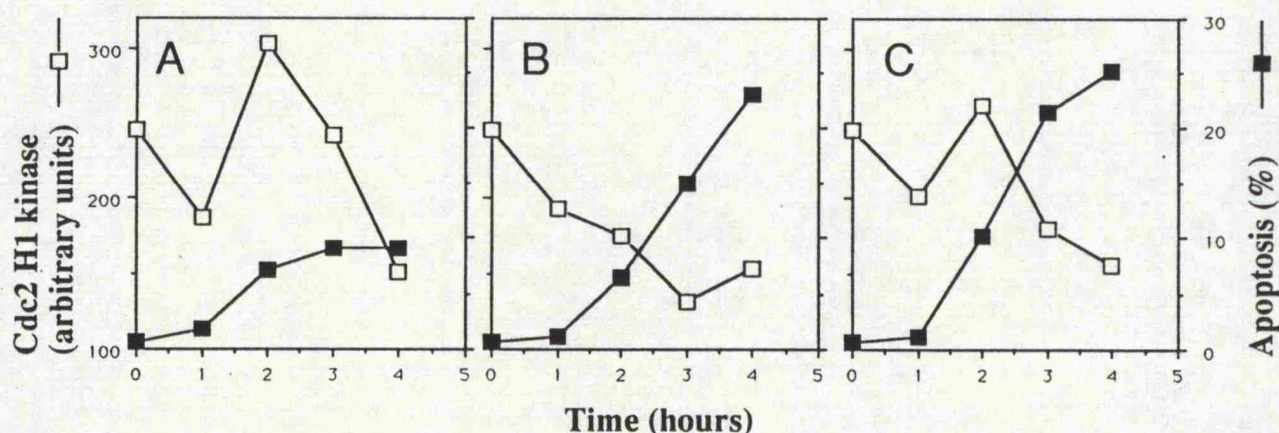
Figure 2. No correlation between Cdc2 kinase activity and the incidence of apoptosis in quiescent thymocytes (F2). Quiescent thymocytes (F2) were then further incubated for 4 h either alone, with etoposide (10 μM) or with dexamethasone (0.1μM). The level of Cdc2 kinase activity ($\square$—$\square$) and attendent apoptosis ($\blacksquare$—$\blacksquare$) in control (a), etoposide (b) and dexamethasone (c) treated quiescent thymocytes were assessed. In control and dexamethasone treated cultures (a & c) there was a modest increase in Cdc2 kinase activity at 2 h, after which there was a progressive decrease. In contrast Cdc2 kinase activity fell in the presence of etoposide (b). Apoptotic cells detected by flow cytometry were clearly observed in control cells (a) after 2h. Both etoposide (b) and dexamethasone (c) caused a clear induction of apoptosis. The results shown are from one experiment typical of three.

Cdc2 kinase activity in lysates from etoposide-treated cells showed a progressive decline inconsistent with a role for this kinase in the onset of apoptosis. In control and dexamethasone-treated cells there was a modest increase in Cdc2 kinase activity after 2 hours of culture (Fig. 2), coincident with the appearance of apoptotic cells. Two lines of evidence suggest that this small peak of Cdc2 activity derives from a minor ($\simeq$ 6%) sub-population of proliferating cells in the F2 fraction, rather than from the quiescent/apoptotic majority. Firstly, treatment of F2 cells with dexamethasone after fractionation substantially increased the level of apoptosis without significantly altering the profile of Cdc2 activity (Fig. 2c). In addition the peak of Cdc2 activity occurs when most of the cycling cells are in G_2/M (Fig. 3a). Secondly, treatment with etoposide, an agent known to block proliferating cells in G_2 (Henwood and Brigden 1990, Lock and Ross 1990), eliminated the peak of precipitable Cdc2 activity at two hours (Fig. 2b) and the entry of cycling cells into G2/M (Fig. 3b).
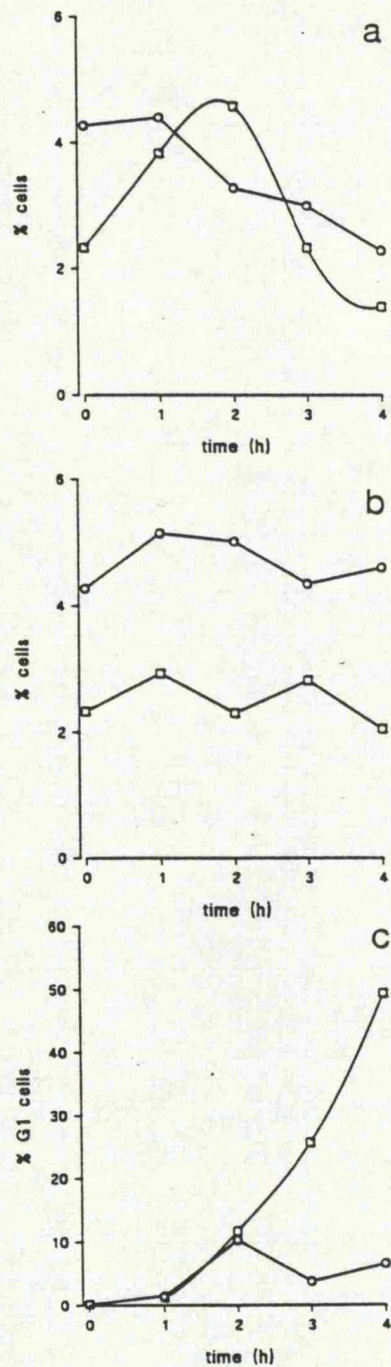
Figure 3. Cdc2 kinase activity peaks at 2 h and correlates with the number of cells in G_2/M phase. Quiescent thymocytes were isolated and incubated either alone (a) or with etoposide (10μM) (b) for up to 4 h. Cells were stained with propidium iodide and the percentage of cells in either S phase (O—O) or G_2/M (□—□) phase of the cell cycle was assessed by flow cytometry. In control cultures the percentage of cells in G_2/M peaked at 2 h and then fell whereas the percentage of cells in S phase fell progressively(a). In contrast the percentage of cells in both S and G_2/M phases remained constant following etoposide treatment (b). Flow cytometry was also used to determine the percentage of cells which possessed hypodiploid DNA (c).

Etoposide (□—□) caused a time dependent increase in the percentage of hypodiploid cells compared to control cells (O—O). The results are from one experiment.

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TLCK inihibited apoptosis but enhanced Cdc2 activity.

To further investigate the possible involvement of Cdc2 kinase in apoptosis, the serine protease inhibitor, N-tosyl-L-lysiny chloromethyl ketone (TLCK), a known inhibitor of apoptosis (Bruno et al. 1992) was used. The data confirmed and extended this earlier study, and demonstrated that TLCK inhibited dexamethasone- and etoposide-induced apoptosis, as assessed both by flow cytometry (Fig. 4) and agarose gel electrophoresis (Fig. 5). In control and dexamethasone-treated cells, Cdc2 kinase activity was elevated by addition of TLCK (Fig. 4).
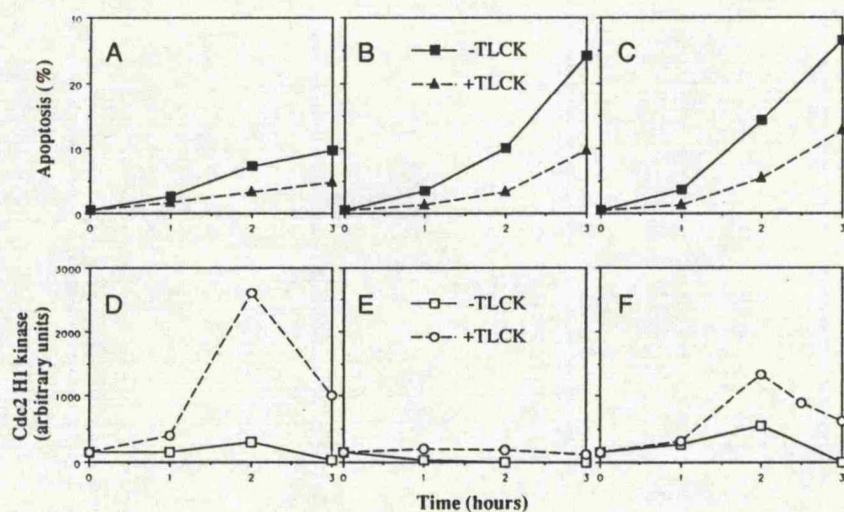
Figure 4. TLCK potentiates Cdc2 kinase activity but blocks apoptosis in quiescent thymocytes (F2). Quiescent thymocytes (F2) were isolated and incubated with dexamethasone (0.1μM) or etoposide (10μM) in the presence or absence of TLCK for up to 3 h. The effects of TLCK on the incidence of apoptosis (a,b & c) and on Cdc2 kinase activity (d,e & f) were assessed. Cells were incubated either alone (a & d), with etoposide (b & e) or with dexamethasone (c & f) to induce apoptosis in the presence (Δ) or absence (■) of TLCK (50 μM). TLCK inhibited the basal level of apoptosis (a) and that induced by either etoposide (b) or dexamethasone (c). In control (d) and dexamethasone (f) treated cultures, there was a modest increase in Cdc2 kinase activity at 2 h after which there was a decrease. TLCK both potentiated the modest increase in Cdc2 kinase activity observed at 2 h in control (d) and dexamethasone (f) treated cultures and inhibited the decrease observed in the presence of etoposide. The results shown are from one experiment typical of three.

Figure 5. TLCK inhibits internucleosomal cleavage of DNA in quiescent (F2) thymocytes. Quiescent thymocytes (F2), were incubated either alone (lane 2), with dexamethasone (0.1μM) (lane 3) or etoposide (10μM) (lane 5) for 4h. Total DNA from 2×10^6 cells was then run on an agarose gel to detect internucleosomal cleavage of DNA (16). The numbers at the left hand side of the figure represent the size of the standards in base pairs (bp). Spontaneous apoptosis resulted in detectable internucleosomal cleavage of DNA (lane 2), which was significantly increased by both dexamethasone and etoposide (lanes 3 & 5 respectively). Coincubation of dexamethasone- and etoposide- treated cells with TLCK (50μM) resulted in a marked inhibition of internucleosomal cleavage of DNA (lanes 4 & 6 respectively). This result was in good agreement with the effects of inhibition by TLCK of dexamethasone- and etoposide- induced apoptosis as detected by flow cytometry (Fig. 3). Lane 1 contains control quiescent cells at zero time.
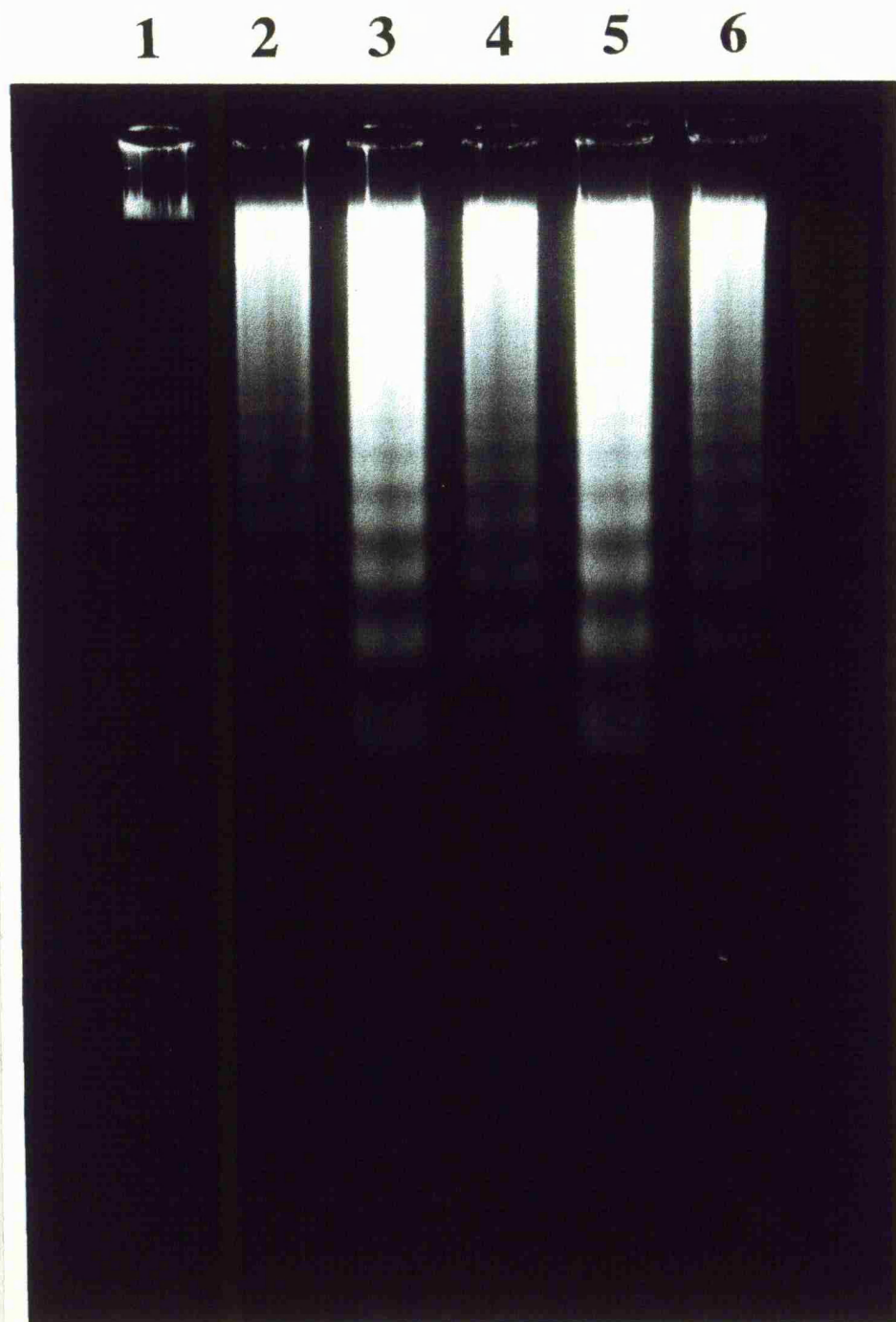
DISCUSSION

These results contrast with those recently published by Shi *et al.*, who describe Cdc2 activation during cell death induced by a cytotoxic T-lymphocyte (CTL) granule serine protease, and reduced sensitivity to apoptotic stimuli of cells with reduced Cdc2 activity. This may be due to the enhanced sensitivity of proliferating cells to CTL-mediated death (Nishioka and Welsh 1994). These authors propose that apoptotic chromatin condensation and lamina disassembly are brought about by the same Cdc2-mediated changes that occur in mitosis. The data shows that quiescent thymocytes that are highly susceptible to apoptotic stimuli lack significant Cdc2 activity. In addition there was no correlation between the induction of apoptosis by dexamethasone or etoposide and Cdc2 activity.

TLCK, while a potent inhibitor of apoptosis alone increased Cdc2 activity. This is in line with the previously documented effect of TLCK addition to surf clam egg extracts, in which the protease inhibitor stabilizes active Cdc2/cyclin kinase complexes by blocking cyclin degradation (Luca and Ruderman 1989). Coincubation of TLCK with etoposide did not give rise to an elevated level of Cdc2 activity (Fig 4e), presumably because the minor proliferating sub-population was arrested in G2. These results further substantiate the conclusions that the Cdc2 activity measured in the F2 population is due to residual proliferating cells, and that there is no significant Cdc2 activity attributable to F2 cells that are engaged in transit from quiescence to apoptosis.

The chromatin condensation and lamina disassembly that occur during apoptosis in these cells must be brought about by alternative, Cdc2-independent mechanisms. Precipitation with p13[suc1] beads gave patterns of H1 kinase activity identical to those seen with Cdc2 immunoprecipitates (data not shown). Thus closely-related Cdc2-like kinases such as Cdk2, which are known to be precipitable with p13[suc1], are also excluded from a possible role in apoptosis in thymocytes. It is possible that under some circumstances Cdc2 activity triggers apoptosis, explaining why cells passing through a defective motosis may undergo apoptosis. However the suggstion that mitotic catastrophe is a common feature of T cell death (Penninger and Mak 1994) is not consistent with the data presented here which shows that Cdc2 kinase activation is not a prerequiste for apoptosis and therefore does not lie within a common apoptotic death pathway. Further support for this conclusion comes from the observation that the chromatin condensation and lamin degradation seen during fibroblast apoptosis does not reqire cdc2 kinase activation (Oberhammer et al. 1994).

Other proteins linked to the cell cycle paly a role in apoptosis; p53 regulates the cell cycle, inducing a G_1 arrest following DNA damage (Lane 1992) and is required for apoptosis induced by the DNA damaging agents etoposide and irradiation (Lowe et al. 1993: Clarke et al. 1993). Cyclin D1 associates with cyclin dependent kinases cdk2, 4 and 5 and controls commitment to S phase. The gene *bcl-1*, which encodes cyclin D1 is an oncogene implicated in B-cell lymphoma (Marx 1994) and in addition to its potential oncogenic role, cyclin D1 is increased during programmed cell death in post mitotic neurones (Freeman et al. 1994). Therefore while results in this chapter rule out an involvement for cdc2 and cdk2 in a common apoptotic pathway a central role in apoptosis for other proteins so far only associated with the cell cycle play cannot be excluded.

CHAPTER 5.
THE EFFECTS OF PROTEASE INHIBITORS ON THYMOCYTE APOPTOSIS

INTRODUCTION

There is a growing realisation that apoptosis acts as a counter balance to cell proliferation and is of vital importance for normal development and tissue homeostasis (Arends and Wyllie, 1991). In addition to its physiological role, apoptosis can be induced by a diverse array of pathogenic stimuli including chemicals, radiation and viruses (Arends and Wyllie, 1991; Sellins and Cohen, 1987; Takizawa et al. 1993). These findings have led to increased efforts to identify the genes required for apoptosis and the subsequent biochemical events. Many studies have shown a major role for DNA degradation in apoptosis. More recently, protein degradation has also been implicated in apoptosis in both invertebrate and mammalian experimental systems. In the nematode, *Caenorhabditis elegans*, the gene *ced-3* is essential for apoptosis and encodes a protein similar to a mammalian protease interleukin-1β-converting enzyme (Yuan et al. 1993). Further work has identified a family of *ced-3* related mammalian genes and their over expression of either *ced-3* or ICE in rat fibroblasts results in apoptosis (Yuan et al. 1993; Miura et al., 1993; Kumar et al. 1994; Wang et al. 1994; Fernandes-Alnemri et al. 1994). Thymocytes express *ich-1*$_L$ mRNA which encodes a Ced-3/ICE-like protease, whose over expression in rat fibroblasts induces apoptosis (Wang et al. 1994). Thymocyte apoptosis has also been shown to be accompanied by calcium dependent proteolysis and a role for calpain, a calcium dependent neutral protease, has been suggested (Squier et al., 1994). In addition a range of chemicals known to inhibit proteolysis can prevent internucleosomal DNA cleavage in thymocytes (Bruno et al., 1992). Protease activity is also required for apoptosis in T-cell receptor mediated cell death (Sarin et al. 1993), cytotoxic T cell killing (Shi et al. 1992), TNF mediated cytotoxicity (Ruggiero et al., 1987; Suffys et al. 1988) and for apoptosis in a tumour cell line (Kaufman, 1989). Thus a role for proteolysis appears to be a common feature of apoptosis in a number of different systems.

The induction of apoptosis in thymocytes is one of the best characterised experimental models of apoptosis, both morphologically and biochemically. Briefly, the cell shrinks (Thomas and Bell, 1981) and the chromatin condenses with an attendant cleavage of DNA (Wyllie, 1980). Initially DNA is cleaved to 300-200 and 50-30 kilobasepair size fragments which are then further degraded to produce oligonucleosomes and the typical DNA ladder pattern (Cohen et al., 1994). The size of the kilobase pair fragments may reflect higher order chromatin structure and the accessibility of cleavage sites just as the DNA ladder reflects nucleosome structure (Filipski et al., 1990). Walker et al. proposed that fragments of 50-30 kbp could be a result of cleavage at the base of chromatin loops and that 250-200 kbp fragments represent rosettes of six loops (Walker et al., 1991). Recently it has been

proposed that isolated thymocyte nuclei degrade their DNA in a Ca^{2+}/Mg^{2+} dependent fashion and that the sequence of events are very similar to those seen in intact cells following apoptotic stimuli (Sun and Cohen, 1994). Therefore isolated thymocyte nuclei may serve as a model of apoptotic chromatin degradation.

The aim of the present study was to ascertain the role of proteolysis in the sequence of events leading to apoptosis in thymocytes. To achieve this, the effects of a number of protease inhibitors on apoptosis induced by different agents was assessed. The apoptotic stimuli used included dexamethasone (a synthetic glucocorticoid) (Wyllie, 1980), etoposide (a DNA topoisomerase II inhibitor) (Walker et al., 1991; Sun et al. 1994), thapsigargin (a microsomal Ca^{2+} pump inhibitor) (Jiang et al., 1994) and staurosporine (a kinase inhibitor) (Jacobson et al., 1993) and a variety of techniques (flow cytometry, DNA electrophoresis and cell sizing) used to assess apoptosis. A number of protease inhibitors, including N-tosyl-L-lysinyl chloromethylketone (TLCK), N-tosyl-L-phenylalaninyl chloromethyl ketone (TPCK), benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethyl ketone (z-VAD.FMK) and N-acetyl-leucinyl-leucinyl-norleucinal (ALLN), were used. TLCK and TPCK prevent internucleosomal cleavage of DNA in both thymocytes and a tumour cell line. Both these compounds react with a histidine residue in the active site of serine proteases and irreversibly inhibit proteolytic activity. TLCK inhibits trypsin-like proteases which require a substrate with a basic amino acid in the P1 position and are therefore not inhibited by TPCK. TPCK inhibits chymotrypsin-like proteases which require aromatic amino acids in the P1 position and are therefore not inhibited by TLCK (Powers and Harper, 1986). ICE-like proteases require an aspartate residue in the P1 position (Thornberry et al., 1992) and are therefore inhibited by (z-VAD.FMK). Four other, Asp-containing peptide inhibitors of ICE were also tested; N-acetyl-tyrosinyl-valinyl-alaninyl-aspartyl aldehyde (YVAD.CHO)(Thornberry et al. 1992), N-acetyl-tyrosinyl-valinyl-alaninyl-aspartic acid methyl ester (YVAD.COCH$_3$), benzyloxycarbonyl-valinyl-alaninyl- δ-ethyl ester aspartyl [(2,6-dichlorobenzyl)oxy] methyl ketone (z-VAD.DCOMK) (Thornberry et al. 1994) and N-acetyl-tyrosinyl-valinyl-alaninyl-aspartic acid chloromethyl ketone (YVAD.CMK) (Lazebnik et al. 1994). Finally two cell permeable, reversible inhibitors of calpain, ALLN and benzyloxycarbonyl-valinyl-phenylalaninyl aldehyde (MDL-70,128) were used (Crawford, 1990). The following chapter is divided into two sections, the first describing the effects of TLCK and TPCK and the second describing the effects of both the calpain inhibitors and ICE inhibitors.

MATERIALS AND METHODS

Materials

All media and serum were from Gibco (Paisley U.K.). Pronase, TLCK, TPCK and ALLN
were from Boehringer Mannheim (Mannheim, Germany). Z-VAD.FMK was from Enzyme
Systems, Inc (Dublin, USA). YVAD.CMK was from Bachem (Bubendorf, Switzerland).
YVAD.CHO, YVAD.COCH$_3$ and z-VAD.DCOMK were gifts from Professor Lee Rubin
(Eisai Laboratory, UCL). MDL-70,128 was from Marion Merrel Dow (Cincinnati, USA).
All other chemicals were from Sigma Chemical Company (Poole, U.K.).

Preparation and incubation of thymocyte suspensions

Suspensions of thymocytes from immature male F344 rats [4-5 weeks old, 65-85 g) were
prepared as described previously (chapter 2). To induce apoptosis, thymocytes (2×10^7 cells
ml^{-1}) were incubated at 37^0 for 4 h in RPMI 1640 medium supplemented with 10% foetal
bovine serum with either dexamethasone (0.1μM), etoposide (10μM), staurosporine (1μM)
or thapsigargin (0.05μM). In experiments with protease inhibitors, thymocytes were
incubated for 1 h with each inhibitor prior to treatment with apoptotic stimuli. Thymocytes
were either preincubated with TLCK for 1 h prior to exposure to the apoptotic stimuli or
were cotreated with TLCK or TPCK and dexamethasone or etoposide for 4 h. In
experiments to investigate whether the TLCK-sensitive target was pre-existing or was
synthesised in response to apoptotic stimuli, TLCK (100μM) was incubated without cells for
up to 1 h in culture medium before dilution with a cell suspension to give a final TLCK
concentration of 50μM and a final cell density of 2×10^7 cells ml^{-1}. These cultures were then
further incubated for 4 h with either dexamethasone or etoposide.

Apoptosis assessed by flow cytometry

Thymocytes (1×10^6) were stained with Hoechst 33342 and propidium iodide. The number
of viable apoptotic and normal cells were assessed by flow cytometry (Chapter 2). All
protease inhibitors were used at nontoxic concentrations as assessed by exclusion of
propidium iodide.

DNA analysis and cell size

Cells (5×10^6) were lysed and high molecular weight DNA separated from degraded
chromatin as described previously (Wyllie, 1980). The amounts of DNA in the supernatant
(degraded) and the pellet (high molecular weight) were quantified using the diphenylamine
reaction (Burton, 1956). Conventional and field inversion gel electrophoresis (FIGE) were
carried out as previously described (Chapter 2).

Cell sizing and electron microscopy were carried out as described (Chapter 2).

RESULTS SECTION I
THE EFFECTS OF TLCK AND TPCK

TLCK and TPCK inhibit internucleosomal cleavage of DNA.

Internucleosomal cleavage of DNA detected by agarose gel electrophoresis has often been considered the biochemical hallmark of apoptosis (Arends and Wyllie, 1991; Compton and Cidlowski, 1992; Wyllie, 1980). Control cells show a low level of internucleosomal cleavage consistent with a low level of spontaneous apoptosis. After incubation for 4h, both dexamethasone ($0.1\mu M$) and etoposide ($10\mu M$) induced an increase in DNA laddering (fig.1, lanes 2 and 6 respectively). TPCK ($25\mu M$) and TLCK ($50\mu M$) both inhibited dexamethasone and etoposide induced internucleosomal cleavage of DNA (fig.1, lanes and respectively). TLCK also inhibited internucleosomal cleavage induced by staurosporine ($1\mu M$) and thapsigargin ($0.05\mu M$) (data not shown).

Figure 1. TLCK and TPCK both inhibit internucleosomal cleavage.
Cells ($2x10^6$/well) were loaded and electrophoresis carried out as described in Materials and Methods. Thymocytes were incubated for 4 h either alone (lane 1), with dexamethasone ($0.1\mu M$) alone (lane 2), or in the presence of TPCK ($25\mu M$) (lane 3), or TLCK ($50\mu M$) (lane 4). Thymocytes were also incubated with etoposide ($10\mu M$) alone (lane 5), or in the presence of TPCK ($25\mu M$) (lane 6) or TLCK ($50\mu M$) (lane 7). The distance migrated by 123 basepair marker is indicated.
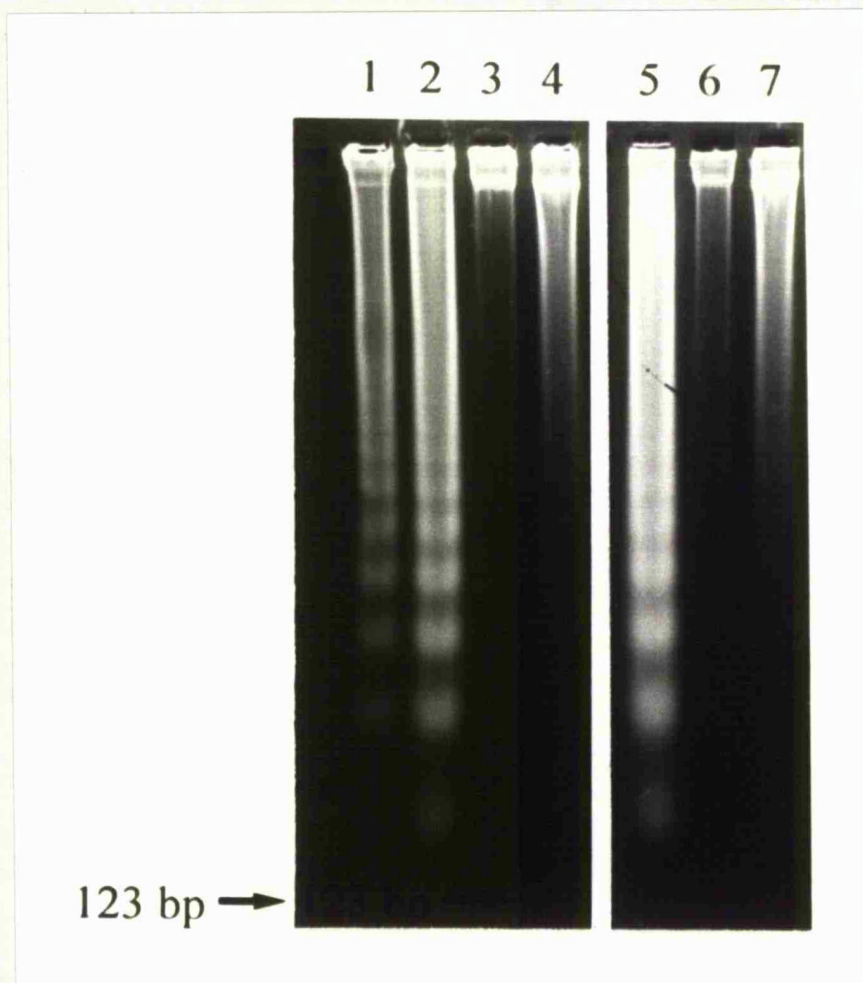
DNA fragmentation is quantifiable by measurement of the percentage of diphenylamine reactive material present in the supernatant fraction obtained after centrifugation of lysed cells. In agreement with previous studies (Wyllie, 1980; Sun et al. 1994), both dexamethasone and etoposide caused an increase in DNA fragmentation (Table 1). These increases were abrogated by both TLCK (50μM) and TPCK (25μM).

Treatment	% DNA fragmentation
Control	6.8 ± 1.6
Dexamethasone	28.0 ± 2.4
Dexamethasone + TLCK	8.6 ± 1.8
Dexamethasone + TPCK	11.0 ± 1.5
Etoposide	39.8 ± 3.6
Etoposide +TLCK	16.3 ± 2.5
Etoposide +TPCK	9.3 ± 1.4
TLCK	2.0 ± 0.8
TPCK	9.4 ± 1.2

TABLE 1. Inhibition of dexamethasone and etoposide induced DNA fragmentation by TLCK and TPCK.
Cells were incubated for 4 h with either dexamethasone (0.1μM) or etoposide (10μM) to induce apoptosis and the ability of TLCK (50μM) and TPCK (25μM) to inhibit DNA fragmentation. The data represent the mean (±S.E.M.) of at least 3 experiments.

TLCK inhibits the appearance of apoptotic morphology.

Thymocytes were incubated for 1 h in the presence of TLCK (50μM) prior to exposure to apoptotic stimuli for 4 h. After this time cells were fixed and examined by electron microscopy. Dexamethasone (0.1μM) (Fig.2a), etoposide (10μM) (Fig.2c) or thapsigargin (0.05μM) (data not shown), all increased the number of cells showing typical apoptotic morphology; chromatin condensed to one pole of the nucleus, nucleolar disintegration, cytoplasmic vacuolation and cell shrinkage compared to control cells (data not shown). TLCK (50μM) inhibited both the cytoplasmic and nuclear changes of apoptosis induced by dexamethasone (Fig. 2b) and etoposide (Fig.2d). While

Figure 2. TLCK prevents the morphological changes of apoptosis

Thymocytes were incubated for 1 h either alone (a and c) or with TLCK (50μM) (b and d) prior to treatment for 4h with dexamethasone (0·1μM) (a and b) or etoposide (10μM) (c and d). Bars represent 2μm.
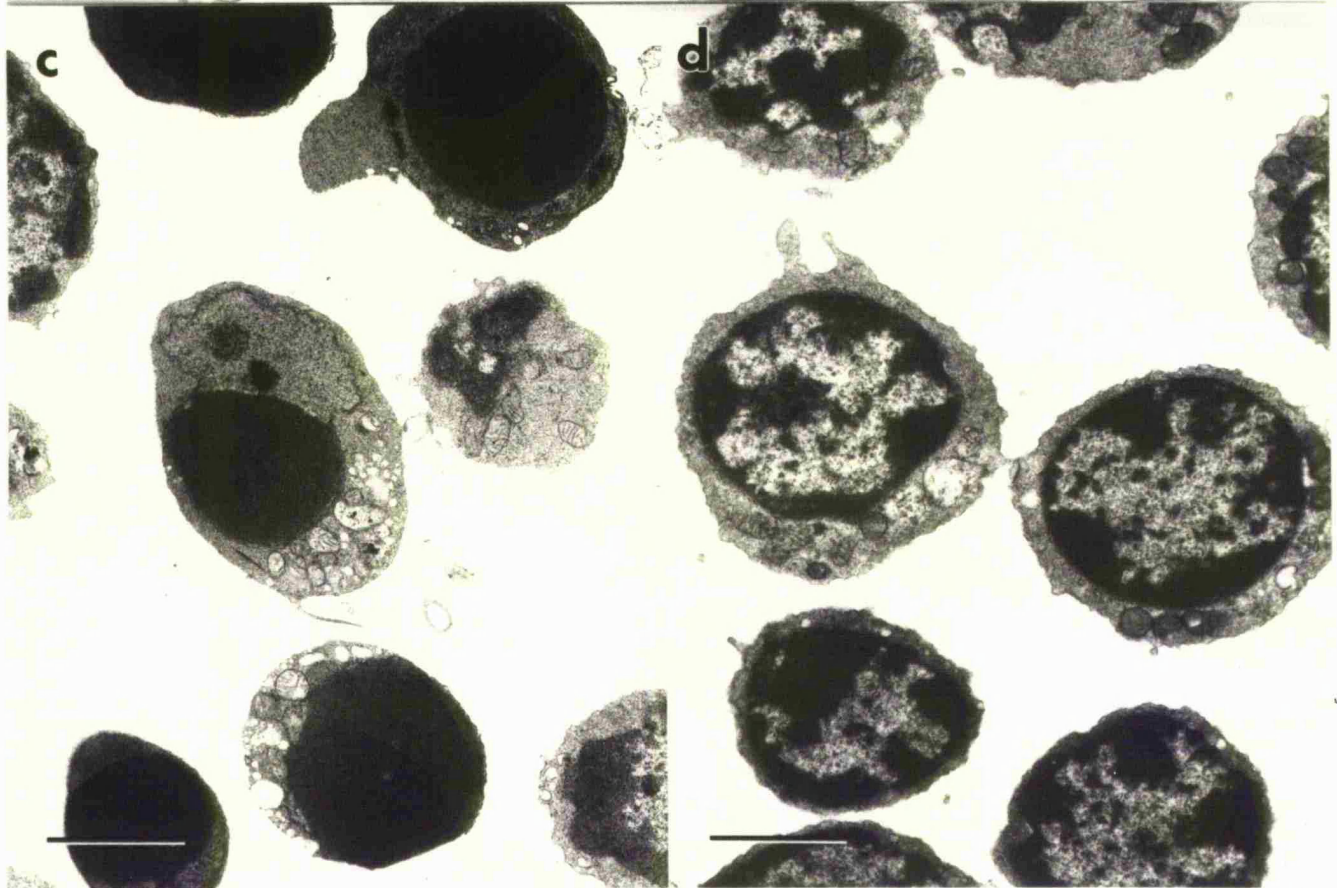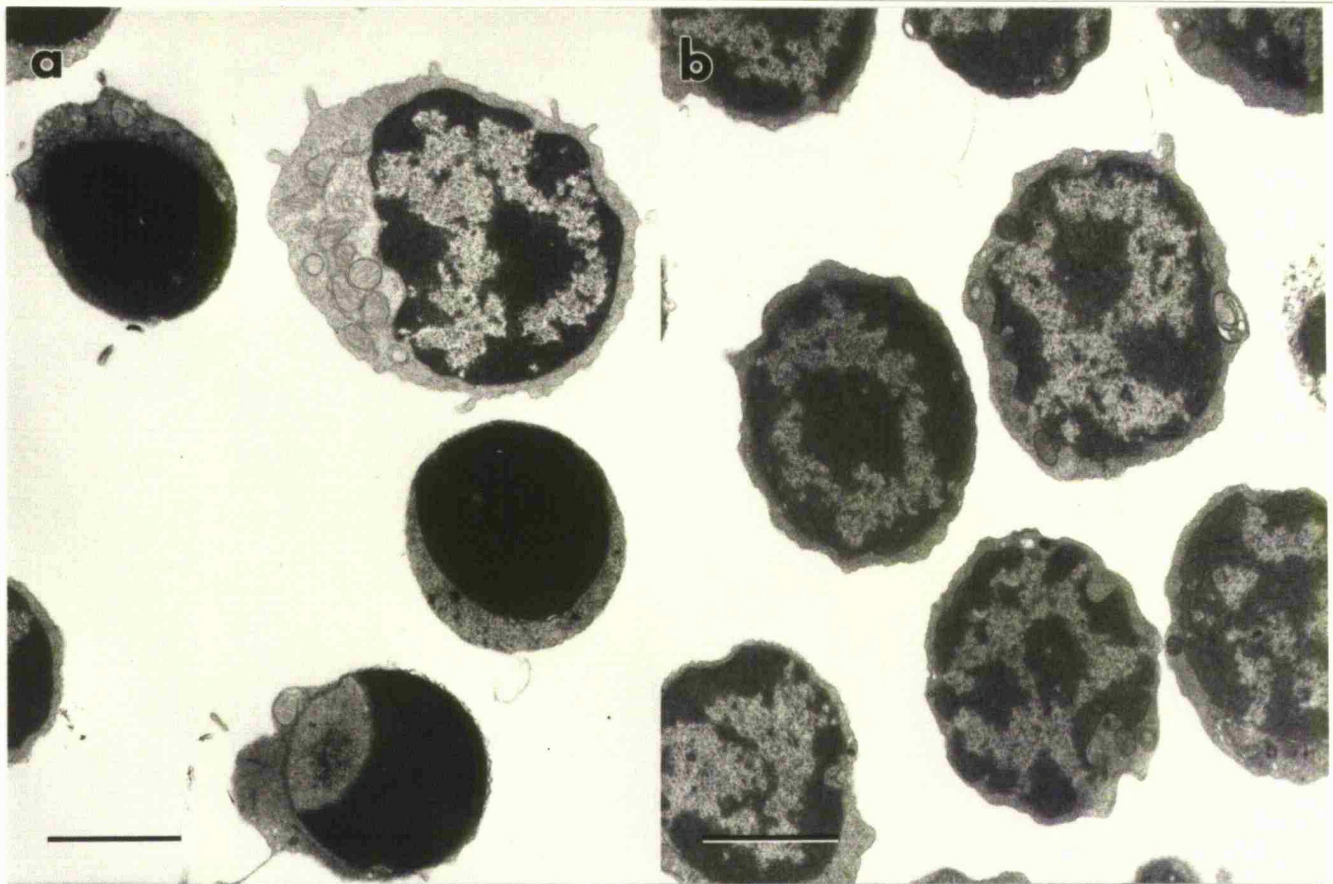
TLCK prevented both nuclear and cytoplasmic changes of apoptosis induced by thapsigargin it had little or no effect on mitochondrial changes induced by thapsigargin (see Fig. 15a). TLCK alone did not induce any morphological changes typical of apoptosis. However
TLCK clearly arrested cycling thymocytes in mitosis and in some cases prevented cytokinesis (data not shown).

TLCK inhibits apoptosis detected by flow cytometry.

Thymocytes were incubated for 1 h in the presence of TLCK (6.25-100μM) prior to exposure to apoptotic stimuli. After a further 4h incubation apoptosis was assessed by flow cytometry. As previously discussed (chapter 1) the increased Hoechst 33342 staining of apoptotic cells allows quantitation of the incidence of apoptosis (Sun et al., 1992). In agreement with previous results, both dexamethasone and etoposide caused an increase in high blue fluorescent cells (Sun et al.,1992; Cohen et al., 1992b; Sun et al., 1994) (Table 2).

Treatment	% Apoptotic cells
Control	10.4 ± 0.3
Dexamethasone	25.6 ± 1.2
Dexamethasone +TLCK	7.7 ± 1.1
Etoposide	35.7 ± 4.3
Etoposide +TLCK	13.7 ± 1.9
Thapsigargin	39.5 ± 2.0
Thapsigargin +TLCK	16.2 ± 2.8
Staurosporine	23.7 ± 0.7
Staurosporine +TLCK	15.3 ± 0.7

TABLE 2. TLCK inhibits a common effector of thymoctye apoptosis induced by diverse stimuli.
Cells were incubated for 1 h with TLCK (50μM) and then further incubated for 4 h with either dexamethasone (0.1μM), etoposide (10μM), thapsigargin (0.05μM) or staurosporine (1μM). The percentage of apoptotic cells was then assessed by flow cytometry (10). The data represent the mean (±S.E.M.) of at least 3 experiments.
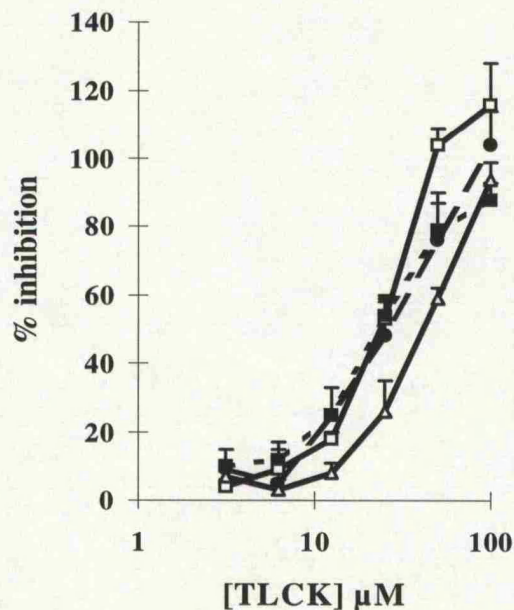
Figure 3. TLCK inhibits apoptosis induced by diverse stimuli.
Thymocytes were incubated for 1 h with TLCK (6.25-100μM) and then further incubated for 4 h in the presence of dexamethasone (0.1μM)(
□———□) , etoposide (10μM)(■----■), thapsigargin (50nM)(●----●)or staurosporine (1μM)(△———△). Apoptosis was assessed by flow
cytometry as descibed in materials and methods. The effect of TLCK is expressed as % inhibition, 0% defined as the inhibition in the absence
of TLCK and 100% as inhibition to control levels of apoptosis. The incidence of apoptosis caused by each agent was; dexamethasone, 25.3% ±
1.3; etoposide, 32.2% ± 3.7; thapsigargin, 38.3 ± 3.1; and staurosporine, 23.7 ± 1.4. The incidence of apoptosis in control cultures was
10.6% ± 0.6. The data are the means of 3 experiments ± SEM.

The other apoptotic stimuli, staurosporine (1μM) and thapsigargin (0.05μM) also caused an
increase in high blue fluorescent cells (Table 2). The increase induced by all four stimuli was
inhibited by TLCK (50μM) (Table 2). TLCK inhibition was concentration dependent (Fig.3)
and the concentration dependence was similar for dexamethasone, etoposide and
thapsigargin. Staurosporine required a slightly higher concentration of TLCK for inhibition
than the other three agents (Fig.3).

TLCK inhibits the formation of large kilobasepair fragments of DNA.
Following incubation of rat thymocytes for 4 hours with dexamethasone or etoposide, a
marked increase in the formation of large fragments of DNA, particularly of 10-50 kbp, was
detected by FIGE (Fig.4, lanes 4 and 6 respectively). This DNA cleavage was inhibited by
TLCK (50μM) (Fig.4, lanes 5 and 7 respectively). TLCK (50μM) alone caused a slight
decrease in the large fragments (10-50 kbp) observed in control cells (Fig. 4 compare lanes 3
and 8). Thapsigargin and staurosporine also showed an increased formation of large
fragments which was inhibited by TLCK (data not shown).

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Figure 4. TLCK, but not TPCK prevents the formation of large kilobase pair sized fragments of DNA.
A) Thymocytes were incubated for 4 h either alone (lane 3), with dexamethasone (0.1μM) alone (lane 4) or in the presence of TLCK (50μM) (lane 5), with etoposide (10μM) alone (lane 6) or in the presence of TLCK (50μM) (lane 7) or with TLCK (50μM) alone (lane 8). **B)** Thymocyes were also incubated for 4 h with TPCK (25μM). Size markers, 0.1-100 kbp (lane 1) and 245-2200 kbp (lane 2) are shown. Agarose plugs containing 1×10^6 cells were prepared and FIGE carried out as described in Materials and Methods.

Figure 5. The effect of TLCK and TPCK on dexamethasone induced cell shrinkage.
Thymocytes were incubated for 4 h either alone (—) or in the presence of dexamethasone (0.1μM) (······) and cell volume assessed as described in Materials and Methods. The volumes of normal and apoptotic cells are shown in fl. A) Thymocytes were also incubated with dexamethasone in the presence of TLCK (50μM) (----). The size distribution of cell treated with TLCK alone was identical to that of cells treated with dexamethasone plus TLCK and is not shown for clarity. B) The size distribution of cells treated with TPCK (25μM) alone (----) was identical to that of cells treated with dexamethasone plus TPCK.
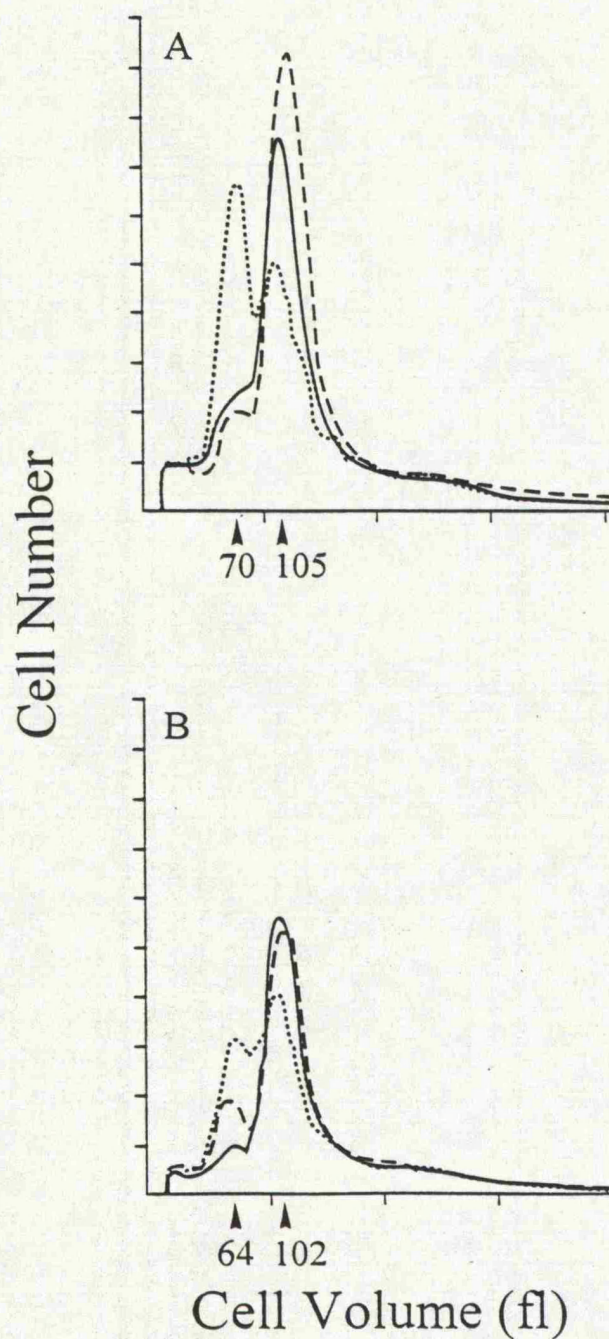
TLCK inhibits cell shrinkage induced by dexamethasone and etoposide.

Thymocytes undergoing apoptosis show a marked decrease in cell volume (Thomas and Bell, 1981; Wyllie and Morris, 1982). Following dexamethasone treatment a proportion of the cells shrink, a change that was prevented by TLCK (50μM) (Fig. 5A). Etoposide also caused cell shrinkage that was inhibited by TLCK (50μM) (data not shown).

A TLCK sensitive target was present in thymocytes prior to the induction of apoptosis.

Pre-incubation of media with TLCK (100μM) alone demonstrated that TLCK became progressively less effective at inhibiting apoptosis confirming the known instability of TLCK above neutral pH (Fig.6). The length of incubation of TLCK with medium alone clearly affected its ability to subsequently inhibit DNA fragmentation (Fig. 6). Thus after 1 h pre-incubation, insufficient TLCK remained to exert any inhibitory effect on either dexamethasone- or etoposide- induced apoptosis (Fig. 6). Only when TLCK was pre-incubated for 15 min or less was there sufficient remaining to inhibit dexamethasone- or etoposide-induced DNA fragmentation (Fig.6) or the appearance of apoptotic cells detected by flow cytometry (data not shown). In contrast when cells rather than medium were pre-treated with TLCK for 1 h and subsequently exposed to apoptotic stimuli, marked inhibition of apoptosis was observed (Table 2). These results suggested that TLCK bound to and inactivated its target, a putative protease, which was already present in thymocytes and which was not synthesised in response to apoptotic stimuli.
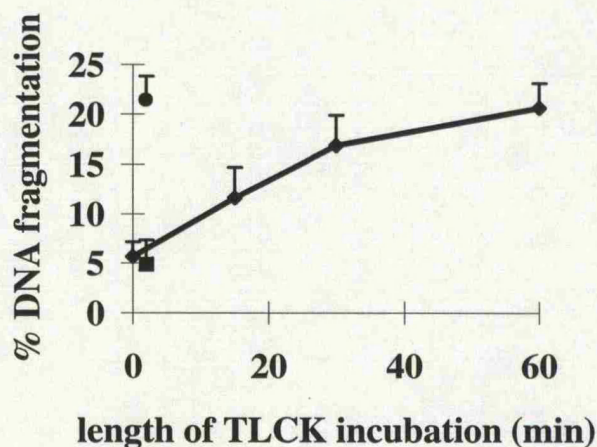
Figure 6. The TLCK sensitive protease is already present and not synthesized in response to apoptotic stimuli. TLCK (100μM) was incubated in culture medium for the indicated times before being added to cells to a final concentration of 50μM with either dexamethasone (0.1μM) (♦—♦) and further incubated for 4 h. Some cells were exposed to only dexamethasone (0.1μM) (■) for 4 h. After this time, DNA fragmentation was measured as described (3,27). Results are the mean (±S.E.M.) of 3 experiments. Essentially the same results were obtained if etoposide was used to induce apoptotsis or if apoptosis was assessed by flow cytometry (data not shown).

Figure 7. The effect of TLCK on Mg2+ and Ca2+/Mg2+ dependent DNA degradation in isolated nuclei.
Thymocytes were incubated for 1 h either alone or with TLCK (50µM). Nuclei from cells incubated alone were isolated and incubated in the absence of (lane 1) or presence of (lane 2) Mg^{2+} or Ca^{2+} and Mg^{2+} (lane3). Nuclei from cell treated with TLCK were also incubated in the absence of (lane 4) or presence of (lane 5) Mg^{2+} or Ca^{2+} and Mg^{2+} (lane 6). DNA degradation was assessed by FIGE (A) or conventional agarose gel electrophoresis (B).

A
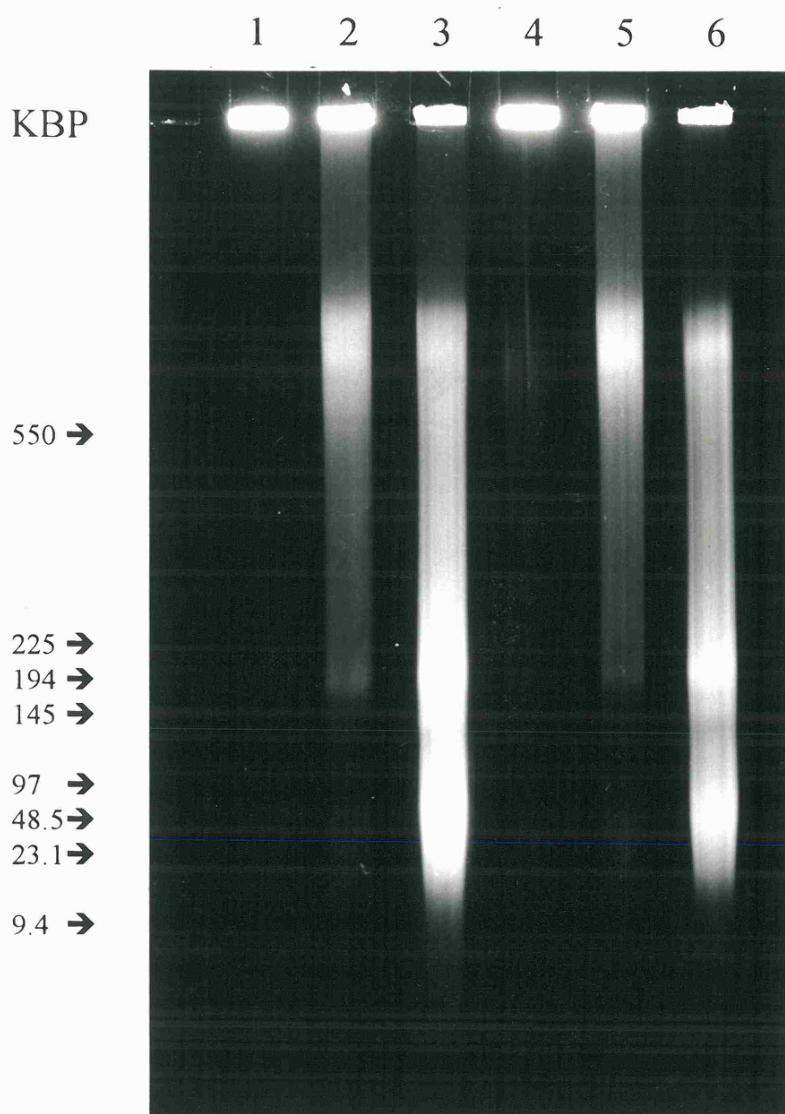
B
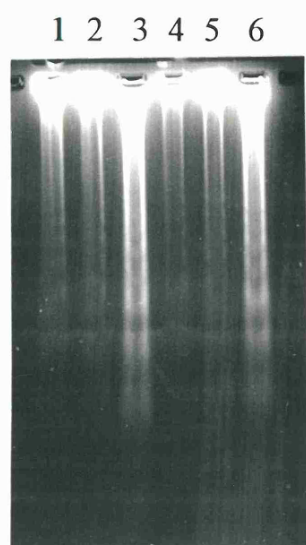
Lack of inhibition of DNA fragmentation by TLCK in iso lated nuclei.

To ascertain whether proteolysis was involved before the initial cleavage of DNA that produces large fragments, the effects of TLCK on isolated rat thymocyte nuclei were investigated. TLCK binds and inactivates its target within 30 minutes (shown above). To ensure a relevant TLCK concentration when testing the effects of TLCK on Ca^{2+}/Mg^{2+} dependent chromatin degradation, cells were pre-treated with TLCK before nuclei were isolated. The DNA of isolated thymocyte nuclei was degraded in a Ca^{2+}/Mg^{2+} dependent fashion as previously reported (Fig. 7 lane 3). The data show that thymocyte nuclei isolated from cells treated with TLCK degrade their DNA in the same way as nuclei isolated from control cells (Fig. 7, lanes 2 and 3 respectively). These results suggest that in intact cells TLCK inhibits apoptosis at a stage prior to DNA fragmentation, and that proteolysis is an early event and may be important in triggering apoptosis.

TPCK induces changes characteristic of early apoptosis

The effects of TPCK on large fragment formation were very different from those of TLCK. TPCK (25µM) alone caused an increase in large fragments of 10-50 kbp and ~200 kbp (Fig. 4B) compared to control cells, similar to that seen when thymocytes were treated with dexamethasone in the presence of zinc (Cohen et al., 1992). These thymocytes displayed a distinct ultrastructure, characterised by condensed chromatin abutting the nuclear membrane (Cohen et al. 1992b). Ultrastructural examination of cells following 4h incubation with TPCK (25µM) revealed that most thymocytes (80%) showed a very similar morphology (Fig. 8). Several of the cells treated with TPCK showed disintegration of the nucleolus together with the formation of cytoplasmic vacuoles. Consistent with the cytoplasmic morphology, TPCK (25µM) alone caused a decrease in cell volume in a proportion of the cells (Fig.5B). Incubation of thymocytes for 4 h with TPCK (25µM) resulted in the induction of apoptosis (22.2 % ± 2.2, Mean ± SEM., n=3), as assessed by flow cytometry, compared to control cells (9.3% ± 0.3). These studies demonstrated that TPCK induced both biochemical and morphological changes associated with early nuclear changes of apoptosis without producing internucleosomal cleavage.

Figure 8. TPCK causes perinuclear condensation of chromatin
A. Thymocytes incubated in the control medium exhibited normal ultrastructure with diffuse aggregations of centrinuclear and perinuclear heterochromatin.
B. Cells incubated in the presence of TPCK (25µM) showed condensation of heterochromatin into discrete, sharply-defined clumps (arrows). Several of these cells showed disintegration of the nucleolus (*) together with the formation of cytoplasmic vacuoles which fused with the cell membrane (arrowheads). Bars = 2µm.
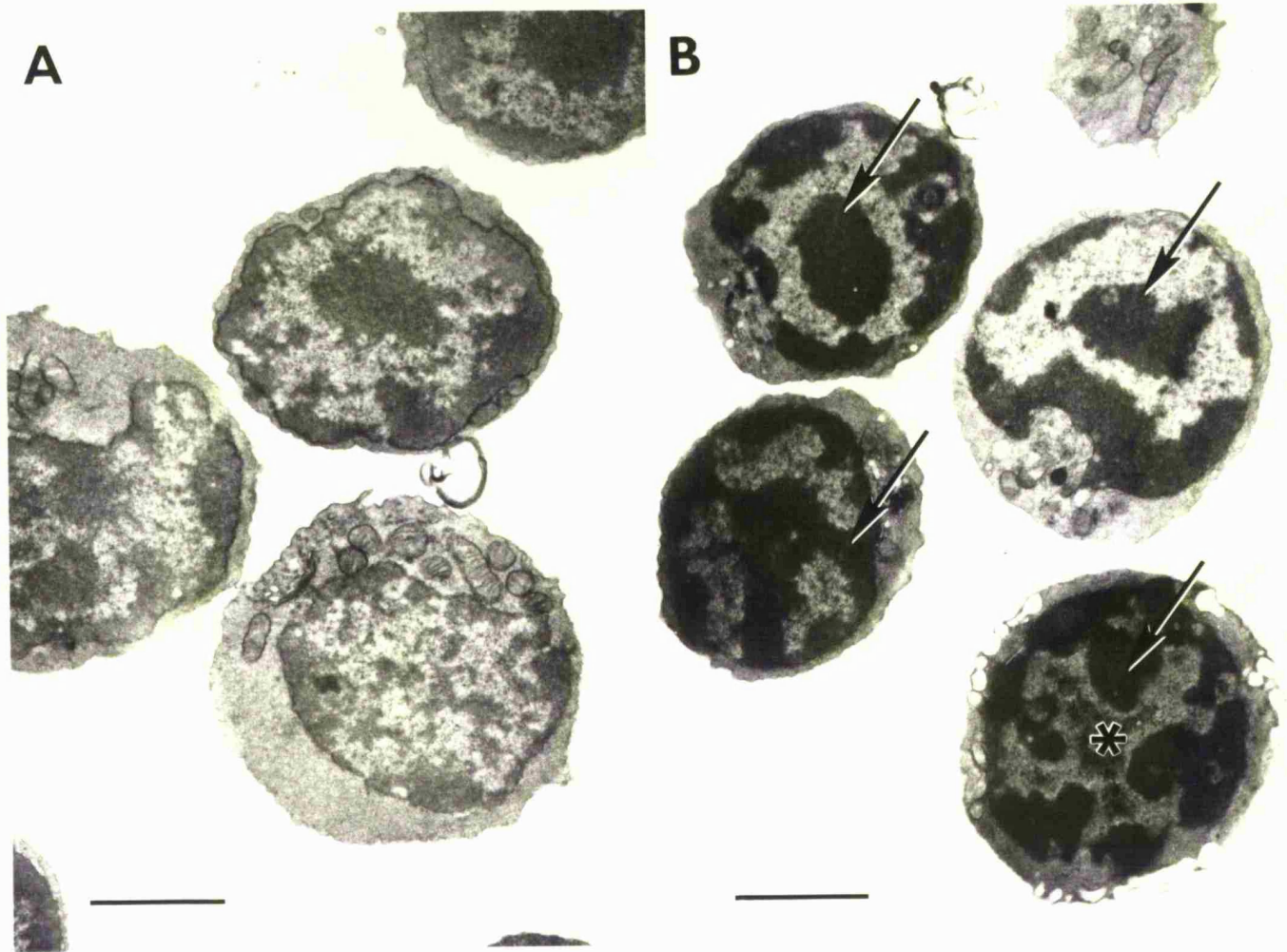
DISCUSSION

A pre-existing TLCK-sensitive target is a common effector of thymocyte apoptosis

Apoptosis was induced in thymocytes by four stimuli with different mechanisms of action (Table 2). TLCK inhibited all the characteristic changes of apoptosis induced by these stimuli i.e. both large fragment formation (Fig. 4) and internucleosomal cleavage (Fig.1) of DNA, cell shrinkage (Fig.5A) and plasma membrane changes (Table 2 & Fig. 3). Thus a TLCK-sensitive target is required at an early stage of thymocyte apoptosis prior to both the nuclear and cytoplasmic changes of apoptosis. The data obtained following preincubation of media or cells for 1 h with TLCK (Fig. 6 and Table 2) demonstrated that the TLCK-sensitive target was pre-existing and not synthesised in response to apoptotic stimuli.

As transcription and translation are often required for thymocyte apoptosis (Wyllie et al. 1984), the possibility that TLCK was affecting these was examined by comparing its effects to those of cycloheximide. Apoptosis induced by dexamethasone, etoposide and thapsigargin was inhibited by both TLCK and cycloheximide, whereas that induced by staurosporine was inhibited only by TLCK (data not shown). These data support a model in which the apoptotic machinery is pre-existing and staurosporine induces apoptosis by acting downstream of *de novo* protein synthesis.

We and others have shown that there is an initial degradation of DNA to fragments of $\geq$ 700, 200-300 and 30-50 kbp in size (Walker et al., 1991; Brown et al., 1993; Oberhammer et al., 1993). These large fragments are precursors of DNA ladders and are associated with cells displaying early apoptotic morphology (Brown et al., 1993; Oberhammer et al., 1993; Cohen et al. 1994). Differences have been observed in the sizes of the large fragments reported by the various groups possibly due to the electrophoretic systems used or the preparation of the DNA samples. The data presented here shows that TLCK inhibited the formation of these large fragments (Fig.4) consistent with proteolysis being required either for the initial cleavage of intact DNA to kilobase pair fragments or at some earlier stage in the apoptotic process. To distinguish between these possibilities, isolated thymocyte nuclei, which exhibit both a Mg^{2+} and a Ca^{2+}/Mg^{2+}-dependent DNA degradation, were used as a model of the nuclear changes seen during apoptosis (Sun and Cohen, 1994). With these nuclei, TLCK inhibited neither the formation of large fragments nor internucleosomal cleavage (Fig.7). These data were inconsistent with the first possibility and therefore proteolysis was required at an early stage of apoptosis, prior to the formation of kilobase pair fragments of DNA.

TPCK inhibits DNA laddering but itself induces early apoptotic changes

TPCK exerted markedly different effects compared to TLCK. TPCK inhibited internucleosomal cleavage induced by dexamethasone and etoposide (Fig.1) but alone caused the formation of large DNA fragments (Fig. 8). Weaver et al. (1993) showed that the induction of internucleososmal cleavage of DNA by dexamethasone or teniposide but not the formation of large fragments in rat thymocytes was prevented by TPCK. On ultrastructural examination of TPCK-treated cells, formation of cytoplasmic vacuoles and condensation of chromatin into sharply defined clumps abutting the nuclear membrane were observed(Fig.9). Both the pattern of DNA fragmentation and the morphology are typical of early nuclear changes of apoptosis (Wyllie, 1980; Kerr et al., 1987; Cohen et al., 1992b; Cohen et al. 1993). Consistent with the alterations in cytoplasmic morphology, TPCK alone induced shrinkage in a proportion of cells (Fig.5B). TPCK also increased the percentage of high blue fluorescent cells reflecting an altered membrane permeability (Ormerod et al. 1993). These data demonstrate that the effects of TLCK and TPCK are different and that TPCK alone induces many of the early changes of apoptosis and only inhibits the terminal stages of DNA fragmentation i.e. internucleosomal cleavage and full chromatin condensation.

A trypsin-like protease is required for early apoptotic changes in thymocytes.

In this chapter no direct evidence of proteolysis is presented. However, TLCK and TPCK at the concentrations used, inhibit intracellular proteolysis (Henkel et al. 1993). Under identical conditions, TLCK stabilised cdc-2 kinase activity, consistent with an inhibition of cyclin degradation (Norbury et al., 1994). While intracellular proteases are the most likely targets of these inhibitors, other cellular targets cannot be totally excluded. As TLCK and TPCK both inhibit cysteine proteases (Powers and Harper 1986), it is possible that they exert their effects by inhibition of a cysteine protease similar to ICE. The known substrate specificity of ICE (Thornberry et al. 1992) together with a recent report that a TLCK- and TPCK-insensitive ICE-like protease is required for the nuclear changes of apoptosis (Lazebnik et al., 1994) make it unlikely that the effects described in the present study are due to a direct inhibition of an ICE- like protease. The cysteine protease calpain has also been implicated in thymocyte apoptosis (Squier et al., 1994). Calpain cannot be excluded as the target for TLCK or TPCK. However the substrate specificity of calpain (Sasaki et al. ,1984) and the observation that a calpain inhibitor, ALLN, alone causes the formation of large DNA fragments (Fig. 10B) suggest that calpain is not the target. TLCK inhibits trypsin-like proteases but does not inhibit chymotrypsin-like proteases. Conversely, TPCK inhibits chymotrypsin-like proteases but not trypsin-like proteases (Powers and Harper, 1986). Thus the contrast between the effects of TLCK and TPCK described here support a role for at least two distinct proteases in thymocyte apoptosis. A TPCK-sensitive chymotrypsin-like

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protease is required for the terminal changes of apoptosis i.e. both the internucleosomal cleavage of DNA and the complete condensation of the chromatin and a TLCK-sensitive trypsin-like protease is required early in the apoptotic process. This TLCK-sensitive protease mediates both cytoplasmic and nuclear changes of thymocyte apoptosis induced by diverse stimuli. Therefore this protease, which is pre-existing and is not synthesised in response to apoptotic stimuli, is a common effector of thymocyte apoptosis.

RESULTS SECTION II
INHIBITORS OF CALPAIN AND INTERLEUKIN-1β-CONVERTING ENZYME.

ALLN inhibits some features of apoptosis but induces the formation of kilobase pair sized fragments.

The cysteine protease, calpain has been shown to play a role in both induction and release models of apoptosis and calpain activation is suggested as a committed step in apoptosis (Squier et al. 1994). In thymocytes two peptide aldehyde inhibitors of calpain inhibit both calcium dependent proteolysis, autolysis of calpain and DNA laddering. Therefore an inhibitor of calpain, ALLN (Crawford 1990) which has been shown to inhibit intracellular proteolysis (Sherwood et al. 1993) was used to determine which changes associated with apoptosis require calpain. Cells were pre-incubated with ALLN in order to increase its intracellular concentration. The cells were then further incubated for a further 4 h in the presence of dexamethasone or etoposide, after which apoptosis was assessed by flow cytometry. The appearance of high blue fluorescent cells (Fig. 9A) and cell shrinkage was partially blocked (data not shown) by ALLN (25μM) and no further inhibition observed up to 200 μM. ALLN inhibited both dexamethasone and etoposide induced DNA fragmentation detected by a colourimetric assay in agreement with Squier et al. (data not shown). A small amount of 10-30 kbp fragments was seen in control cells (Fig. 9B lane 1) which was markedly increased in the presence of ALLN (12.5-200 μM) (Fig. 9B lanes 2-6). This increase was maximal at the lowest concentration of ALLN used and was comparable to the increase seen following dexamethasone or etoposide treatment (Fig. 9B, lanes 7 and 8 respectively).

Another calpain inhibitor (MDL-70,128) was tested for its ability to inhibit thymocyte apoptosis. Cells were incubated for 1 hour with MDL (200 μM) to ensure entry into the cell before dexamethasone or etoposide were added. The incidence of apoptosis was assessed 4 hours later by flow cytometry, FIGE and by a DNA fragmentation assay. Neither the appearance of apoptotic cells detected by flow cytometry (Fig. 10A) nor DNA fragmentation assessed by the diphenylamine reaction (Fig. 10B) was inhibited by MDL-70,128 (200μM).

Figure 9. The effects of ALLN assessed by flow cytometry and FIGE.
A) Inhibition by ALLN of apoptosis induced by dexamethasone and etoposide as assessed by flow cytometry. Thymocytes were preincubated for 1 h with increasing concentrations of ALLN and further incubated for 4 h either alone (O), with dexamethasone (●) (0.1μM) or with etoposide (■) (10μM). The data are the means of 3 experiments (±S.E.M.). *B)* The effects on DNA of ALLN alone assessed by FIGE. Thymocytes were incubated for 5 h either alone (lane 1) or with 12.5μM (lane 2), 25μM (lane 3), 50μM (lane 4), 100μM (lane 5) or 200μM ALLN (lane 6). Some cells were incubated alone for 1 h and then further incubated for 4 h with dexamethasone (0.1μM) (lane 7) or etoposide (10μM) (lane 8). Agarose plugs containing 1×10^6 cells were prepared and electrophoresis carried out as described (6).

A

B
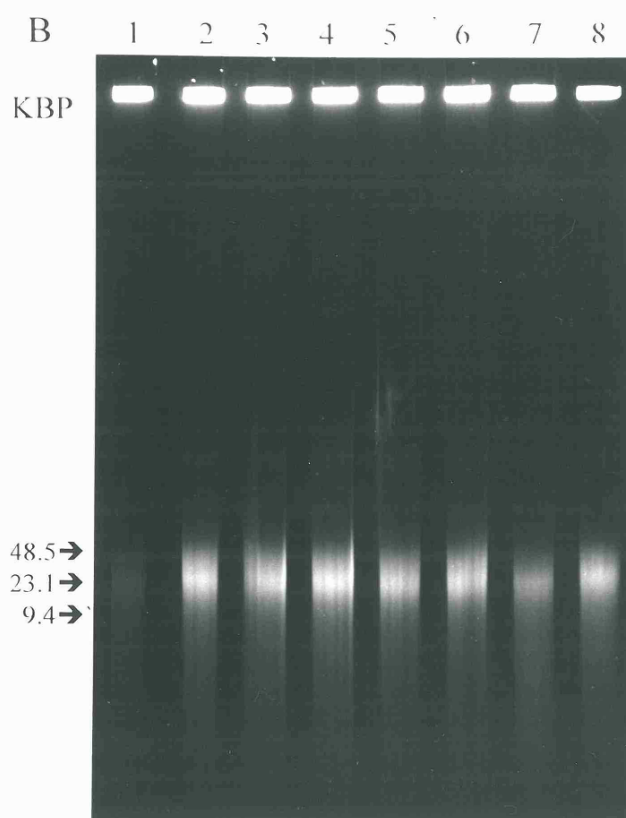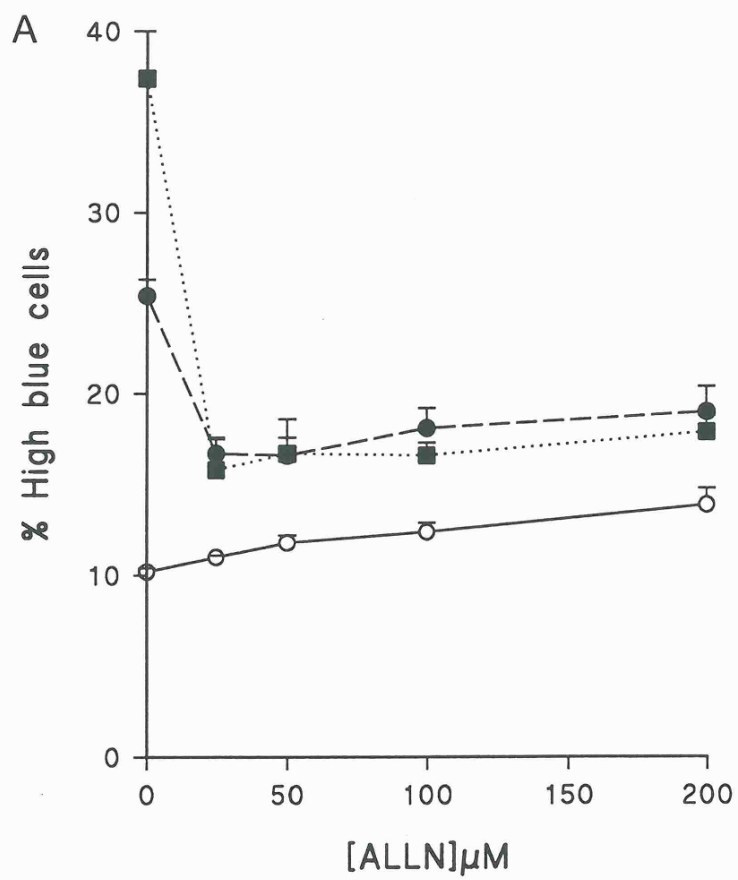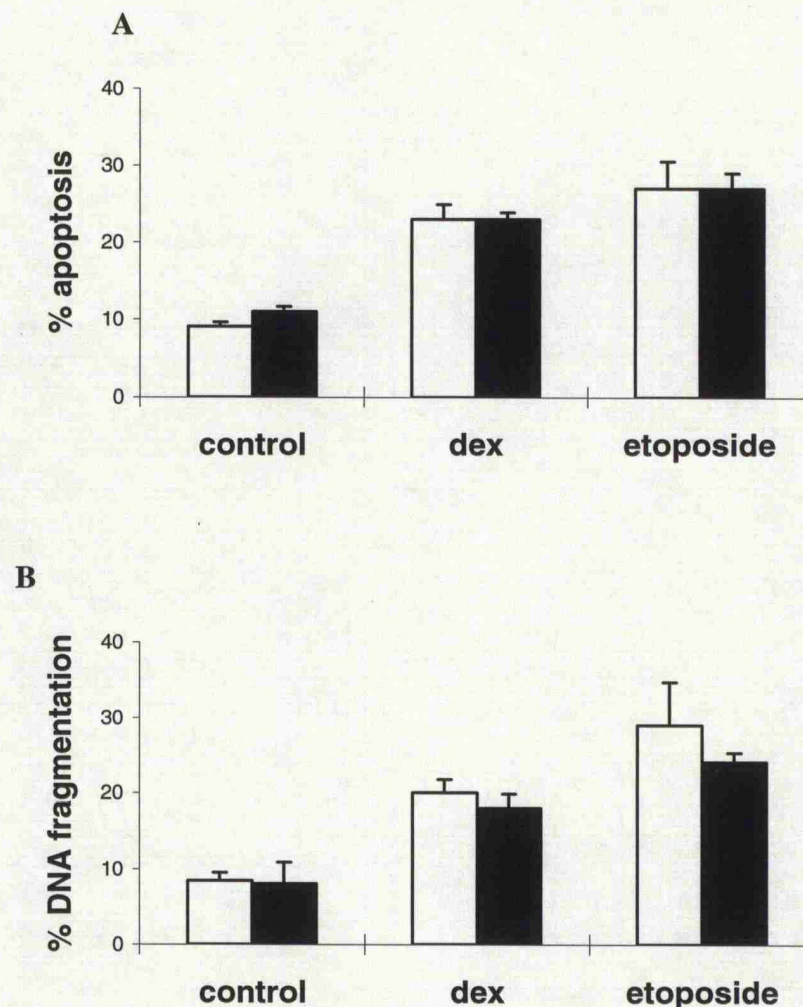
Figure 10. MDL 70,128 did not inhibit thymocyte apoptosis.
Thymocytes were incubated with MDL 70,128 (200μM) for 1 h prior to further incubation with either dexamethasone (0.1μM) or etoposide (10μM) for 4 h. Apoptotsis was assessed by flow cytometry (**A**) or quantitating DNA fragmentation (**B**). The data are the means of 3 experiments ± SEM.

In addition the formation of kilobase pair sized fragments of DNA induced by dexamethasone or etoposide was not inhibited (data not shown).

ALLN induces large fragments in F1 and F2 thymocytes.

ALLN exerts an effect on the cell cycle (Sherwood et al. 1993) in the concentration range that affects thymocytes. Another calpain inhibitor, benzyloxycarbonyl-leucinyl-leucinyl-tyrosinyl diazomethyl ketone inhibits growth of two human cell lines (Mellgren et al. 1994). Therefore the effects of ALLN on populations of predominantly cycling thymocytes or predominantly quiescent thymocytes was examined. Thymocytes were separated into these two

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Figure 11. ALLN causes the formation of large fragments predominantly in F1 thymocytes. Thymocytes were isolated and seperated on discontinuous Percoll gradients as described in Materials and Methods. F1 and F2 thymocytes were then further incubated with ALLN (25μM) for 4 h. The formatiom of large kilobase pair size fragments of DNA was assessed by FIGE.
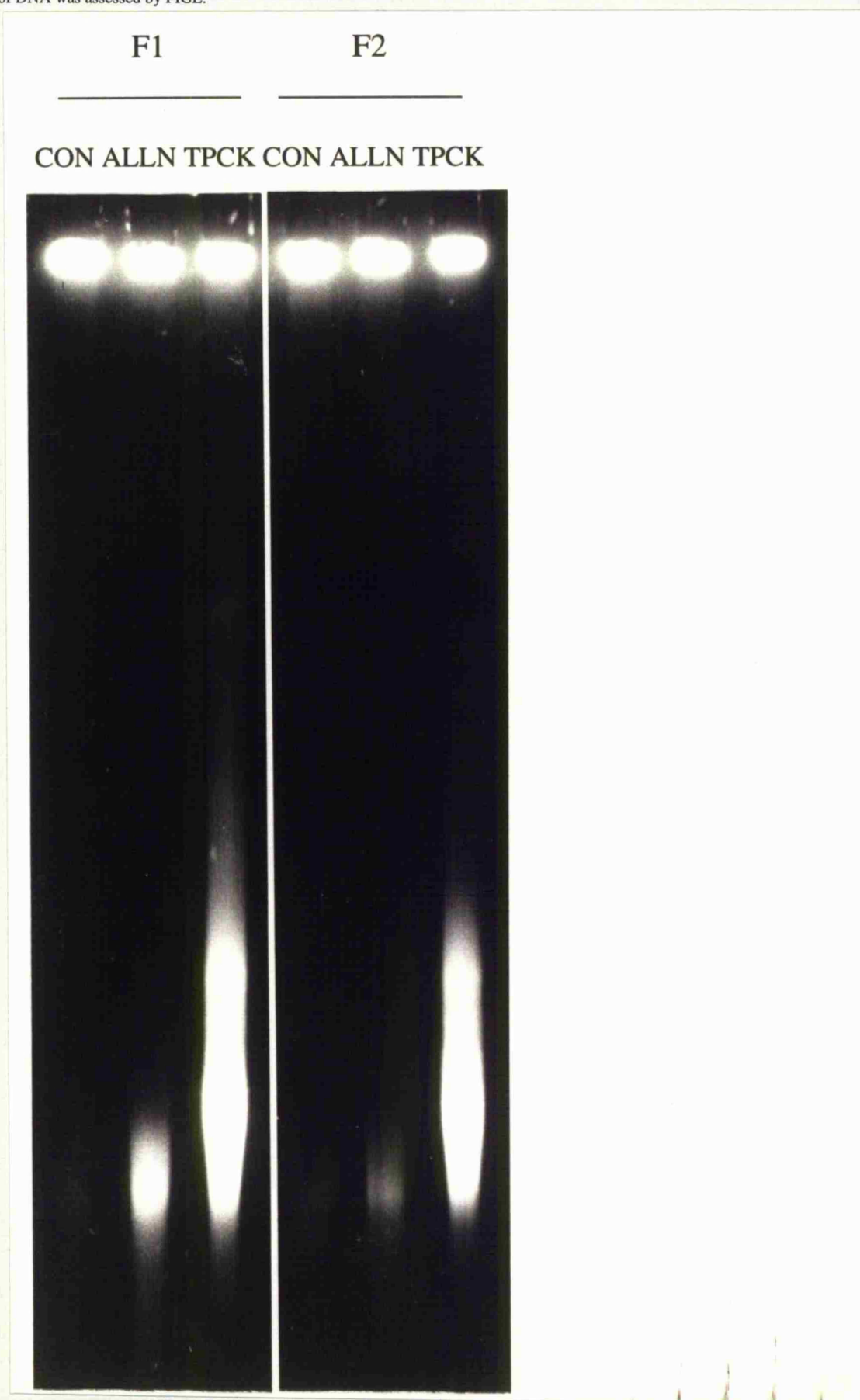
Figure 12. ALLN alone causes the appearance of electron dense membrane bound cytoplasmic bodies.
Thymocytes were incubated either alone (A) or with ALLN (B)(25µM) for 4 h prior to fixation and ultrastructural examination by electron microscopy as described in Materials and Methods. Osmophilic particles are arrowed.
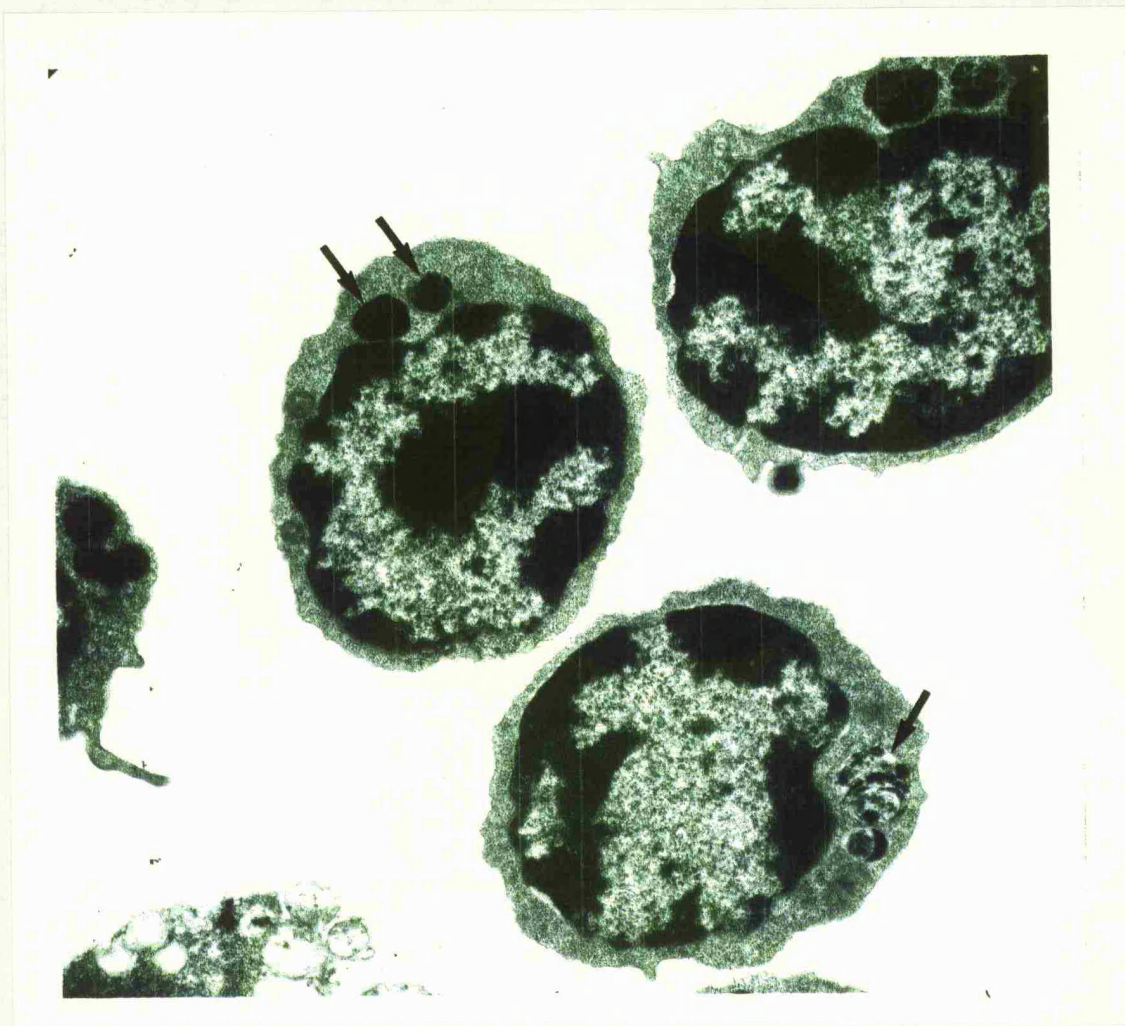
populations by centrifugation on discontinuous Percoll gradients as previously described and F1 (cycling cells) and F2 (quiescent cells) isolated. These populations were then further incubated with ALLN (25 µM) for 5 h and plugs prepared for FIGE. In F1, ALLN caused very extensive DNA fragmentation, producing 10-50 kbp sized fragments (Fig. 11) in the absence of ladders . In F2 there was less, but still marked, DNA fragmentation (Fig.11).

ALLN alone causes the appearance of electron dense membrane bound cytoplasmic bodies.
Ultrastructural examination of cells treated with ALLN alone (2-25µM) revealed that nuclear morphology was normal and many normal mitochondria were visible. However in many cells there were small electron dense particles bounded by a membrane (Fig. 12 arrowed). The origin of these particles is not clear. Their size is similar to that of mitochondria but some cells possess both normal mitochondria and dense particles. ALLN inhibits lysosomal cathepsins so lysosomes may be a target organelle and the electron dense material represent an accumulation of lipid, although how inhibition of these proteases would result in such a marked condensation is unclear.

z-VAD.FMK prevents thymocyte apoptosis induced by three diverse stimuli.
Thymocytes were incubated for 1 h with z-VAD.FMK (50, 100 or 200µM) and subsequently treated with either dexamethasone, etoposide or thapsigargin for a further 4 h. After this time apoptosis was assessed by flow cytometry, conventional agarose gel electrophoresis and FIGE. The effects of z-VAD.FMK on thymocyte ultrastructure were also examined. Z-VAD.FMK inhibited thymocyte apoptosis assessed by flow cytometry in a concentration dependent fashion. Little or no inhibition was seen at 50 µM and maximal inhibition was seen with 200µM irrespective of the apoptotic stimulus used (Fig.13).
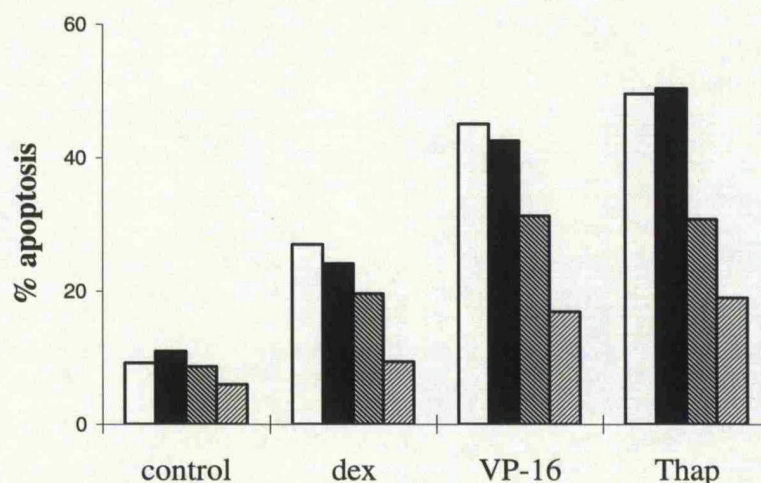
Figure 13. Z-VAD.FMK prevents apoptosis induced by 3 different stimuli.
Thymocytes were incubated with z-VAD.FMK (0µM, open bars; 50µM, solid bars; 100µM , light hatch; or 200µM, dark hatch) for 1 h prior to further incubation with eith dexamethasone (0.1µM), etoposide (10µM) or thapsigargin (50nM) for 4 h. Apoptosis was assessed by flow cytometry as described in Material and Methods.

Figure 14. z-VAD.FMK prevents DNA fragmentation.
Thymocytes were incubated either alone or with z-VAD.FMK (50,100, 200μM) for 1
h prior to further incubation for 4 h with either etoposide (10μM) or thapsigargin
(50nM). a) the formation of large kilobase pair sized fragments was detected by FIGE,
b) internucleosomal cleavage of DNA was detected by conventional agarose gel
electrophoresis.

STD Con   **Etoposide**   **Thapsigargin**

z-VAD.FMK (μM)   0   0   50   100   200   0   50   100   200

(a)

KBP

48.5 →

9.8 →

(b)
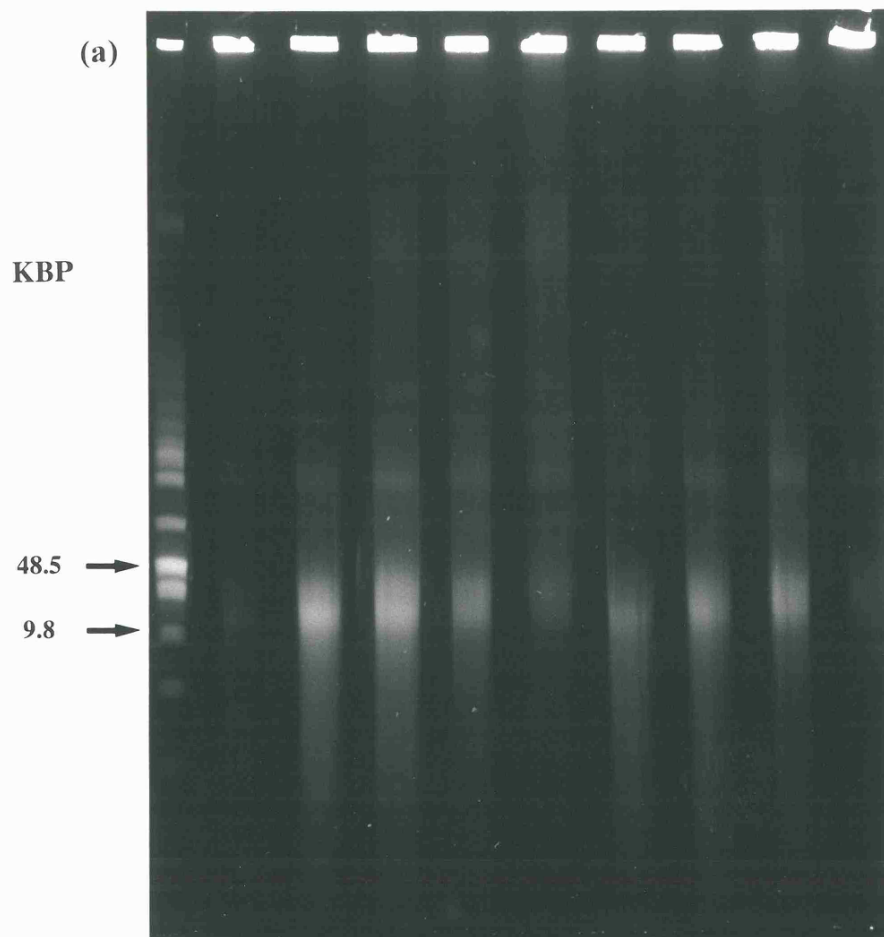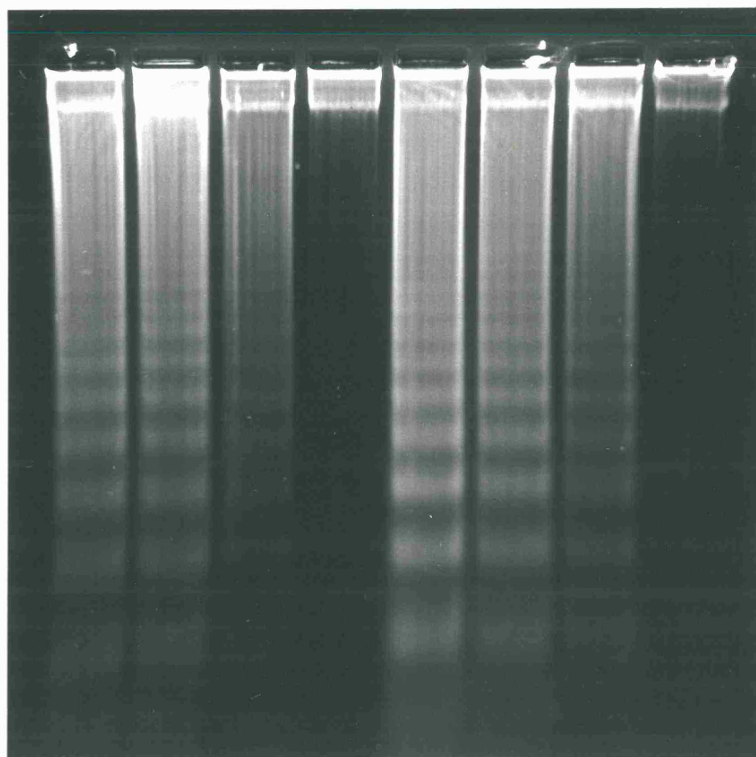
z-VAD.FMK (μM)   0   50   100   200   0   50   100   200

Etoposide   Thapsigargin

Figure 15. z-VAD.FMK prevents the morphological changes of apoptosis
Thymocytes were incubated either alone (a) or with z-VAD.FMK (200μM) (b) for 1 h prior to further incubation for 4 h with thapsigargin (50nM). z-VAD.FMK had essentially the same effect on the morphological changes of apoptosis induced by dexamethasone or etoposide. Bars represent 2μm.
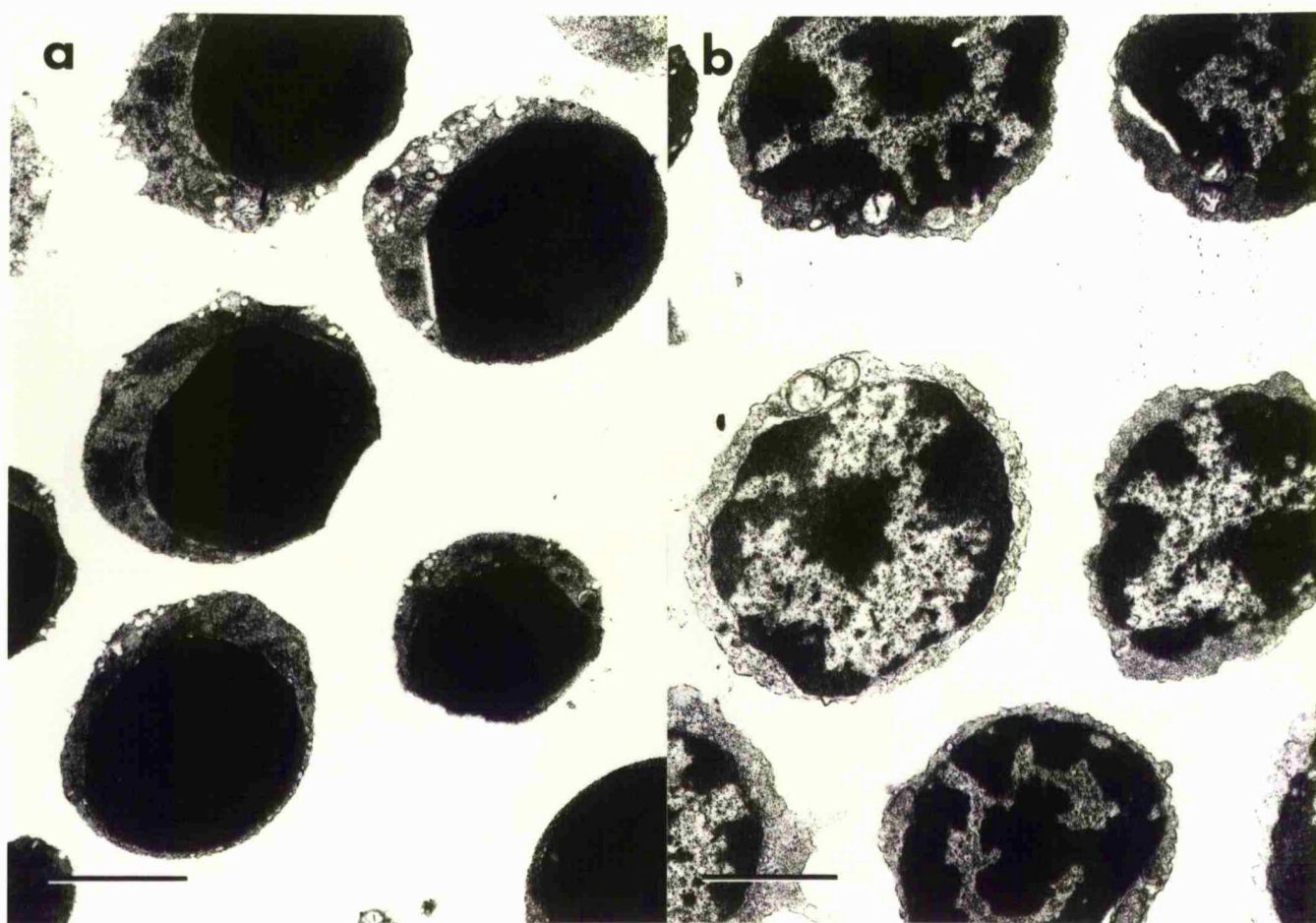
The effects of z-VAD.FMK on DNA fragmentation was assessed by both FIGE and conventional agarose gel electrophoresis. Etoposide and thapsigargin induced both the formation of large kbp sized fragments of DNA and DNA laddering. Both forms of DNA fragmentation were inhibited by z-VAD.FMK (Fig.14A & B). The inhibition was concentration dependent and correlated well with the inhibition of apoptosis assessed by flow cytometry. Similar inhibition of dexamethasone induced changes was observed (data not shown).

Morphological changes of apoptosis are inhibited by z-VAD.FMK

At a concentration of 200μM z-VAD.FMK inhibited apoptosis by biochemical criteria. At this concentration, ultrastructural examination revealed that z-VAD.FMK also prevented the typical morphological changes of apoptosis induced by dexamethasone, etoposide or thapsigargin (Fig. 15).

Four ICE inhibitors did not prevent dexamethasone or etoposide induced apoptosis.

Thymocytes were incubated for 1 h in the presence or absence of either YVAD.CHO (200μM), YVAD.COCH$_3$ (200μM), z-VAD.DCOMK (50μM) (at higher concentrations these three inhibitors precipitated in culture medium) or YVAD.CMK (12-200μM). Either dexamethasone or etoposide was subsequently added and the incidence of high blue fluorescent cells assessed by flow cytometry 4 h later. Both dexamethasone and etoposide caused an increase in the percentage of high blue fluorescent cells (Fig. 16). YVAD.CHO, YVAD.COCH$_3$ or YVAD.DCOMK did not inhibit dexamethasone induced apoptosis assessed by flow cytometry (Fig.16). YVAD.CHO and YVAD.COCH$_3$ did not inhibit apoptosis induced by etoposide (Fig.16) (there was insufficient YVAD.DCOMK to test against etoposide induced apoptosis).
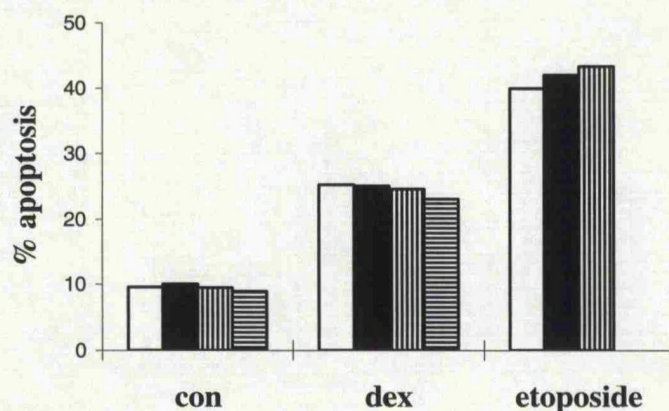
Figure 16. Three ICE inhibitors do not block apoptosis.
Thymocytes were incubated for 1 hour with YVAD.CHO (200μM), (solid bars); YVAD.COCH$_3$ (200μM), (vertically striped bars); or YVAD.DCOMK (50μM), (horizontally striped bars) before futher incubation with either dexamethasone (0.1μM) or etoposide (10μM) for 4 h. Apoptosis was assessed by flow cytometry and the data is the mean of 2 experiments (YVAD.CHO and YVAD.COCH$_3$) or from 1 experiment (YVAD.DCOMK).

YVAD.CMK was tested at a range of concentrations; up to 200μM YVAD.CMK did not prevent either dexamethasone or etoposide induced apoptosis (Fig. 17). The effect of YVAD.CMK on the formation of large kilobase pair sized fragments was assessed by FIGE. This showed that the formation of 10-50 kbp sized fragments induced by both dexamethasone and etoposide was not inhibited by YVAD.CMK (data not shown).

Figure 17. YVAD.CMK dod not inhibit the appearance of high blue cells. Thymocytes were incubated either alone or with YVAD.CMK (12.5-200μM) for 1 h prior to further incubation alone (O——O)with dexamethasone (0.1μM) (●——●) or with etoposide (10μM) (■——■) for 4h. After this time apoptosis was assessed by flow cytometry and the Hoechst/PI method. The data is from one experiment.
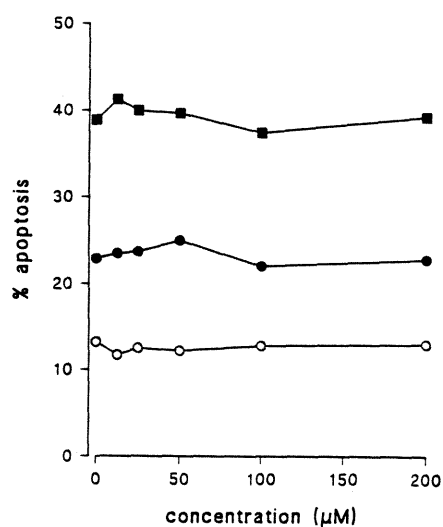
DISCUSSION

ALLN differentially affected the nuclear and plasma membrane changes of apoptosis.
The data confirm an earlier report that ALLN inhibited DNA fragmentation (Squier 1994). The assay for DNA fragmentation relies on detecting DNA fragments that are not sedimented by centrifugation. The detection of DNA fragmentation in this way correlates well with DNA laddering but does not correlate with the formation of larger (10-50 kbp) sized fragments (Cohen et al. 1992b; Sun et al. 1994; Brown et al. 1993). ALLN also inhibited both increased Hoechst 33342 staining (Fig. 10A) and cell shrinkage induced by dexamethasone and etoposide. Like TPCK, ALLN alone caused the formation of kilobase pair sized fragments of DNA (Fig. 10B) typical of early apoptosis. Thus the appearance of some features of the apoptotic phenotype (10-50 kbp sized fragments) occurred in the absence of other features (increased Hoechst 33342 staining and cell shrinkage). The formation of 10-50 kbp sized fragments was greater in F1 thymocytes than in F2 thymocytes. F1 consists of predominantly cycling cells and the formation of DNA breaks may reflect the ability of ALLN (Sherwood et al. 1993) and another calpain inhibitor, benzyloxycarbonyl-leucinyl-leucinyl-tyrosinyl diazomethyl ketone (Mellgren et al. 1994) to induce cell cycle arrest in other cell types. Ultrastructural examination of thymocytes treated with ALLN alone revealed that ALLN had no observable effect on nuclear morphology but caused the appearance of cytoplasmic granules. Co-treatment of thymocytes with dexamethasone and zinc (Cohen et al., 1992; Brown et al., 1993) or treatment with TPCK (Fig. 9) produces both the formation of 10-50kbp sized fragments of DNA accompanied by an early apoptotic morphology. The effects of ALLN show that DNA fragmentation to 10-50 kbp fragments is not necessarily accompanied by these early morphological changes in the nucleus supporting a previous observation that DNA cleavage and chromatin condensation can occur independently (Oberhammer et al. 1993; Sun et al. 1994). The granules were very osmophilic and contained membranes. While no definitive statement can be made they are probably mitochondria or mitochondria within lysomes. This effect may be linked to the inhibition of lysosomal cathepsins by ALLN (Hiwasa et al. 1990; Sasaki et al. 1990).

The data is compatible with a model in which the protease inhibitors act at different stages of apoptosis and that more than one protease is required for completion of the apoptotic process (Fig. 18). The effects of ALLN show that the formation of 10-50 kbp sized fragments of DNA can be dissociated from both the plasma membrane changes and morphological changes of apoptosis. This suggests that an apoptotic pathway induced by both dexamethasone and etoposide diverges into independent pathways each leading to the changes in nuclear morphology, DNA cleavage or plasma membrane changes of apoptosis.

Some support for this model comes from the observation that thymocytes, exposed to dexamethasone or etoposide in the presence of zinc, displayed increased Hoechst 33342 staining in the absence of DNA ladders (Cohen et al, 1992; Sun et al., 1994), that chromatin condensation can be dissociated from DNA cleavge (Sun et al. 1994a) and that the cytoplasmic changes of apoptosis occur in the absence of a nucleus (Jacobson et al. 1994). In this model, TPCK induced both early nuclear changes and plasma membrane changes of apoptosis but prevented the final steps of DNA degradation (DNA laddering). In contrast, TLCK inhibited both nuclear and membrane changes induced by dexamethasone or etoposide supporting a common effector responsible for the nuclear and plasma membrane changes of apoptosis. Whilst the data support this model the posibility that there are distinct targets for TLCK at early stages within these separate pathways cannot be excluded. This model is in good agreement with one recently proposed by Jacobson et al. (1994) in which the events of programmed cell death were controlled by a cytoplasmic effector responsible for activation of parallel pathways leading to apoptotic changes.

ALLN will inhibit intracellular proteases other than calpain. Lysosomal cathepsins, also cysteine proteases, are inhibited (Hiwasa et al. 1990; Sasaki et al. 1990) as are some activities of the multi-catalytic protease (proteosome) (Figueiredo-Pereira et al. 1994) and endoplasmic reticulum localised proteolysis of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Sherwood et al. 1993). Squier et al. (1994) report that inhibitors of cathepsins do not inhibit thymocyte apoptosis, in agreement with results presented here. In a recent study, the effective concentrations of ALLN against the proteolytic activities of calpain and some activities of the multicataytic protease were compared. Maximal inhibition of calpain activity was observed at 10 μM ALLN calpain whereas 50 μM ALLN was required for maximal inhibition of the chymotrypsin-like activity of the multi-catalytic protease (Figueiredo-Pereira et al. 1994). This evidence, and the lack of inhibition of thymocyte apoptosis by the second calpain inhibitor MDL-70,128, suggests that care must be taken when describing calpain as the relevant target of ALLN in thymocyte apoptosis.

ICE inhibitors and thymocyte apoptosis.
The experiments described in this chapter examined the effects of 5 ICE inhibitors. Dexamethasone, etoposide and thapsigargin induced ultrastructural changes in the nucleus and cytoplasm typical of apoptosis which were prevented by z-VAD.FMK (200 μM) (Fig. 15). Z-VAD.FMK (200 μM) inhibited thymocyte apoptosis by all criteria used; flow cytometry (Fig. 13), conventional agarose gel electrophoresis and FIGE (Fig. 14). TLCK has the same biochemical and morphological effects (Section I). Thus the inhibition of biochemical changes of apoptosis correlated closely with the inhibition of morphological

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Figure 18. Proposed sites of action of protease inhibitors on apoptosis.
TLCK inhibited dexamethasone (DEX) and etoposide induced apoptosis as assessed by the techniques shown in the boxes. In contrast, TPCK caused the formation of large DNA fragments, induced membrane changes and morphological changes associated with apoptosis. For this reason TPCK has been placed with dexamethasone and etoposide. ALLN also caused the formation of large DNA fragments but prevented the membrane changes induced by dexamethasone and etoposide. Both TPCK and ALLN prevented the conversion of large DNA fragments to oligonucleosomes. These results are compatible with distinct cytoplasmic and nuclear pathways differentially effected by TPCK and ALLN but inhibited at a common effector site by TLCK.
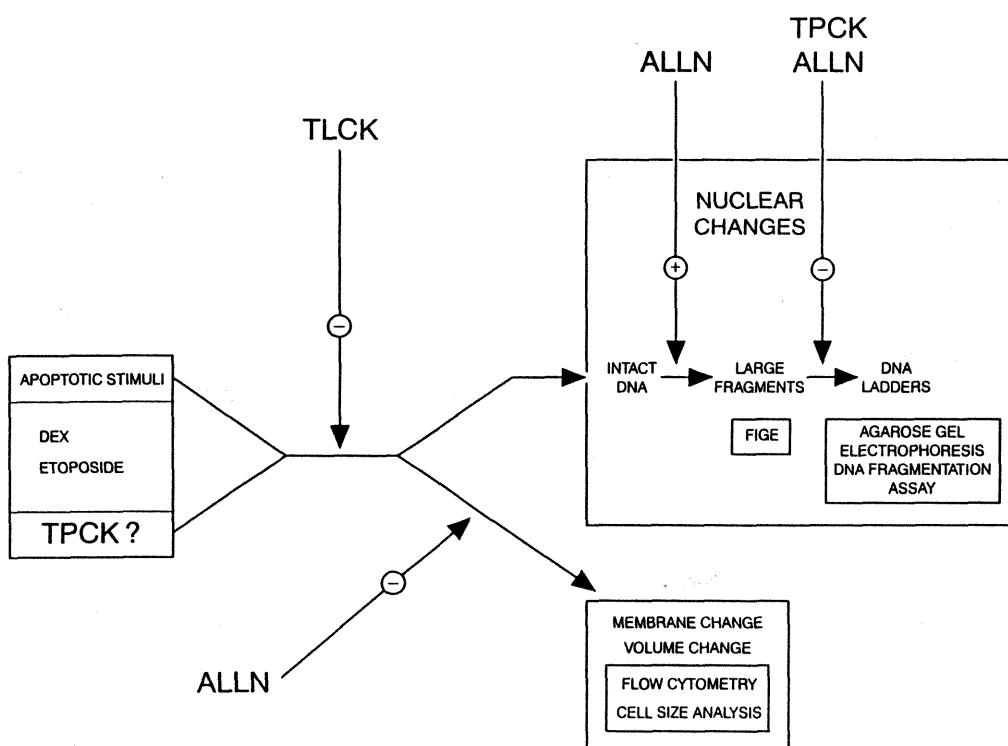
changes of apoptosis. The substrate specificity of ICE means that TLCK will inhibit neither this protease (Thornberry et al. 1992) nor the ICE-like protease (prICE) in a cell free system (Lazebnik et al. 1994). Likewise, z-VAD.FMK will not inhibit trypsin-like proteases which require a basic amino acid in the P1 position (Powers and Harper 1986). Therefore the data are consistent with at least two proteases acting as common mediators of thymocyte apoptosis at a very early stage of the process.

Four other inhibitors tested (YVAD.CHO, YVAD.COCH$_3$, z-VAD.DCOMK and YVAD.CMK) did not block either dexamethasone or etoposide induced apoptosis. No direct evidence is presented to show that they gained access to the intracellular environment although YVAD.CHO has previously been shown to inhibit interleukin-1β maturation in intact cells (Thornberry et al. 1992). Members of the ced-3/ICE-like protease family are descibed as such largely because they share a requirement for aspartic acid in the P1 position. While all the inhibitors employed in these studies possessed such a P1 residue the ability of only one (and only at high concentrations) to inhibit thymocyte apoptosis suggests that there are other constraints on substrate recognition within this family of proteases. This is supported by the observation that the effects of an ICE-like protease, prICE responsible for poly(ADP-ribose) polymerase breakdown and chromatin condensation in isolated nuclei were not mimicked by ICE (Lazebnik et al. 1994).

CHAPTER 6
A HIERARCHY OF PROTEASES IN APOPTOSIS

The evidence that proteases play a role in apoptosis has come from two distinct approaches; the identification of the genes required for programmed cell death and use of inhibitors of proteolysis to prevent apoptosis. As a consequence a number of proteases have been identified in a variety of systems.

Members of the Ced-3/ICE-like family.

The genetic approach has identified what is now a growing family of related proteases (Table 1).

Table 1. Members of the ced-3/ICE family.

ICE-like protein	Ref
Ced-3	Yuan et al. 1992
Nedd-2	Kumar et al. 1994
Ich-1$_L$	Wang et al. 1994
Ich-1$_S$	Wang et al. 1994
CPP32	Fernandes-Alnemri et al. 1994
prICE	Lazebnik et al. 1994

The gene *ced-3* is required for programmed cell death in the nematode *C. elegans* and the protein encoded has homology with the mammalian cysteine protease, interleukin-1β-converting enzyme (ICE) (Yuan et al. 1993). Over expression of either *ced-3* or ICE in rat fibroblasts results in apoptosis (Yuan et al. 1993), which can be inhibited by the *crmA* gene which encodes a serpin that inhibits ICE (Ray et al. 1992; Gagliardini et al. 1994). More recently other ICE/*ced-3* related mammalian genes have been identified (*nedd-2*, Kumar et al.1994; *ich-1$_L$*, Wang et al. 1994; CPP32, Fernandes-Alnemri et al. 1994) which induce apoptosis. Therefore proteolysis is a modulator of apoptosis (or a control point in apoptosis) rather than occurring as a consequence. In addition an ICE-like protease activity (prICE) has been identified which induces apoptotic changes in isolated nuclei (Lazebnik et al., 1994) giving biochemical support to the role of ICE-like proteases. The gene *ich-1* is related to both *ced-3* and ICE but alternative splicing yields two distinct mRNAs, *ich-1$_L$* and *ich-1$_S$* (Wang et al.1994). *Ich-1$_L$* encodes a protein which shares homology with both the p20 and p10 subunits of ICE and over expression causes cell death. *Ich-1$_S$* is a truncated version of *ich-1$_L$* and its over expression suppresses cell death in fibroblasts induced by serum withdrawal. In isolated nuclei prICE induces proteolysis of poly (ADP-ribose) polymerase and chromatin condensation, both features of apoptosis (Wyllie 1980; Kaufman, 1989) (Fig. 1). However, in the same model, purified ICE cannot produce the same changes (Lazebnik

et al., 1994). Thus, not only can ICE/*ced-3* related genes act as both executors of and saviours from apoptotic death, but, in at least one instance, function is not conserved. The last statement assumes that ICE-like proteolytic activity is not only required but also that it is sufficient for apoptosis. This may or may not be true, so the inability of ICE to substitute for prICE may reflect a lack of other factors required for apoptosis rather than a lack of conserved function. An in-vivo role for a mammalian ICE-like protease has also been demonstrated. Proteolytic degradation of the extracellular matrix during involution of the mammary gland triggers apoptosis, an effect that can be mimicked in-vitro with CID-9 mammary epithelial cells (MEC) cultured in the absence of exogenous extracellular matrix. In both instances ICE mRNA was expressed and protein was also detected in cultured MEC cells. In contrast, in lactating breast there is no apoptosis and ICE mRNA is not present, nor is it expressed in MEC cultured on exogenous extracellular matrix. It therefore appears that the extracellular matrix suppresses expression of ICE and consequently apoptosis in MEC (Boudreau et al. 1995).

Proteases and Cytotoxic T-cell killing

Cytotoxic T cells (CTL) and natural killer cells possess cytoplasmic granules used to kill their targets that contain a cocktail of proteins and proteoglycans (Trapani and Smythe 1993). Amongst the proteins there are a number of serine proteases (Tschopp et al. 1988; Hudig et al. 1991; Shi et al. 1992; Odake et al. 1991; Fruth et al. 1987). The initial step is recognition of a target cell followed by the exocytosis of granule contents. One granule protein, perforin, polymerises in the target cell membrane forming a pore through which other granule components enter and as a result the DNA of the target cell undergoes rapid fragmentation. Two of the serine proteases present in the granules, Granzyme A and Granzyme B have been shown to be sufficient for DNA fragmentation (Hayes et al. 1989; Shiver et al. 1992; Shi et al. 1992; Heusel et al.1994). Granzyme A is a serine protease with trypsin-like activity while Granzyme B is a serine protease that, like ICE-like proteases, requires an aspartic acid in the P1 position. Although this suggests that ICE and Granzyme B share similar substrate specificity a recent report has shown that Granzyme B does not cleave the immature form of interleukin-1β (Darmon et al. 1994). Therefore recognition of substrate has additional constraints which are as yet unidentified and in this context it is interesting that an ICE-like protease but not purified ICE can induce apoptotic changes in nuclei (Lazebnik et al. 1994). A central role in CTL killing for serine proteases and trypsin-like proteases in particular is also supported by inhibitor studies which have shown that both

Figure 1.

CTL killing

release from CTL

TARGET CELL

Granzymes (serine proteases)
including tryp-ases, Asp-ases and Met-ases.

Substrates ?

Perforin
p40-TIA
Granzymes

CTL granule fuses with membrane & releases its contents

calcium dependent polymerisation perforin monomers to form a pore in the cell membrane and allow entry of other granule contents

p40-TIA N☐___C

p15-TIA ☐C

$\rightarrow$ DNA fragmentation (Shi et al. 1992)

3 RNA binding domains

Trypsin cleavage site, Phe-Val-Arg

protein/protein interaction?
p15-TIA is sufficient for DNA fragmentation

N☐___ interaction with ADP-ribosylated proteins? (suggested by Tian et al. 199)

TNF-induced death

TNF   TNF-R

Liberation of ceramide
Proteolysis of I κB and activation of NF-κB
phospholipase A2 activation

inhibited by a range of serine protease inhibitors.
(TLCK, TPCK, APNE, TAME, PMSF)
(Ruggiero et al. 1987; Suffys et al 1988)
PAI-2, a serpin confers resistance
(Kumar and Baglioni 1991)

Cytolysis, DNA fragmentation

Lazebnik et al. 1994

NORMAL NUCLEUS $\rightarrow$ APOPTOTIC NUCLEUS

Normal chromatin $\xrightarrow{\text{inhibited by YVAD.CMK}}$ condensed chromatin

PARP (116 kDa) $\xrightarrow{\text{pr-ICE, inhibited by YVAD.CMK}}$ PARP degraded to a range of products. 85 kDa fragment most stable.

Lamin $\xrightarrow{\text{TPCK & TLCK sensitive protease}}$ Lamin degraded

APOPTOTIC NUCLEUS
Condensed chromatin
PARP degraded
Lamin degraded

TCR-induced death and a protease cascade   (Sarin et al. 1993)

Murine T-cell hybridoma $\rightarrow$ TCR mediated rise in $[Ca^{2+}]_i$ $\rightarrow$ calpain activity increased $\rightarrow$ serine protease activity increased $\rightarrow$ apoptosis
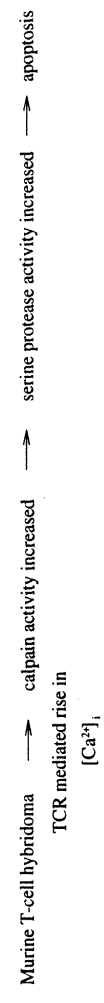
the protease inhibitor TLCK (Chang and Eisen 1980) and isocoumarin protease inhibitors (Hudig et al. 1991) can prevent granule mediated cell death (Figure 1).

Proteases and TNF induced cell death

TNF induced cell death often displays features of apoptosis (Gerschenson and Rotello 1992). TNF binds to a plasma membrane receptor and the receptor-TNF complex is internalised leading to intracellular signalling that culminates in cell death. Although the signalling events are largely unknown, there is evidence that supports a role for proteolysis in the control of TNF action. A wide range of protease inhibitors prevent TNF mediated death (Ruggiero et al. 1987; Suffys et al. 1988). TPCK and TLCK are effective inhibitors of TNF induced cell death and in addition, PAI-2 (plasminogen activator inhibitor-2), a serpin confers resistance to TNF (Kumar and Baglioni 1991). Wright et al. (1994) have purified a 24 kDa protease from U937 cells undergoing TNF induced DNA fragmentation that cleaved synthetic substrates of elastase-like proteases but not those of trypsin-like or chymotrypsin-like proteases. The activity of the protease isolated from apoptotic cells was ~10 times greater than that recovered from normal cells and the purified protein induced DNA laddering in isolated U937 nuclei. TNF liberates ceramide from the cell membrane by activation of either phospatidylcholine specific phospolipase C or acidic sphingomyelinase. Ceramide has been proposed as a mediator of a common apoptotic pathway (Hannun and Obeid 1995). Precisely how TNF brings about cell death is unclear, however, as a result of TNF induced sphingomyelinase activity, the release of ceramide (Machleidt et al. 1994) or some alternate signalling molecule (Betts et al. 1994) increases the activity of the transcription factor NF-κB. This transcription factor is rendered inactive by binding to an inhibitory protein I-κB and dissociation requires both a change in the phosphorylation state of I-κB and proteolysis of I-κB. Thus proteolysis is playing a role in a signalling pathway that may lead to cell death (Figure 1).

Proteases and thymocyte apoptosis

In TCR-mediated apoptosis in both a T-cell hybridoma and peripheral T cells the effects of protease inhibitors has suggested the involvement of more than one protease and that these proteases are involved a different stages of the process (Sarin et al. 1993, Diagram 1). Bruno -et al. (1992) described the inhibition of dexamethasone-induced internucleosomal cleavage in thymocytes by TPCK and TLCK. The relationship between proteolysis and endonuclease activity was further examined by investigating the effects of protease inhibitors on the formation of large kilobase pair sized fragments of DNA. This was carried out in both thymocytes and thymocyte nuclei (Weaver et al. 1993) and isolated hepatocyte nuclei (Zhivotosky et al. 1994; Cain et al. 1995). In intact thymocytes three protease inhibitors (3,4 dichloroisocoumarin, phenylmethanesulphonyl fluoride, and TPCK) prevented the

conversion of large kilobase pair sized DNA fragments into a DNA ladder but did not prevent the formation of fragments greater than 50 kbp in size (Weaver et al. 1993). This data was interpreted as showing a role for serine proteases and that the serine protease is a pre-requisite for DNA laddering. However 3,4 dichloroisocoumarin had no effect on calcium/magnesium dependent DNA fragmentation in isolated thymocyte nuclei. In addition, both 3,4 dichloroisocoumarin and TPCK will inhibit some thiol protease so a role for such proteases could not be excluded.

Data from thymocyte demonstrated that protease inhibitors did not act directly on the calcium and magnesium dependent endonuclease(s) (Weaver et al. 1993). The effects of protease inhibitors on DNA fragmentation have also been examined in isolated hepatocyte nuclei. These nuclei possess Mg^{2+} and Ca^{2+}/Mg^{2+} dependent endonucleases which produce patterns of DNA fragmentation similar to that seen in isolated thymocyte nuclei. TLCK, TPCK, ALLN and N-acetyl-leucinyl-leucinyl-methional did not prevent the initial DNA cleavage to fragments of or greater than 300 kbp in size but did prevent the further degradation of these fragments to 50 kbp and ladders, suggesting a role for proteolysis within the DNA degradation process. The serine protease inhibitor 3,4-dichloroisocoumarin also prevents Ca^{2+}/Mg^{2+} dependent DNA cleavage (Cain et al. 1995) in contrast to the effects in thymocyte nuclei reported by Weaver et al. (1993). Cain et al. (1995) attribute the action of 3,4-dichloroioscoumarin to its thiol reactivity rather an inhibition of a specific protease for 3 reasons. First, the effects of DCI are closely mimicked b the effects of N-ethyl maleimide, a classic thiol blocking agent. Second, co-incubation of 3,4-dichloroioscoumarin with dithiothreitol removed the inhibitory activity of 3,4-dichloroioscoumarin and finally, cadmium and mercury which have a high affinity for thiol groups also blocked Mg^{2+} and Ca^{2+}/Mg^{2+} dependent DNA fragmentation.

The previous chapter describes an investigation of the effects of a number of protease inhibitors on a range of biochemical and ultrastructural changes associated with thymocyte apoptosis. The marked contrast between the effects of TLCK and TPCK on thymocyte apoptosis suggested roles for trypsin-like and chymotrypsin-like proteases at different stages of the apoptotic pathway. TLCK acted at a very early stage of the process, inhibiting all changes associated with apoptosis. TPCK prevented only the terminal biochemical and morphological changes induced by dexamethasone or etoposide. However, at the same concentration TPCK alone caused biochemical and morphological changes associated with early apoptosis. TPCK clearly has two distinct effects and therefore is likely to have two distinct targets in the cell. One target is required for the progression of early apoptotic cells to a fully apoptotic phenotype. The data suggests that this target is nuclear which is consistent with the results obtained from both thymocyte (Weaver et al. 1993) and

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hepatocyte (Zhivotosky et al. 1994) nuclei. Alkylation of the second target by TPCK produces many characteristics of apoptosis. This effect of TPCK may be explained in terms of either specific or non-specific targets. A specific target may be a negative regulator of apoptosis and its modification by TPCK releases the apoptotic machinery which had previously been held in check. Alternatively alkylation by TPCK may be detected as non-specific damage by the affected cell which subsequently opts for controlled death rather than attempt repair.

The effects of z-VAD.FMK support a role for an ICE-like protease early in the apoptotic process prior to the classical biochemical and morphological changes. TLCK does not inhibit ICE-like proteases and z-VAD.FMK does not inhibit trypsin-like proteases, therefore both types of protease are involved at this early stage. Determining which is upstream of the other is impossible with the data currently available. To make this decision the relevant substrates must be identified and their degradation in the presence of inhibitor assessed. While z-VAD.FMK was effective, it inhibited thymocyte apoptosis at concentrations approximately four times greater than those required for complete inhibition of etoposide induced apoptosis in THP.1 cells (personal communication H. Zhu). In addition four other related ICE inhibitors were ineffective. Thus the presence of a P1 aspartic acid is not sufficient for inhibition of apoptosis. Three of the ineffective inhibitors have tyrosine in the P4 position which gives high specificity for ICE but not necessarily for ICE homologs. Interestingly ICE can not substitute for prICE in a cell free model of apoptosis (Lazebnik et al. 1994) nor can Granzyme B, which cleaves at aspartic acid residues and induces DNA fragmentation, cleave interleukin-1β (Darmon et al. 1994). These data may be explained by the existence of isoforms of the ICE-like protease in different cells and these isoforms having different affinities for inhibitors.

A hierarchy of proteases

All of the data so far points to a role for proteolysis at more than one stage of the apoptotic process and that the proteolysis of each step is mediated by distinct proteases. This raises the interesting possibility of a cascade of proteolysis in apoptosis, each protease activating the next. Such an idea has previously been suggested by Sarin et al. (1993). T-cell receptor (TCR) mediated cell death in 2B4 T-cell hybridoma cells and peripheral T-cells is prevented by a range of inhibitors of both serine and cysteine proteases (Sarin et al. 1993). The effectiveness of diverse protease inhibitors suggested that increased intracellular calcium following TCR signalling triggers calpain, a calcium dependent cysteine protease which then activates serine proteases, a chain of events that culminates in apoptosis. By analogy, thymocyte apoptosis also appears to involve different proteases at distinct stages of the apoptotic process and thus requires a protease cascade.

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A distinction must be made between proteolysis induced by a specific agent and proteolysis associated with many different stimuli. Apoptosis has been defined as consisting of private signalling pathways induced by specific stimuli converging on a series of changes common to all apoptotic stimuli (Cohen 1991). Within the common apoptotic pathway there is a cascade of changes leading to the typical morphological and biochemical characteristics of apoptosis (Kerr et al.1987; Brown et al. 1993; Oberhammer et al. 1993; Cohen et al. 1994). To ensure the events studied lie on a common apoptotic pathway and are not limited to a particular agent, a number of apoptotic stimuli with diverse modes of action were used. The inhibition by TLCK and z-VAD.FMK of apoptosis induced by diverse stimuli suggests that both a trypsin and an ICE-like protease are involved at an early stage within the common pathway (Figure 2). Which protease plays the earlier role cannot be defined yet because of the similarity between the two inhibitors in terms of their effects on apoptosis. However both proteases are upstream of a chymotrypsin-like protease which is required for the progression from an early stage of apoptosis to a fully apoptotic state. In addition to its effect within a common pathway, the induction of early apoptotic changes by TPCK suggests that it also triggers a private pathway.
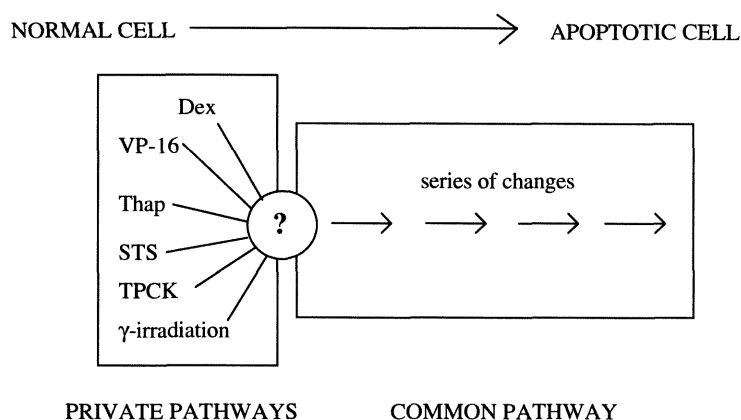
Figure 2. A wide range of agents induce apoptosis in thymocytes, dexamethasone (Dex), etoposide (VP-16), thapsigargin (Thap), staurosporine (STS), TPCK and γ-irradiation. In this model, independent pathways converge on a final common pathway at ?, which may represent either an ICE-like protease, a trypsin-like protease or some effector upstream of these proteases. Downstream there is a series of steps including a TPCK sensitive step required for the conversion of large DNA fragments into ladders.

Is calpain involved?

A role for calpain in thymocyte and myelocyte apoptosis (induction and release models of apoptosis) has been proposed by Squier et al. (1994). These authors present three lines of

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evidence that calpain is an important and early regulator of thymocyte apoptosis. First, inhibitors of calpain (ALLN and MDL-28,170) prevented dexamethasone induced death. Second, following dexamethasone exposure cytosolic, calcium dependent and ALLN sensitive proteolysis was transiently increased. Finally, in response to dexamethasone calpain underwent autoproteolysis, an event associated with its activation. The data presented here may suggest a role for calpain in the process but is complicated by the DNA fragmentation induced by ALLN and by the inhibition of cathepsins by ALLN. The different effects of ALLN also make it difficult to place in a hierarchy of proteases.

Proteins degraded during apoptosis

A number of intracellular proteins undergo proteolysis as a result of apoptosis (Table 2) and some are degraded earlier than others; in HL-60 cells poly (ADP-ribose) polymerase is degraded within 1-2 h followed by degradation of lamin B, then topoisomerase I and II and finally histone H1. Thus degradation of specific proteins may be associated with different stages of apoptosis, reflecting a role for degradation as a control point rather than proteolysis being part of the gross degradation of intracellular macromolecules.

During apoptosis in thymocytes and HL-60 cells degradation of poly (ADP-ribose) polymerase occurred earlier than that of other proteins (Kaufman 1992) and its proteolysis was described as an early marker for apoptosis. In addition poly (ADP-ribose) polymerase is cleaved at an aspartic acid residue by an ICE-like protease in nuclei undergoing apoptotic chromatin condensation (Lazebnik et al. 1994). Poly (ADP-ribose) polymerase is a DNA binding protein that catalyses ADP-ribosylation of itself and other proteins (de Murcia et al. 1995) but its role in apoptosis is unclear. Auto-ADP-ribosylation plays a role in the recognition of DNA strand breaks which are subsequently repaired (Satoh and Lindahl 1992) and activation of poly (ADP-ribose) polymerase is associated with internucleosomal cleavage in HeLa cells (Guano et al. 1994). ADP-ribosylation consumes NAD^+ and can rapidly deplete cell reserves of ATP leading to loss of homeostasis and cell death. Degradation of poly (ADP-ribose) polymerase may prevent inappropriate DNA repair following apoptotic DNA fragmentation thus avoiding a loss of homeostasis and cell lysis. Alternatively, endonucleases responsible for DNA fragmentation may be maintained in an inactive form by ADP-ribosylation but if poly (ADP-ribose) polymerase is degraded the endonuclease is released from this control and DNA cleavage occurs (Rice et al. 1992).

Lamins and topoisomerases are proteins that bind to matrix associated regions of DNA and may play a role in higher order chromatin structure (Pienta et al. 1991) while histone H1 is associated with the linker region between nucleosomes (Hansen and Ausio 1992). Thus all these proteins play a role in controlling chromatin conformation and their degradation may trigger changes in

conformation allowing access to endonucleases responsible for the DNA cleavage typical of apoptosis. In addition lamins and topoisomerases are also components of the nuclear scaffold and their degradation may also alter nuclear architecture allowing endonuclease access to the DNA and result in the morphology typical of apoptosis (Weaver et al. 1993).

However some doubts have been raised about the hypothesis that proteolysis of lamins and poly (ADP-ribose) polymerase are events of a common apoptotic pathway. Ucker et al. (1992) describe both the solubilisation and subsequent proteolysis of lamins in CTL mediated cell killing

Table 2. Proteins known to be degraded in apoptosis

Substrate	cell type	Ref.
Lamins	HL-60, rat thymocytes,	Kaufman 1989
	NIH 3T3	Ucker et al. 1992
		Oberhammer et al. 1994
	cell free system using isolated nuclei	Lazebnik et al. 1994
Topoisomerase I	HL-60, rat thymocytes	Kaufman 1989
Topoisomerase II	HL-60, rat thymocytes	Kaufman 1989
Poly (ADP-ribose) polymerase	HL-60, rat thymocytes	Kaufman 1989
	cell free system using isolated nuclei	Lazebnik et al. 1994
U1-70 kDa	HeLa	Casciola-Rosen et al. 1994
Histone H1	rat thymocytes	Graziev 1991
PAI-2	promyelocytic cell, NB_4	Jensen et al. 1993
Nucleolin	P815 mastocytoma	Pasternack et al. 1991
TIA/TIA-R	CTL targets	Tian et al. 1991
Calpain	murine myelocytes & thymocytes	Squier et al. 1994

as a consequence of death and not a trigger for other chnages which occur. Neither lamins nor poly (ADP-ribose) polymerase were degraded as a result of epidermal growth factor (EGF) induced apoptosis in a breast cancer cell line (Armstrong et al. 1994) and other authors report an actiavtion of poly (ADP-ribose) polymerase during apoptosis (Marks and Fox 1991; Ucker et al. 1992; Guano et al. 1994). Thus while both these proteins may be degraded in some forms of apoptosis, their proteolysis is not required and if it does occur it is likely to be either as part of a private signalling pathway or a dispensable consequence of apoptosis. In either case, poly (ADP-ribose) polymerase is unlikely to be the relevant target for an ICE-like protease within a common apoptotic pathway (Lazebnik et al. 1994).

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Nucleolin, a protein involved in cytoplasmic-nuclear trafficking has been shown to bind Granzyme A (Pasternack et al. 1991), a serine protease from CTL granules which, as previously discussed, is able to induce DNA fragmentation (see discussion of CTL). Nucleolin also undergoes proteolysis by Granzyme A producing an 88 kDa fragment which retains the ability to bind Granzyme A. These authors could not distinguish whether binding to and proteolysis of nucleolin by Granzyme A was required either for the translocation of the protease to the nucleus where it triggered DNA fragmentation or whether the degradation of nucleolin was directly involved in DNA fragmentation. Recently an additional granule protein, TIA-1, capable of inducing DNA fragmentation has been identified (Tian et al. 1991). Two isoforms of TIA-1 exist, a 40 kDa protein (p40) and a 15 kDa (p15) protein. The p40-TIA-1 is structurally related to poly (A) binding proteins and contains 3 RNA binding domains and a glutamine rich C-terminus domain. The p15 isoform appears to be derived from the carboxy-glutamine rich domain of p40 by proteolysis at a trypsin cleavage site (Phe-Val-Arg) present in p40. Both the p40 and p15 proteins can induce DNA fragmentation in digitonin permeabilised thymocytes. A TIA related protein, TIA-R has been identified in cells other than CTLs (Kawakami et al. 1992) raising the possibility that cleavage of endogenous TIA-R by trypsin like proteases may form part of a common apoptotic pathway (Smythe et al. 1994). In CTL killing the CTL supplies both the protein required for DNA fragmentation (TIA-40) and the means for activating it (by Granzyme A mediated proteolysis) (Diagram 1). By analogy, in other cell types undergoing apoptosis the process may be initiated by an increase in TIA-R and its activation by proteolysis.

As described above the serpin PAI-2 can prevent TNF-induced cell death (Kumar and Baglioni 1991). Proteolysis of an intracellular form of PAI-2 has been described as a marker for apoptosis in myeloleukaemia cells (Jensen et al. 1994). PAI-2 is a serpin with some homology to the product of crmA, a serpin which inhibits ICE. The P1 residue of crmA is aspartate (Ray et al. 1992) and that of PAI-2 is arginine so they are unlikely to interact with the same protease. PAI-2 proteolysis during apoptosis produced a 33 kDa protein which retains the ability to bind urokinase plasminogen activator (Jensen et al. 1994). In addition no inhibition by PAI-2 of a protease involved in apoptosis has been proven nor whether proteolysis is a controlling step in cell death or part of gross protein degradation. Thus the role of PAI-2 in apoptosis is unclear.

A wide range of calpain substrates including calpain itself have been identified. These include protein kinases, protein kinase C (PKC) (Kishimoto et al. 1993; Melloni et al. 1986), pp60src tyrosine kinase (Oda et al. 1993), components of the cytoskeleton; talin and filamin (Kwak et al. 1993; Basse et al. 1994), membrane calcium pumps (Salamino et al. 1994), phospholipases (Park et al.1993; Banno et al. 1994) and a number of transcription factors (Hirai et al. 1991; Watt and Molloy 1993). Proteolysis of PKC by calpain may regulate kinase activity as cleavage of the regulatory domain from the catalytic domain produces a constitutively active form of PKC, PKM.

However, the role of protein kinase C in thymocyte apoptosis is a controversial subject as there are conflicting reports of PKC playing a role in either inducing apoptosis (Kizaki et al. 1989a; Kizaki et al. 1989b) or preventing it (McConkey et al. 1990; McConkey and Orrenius 1991, Forbes et al. 1992). Therefore it is difficult to interpret the effects of ALLN on apoptosis in terms of PKC activity. Thymocyte apoptosis, in many instances displays a requirement for transcription and translation. Calpain also cleaves a wide range of transcription factors, c-Fos and c-Jun (Hirai et al. 1991; Watt and Molloy 1993) as well as Pit-1, Oct-1, CP1a and b, c-Myc, ATF/CREB, AP2 and AP3 (Watt and Molloy 1993). It is unclear whether calpain mediated proteolysis removes or modulates the activity of transcription factors, however by affecting transcription, calpain may be acting very early in a common apoptotic pathway.

In summary, the data support a role for a number of proteases in apoptosis and that these proteases are likely to be involved in both private signalling pathways and the final common apoptotic pathway. Within the common pathway there appears to be a hierarchy of proteases, some playing early roles in controlling the process (Yuan et al. 1993; Lazebnik et al. 1994) and others playing later roles during, for example DNA fragmentation (Weaver et al. 1993; Zhivotosky et al. 1994). In addition a number of proteins are degraded during apoptosis (Kaufman 1989; Kaufman et al. 1992; Ucker et al. 1992; Oberhammer et al. 1994; Tian et al. 1991; Jensen et al. 1994; Lazebnik et al. 1994) but of those so far identified none are the relevant substrates of proteases which are at or near the apex of a common apoptotic pathway.

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