IN Volvement of DIFFerent PROT eases in the execution and regulation of Apoptosis in Human Monocytic THP.1 CELLS

By

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INVOLVEMENT OF DIFFERENT PROTEASES IN THE EXECUTION AND REGULATION OF APOPTOSIS IN HUMAN MONOCYtic THP.1 CELLS

Huijun Zhu

ABSTRACT

Ced-3, a cysteine protease, is a key effector of apoptotic cell death in Caenorhabditis elegans. However the role of the Ced-3 mammalian homologues, caspases, and other classes of proteases in apoptosis has not been well understood. This study investigated the involvement of proteases in apoptosis induced by different mechanisms using human monocytic THP.1 cells as a model.

Apoptosis, as assessed by morphological and biochemical changes, including nuclear condensation and fragmentation, internucleosomal DNA cleavage and poly-(ADP-ribose) polymerase (PARP) degradation, was induced by cycloheximide (25 μM), thapsigargin (100 nM), etoposide (25 μM) and staurosporine (0.5 μM). The induction of apoptosis by all these stimuli was enhanced by N-tosyl-L-lysyl chloromethyl ketone (TLCK) (100 μM), a trypsin-like protease inhibitor, except for etoposide, where apoptosis was inhibited. Staurosporine also induced necrotic cell death, which was prevented by TLCK.

N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) (50-75 μM), a chymotrypsin-like protease inhibitor, induced all the characteristic apoptotic changes except the fully nuclear condensation and fragmentation, although it inhibited internucleosomal DNA cleavage induced by other stimuli.

Caspase-2, caspase-3, caspase-6, and caspase-7 were processed/activated during the induction of apoptosis. Caspase inhibitors either with low selectivity or with higher selectivity for caspase-3 both proved to be potent in the inhibition of apoptosis. However staurosporine-induced necrosis was resistant to the inhibition of a caspase inhibitor.

This study demonstrated that apoptosis can be induced or modified by different protease inhibitors in a single cell line, implying that proteases are involved in the regulation of apoptosis at multiple stages. TLCK and TPCK inhibitable proteases may control “upstream” events, whereas caspases have a fundamental role in the execution of apoptosis. This study also provides evidence that apoptosis and necrosis involve different protease activities.
## CONTENTS

**ACKNOWLEDGEMENTS**  ii  
**ABSTRACT**  iii  
**LIST OF FIGURES**  ix  
**LIST OF TABLES**  Xi  
**ABBREVIATIONS**  xii  

### CHAPTER 1. INTRODUCTION

1.1 Morphological features of apoptosis 1  
1.2 Biochemistry of apoptosis 2  
  1.2.1 Signalling events associated with apoptosis 3  
  1.2.2 *De novo* protein synthesis 6  
  1.2.3 DNA degradation 6  
  1.2.4 Protein degradation 9  
1.3 Genetic control of apoptosis 11  
  1.3.1 *Ced-3/ICE* gene family 12  
  1.3.2 *Ced-9/Bcl-2* gene family 16  
  1.3.3 Fas 19  
  1.3.4 *Reaper* 20  
  1.3.5 *p53, c-myc* 20  
1.4 Physiological and pathological roles of apoptosis 22  
  1.4.1 Apoptosis in development 22  
  1.4.2 Apoptosis in the immune system 23  
  1.4.3 Apoptosis and autoimmune disease 24  
  1.4.4 Apoptosis and AIDS 25  
  1.4.5 Apoptosis and neurodegenerative disease. 25  
  1.4.6 Apoptosis and neoplastic diseases 26  
1.5 Detection of apoptosis 28  
1.6 Aims of the study 30
CHAPTER 2 MATERIALS AND METHODS

2.1 Materials 33
2.2 Cell culture 33
2.3 Microscopy 33
2.3.1 Fluorescence microscopy 33
2.3.2 Electron microscopy 34
2.4 Flow cytometry 34
2.4.1 HO342/PI flow cytometry 34
2.4.2 In situ terminal deoxynucleotidyl transferase assay 34
2.4.3 Detection of cell cycle 35
2.5 Agarose gel electrophoresis 35
2.5.1 Conventional gel electrophoresis 35
2.5.2 Field inversion gel electrophoresis 36
2.6 Analysis of protease activity 36
2.6.1 Preparation of cell lysates 36
2.6.2 Fluorimetric measurement of protease activity 37
2.7 Western blot analysis 37
2.8 Statistics 38

CHAPTER 3 EFFECTS OF N-TOSYL-L-LYSINYL CHLOROMETHYL KETONE (TLCK) ON APOPTOSIS

3.1 Introduction 39
3.2 Flow cytometric study of apoptosis 41
3.2.1 TLCK potentiates apoptosis induced by CHX and THG but inhibits that induced by etoposide 41
3.2.2 CHX/TLCK induces classical apoptotic morphology 50
3.2.3 STS induces both necrosis and apoptosis and TLCK prevents necrosis but enhances apoptosis 53
3.2.4 Cell-cycle-related analysis of apoptosis: in situ end labelling (ISEL) 55
3.3 Effects of TLCK on biochemical changes of apoptosis 59
3.3.1 TLCK enhances apoptotic DNA degradation induced by CHX, THG and STS but inhibits that induced by etoposide 59
3.3.2 CHX/TLCK and THG/TLCK induce PARP degradation 60
3.3.3 Time course of large kbp DNA degradation and PARP proteolysis induced by CHX in the absence or presence of TLCK 61

3.4 Putative TLCK target 63
3.4.1 Pre-existing TLCK target 63
3.4.2 Effect of TLCK on cell growth 64

3.5 Discussion 66
3.5.1 Mechanisms of the modulation of apoptosis by TLCK 66
3.5.2 The role of TLCK target in cell death and cell growth 67
3.5.3 The role of TLCK target in necrosis and apoptosis 69

3.6 Summary 70

CHAPTER 4. EFFECTS OF N-TOSYL-L-PHENYLALANYL CHLOROMETHYL KETONE (TPCK) ON APOPTOSIS

4.1 TPCK induces apoptosis 71

4.2 Ultrastructural and biochemical study of TPCK-induced apoptosis 71
4.2.1 TPCK induces intermediate phase of apoptotic morphology 71
4.2.2 TPCK induces formation of large kbp DNA fragments 72
4.2.3 TPCK induces processing/activation of caspases and proteolysis of PARP and lamins 72

4.3 TPCK inhibits internucleosomal cleavage of DNA 76

4.4 TPCK modifies the induction of apoptosis by CHX and etoposide in a similar manner to TLCK 77

4.5 Discussion 79
4.5.1 Implication of multiple targets of TPCK in the regulation of apoptosis 79
4.5.2 Different patterns of DNA degradation in apoptosis 81

4.6 Summary 82
CHAPTER 5 EFFECTS OF CASPASE INHIBITORS ON APOPTOSIS

5.1 Introduction 83

5.2 Z-VAD.FMK inhibits apoptosis 84
5.2.1 Flow cytometric assessment 84
5.2.2 Z-VAD.FMK inhibits biochemical changes of apoptosis 86
5.2.3 Z-VAD.FMK inhibits apoptosis assessed ultrastructurally 91

5.3 Z-VAD.FMK failed to prevent cell death induced by STS 91

5.4 Z-DEVD.FMK inhibits apoptosis 94
5.4.1 Inhibition of PARP degradation 94
5.4.2 Inhibition of DNA degradation 95

5.5 Z-VAD.FMK and Z-DEVD.FMK inhibit caspase-3 processing in a different manner 97

5.6 YVAD.CMK is a potent inhibitor of lamin degradation 97

5.7 Discussion 100
5.7.1 Multiple caspases are active in apoptotic cells, while Z-VAD.FMK and Z-DEVD.FMK prevent apoptosis by inhibiting different caspases 100
5.7.2 The YVAD.CMK target is responsible for lamin cleavage 102
5.7.3 Ordering the apoptotic pathway 102
5.7.4 Z-VAD.FMK-inhibitable caspase may not be involved in necrotic cell death 104

5.8 Summary 104

CHAPTER 6 ALTERATION OF PROTEASE ACTIVITIES IN THE INDUCTION OF APOPTOSIS

6.1 Introduction 106

6.2 Boc-Val-Leu-Lys- but not Suc-Ala-Ala-Pro-Phe-cleavage activity is inhibited by TPCK 107

6.3 DEVD- but not YVAD- cleavage activity is associated with the induction of apoptosis 108

6.4 Discussion 111
CHAPTER 7. GENERAL DISCUSSION

7.1 The role of proteases in the execution of apoptosis

7.1.1 Caspases control the execution phase of apoptosis

7.1.2 Contribution of proteolysis to nuclear events of apoptosis

7.2 Multiple proteases are involved in the regulation of apoptosis at early stages

7.3 Future work
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Fig.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The structural organization of chromatin</td>
</tr>
<tr>
<td>1.2</td>
<td>Research programme</td>
</tr>
<tr>
<td>3.1</td>
<td>The structures of TLCK and TPCK</td>
</tr>
<tr>
<td>3.2</td>
<td>HO342/PI flow cytometric analysis of apoptosis</td>
</tr>
<tr>
<td>3.3</td>
<td>Fluorescence microscopic analysis of apoptosis</td>
</tr>
<tr>
<td>3.4</td>
<td>Separation of apoptotic and normal cells by flow cytometry</td>
</tr>
<tr>
<td>3.5</td>
<td>Concentration-dependent effects of TLCK on the induction of apoptosis by CHX and etoposide</td>
</tr>
<tr>
<td>3.6</td>
<td>Ultrastructures of normal and apoptotic THP.1 cells</td>
</tr>
<tr>
<td>3.7</td>
<td>Morphological examination of STS induced-cell death</td>
</tr>
<tr>
<td>3.8</td>
<td>Kinetics of the induction of necrotic and apoptotic cells by STS or by STS/TLCK</td>
</tr>
<tr>
<td>3.9</td>
<td>Analysis of cell cycle-related apoptosis</td>
</tr>
<tr>
<td>3.10</td>
<td>Analysis of STS- and STS/TLCK- induced cell death by flow cytometry using ISEL and HO342/PI methods</td>
</tr>
<tr>
<td>3.11</td>
<td>TLCK enhances internucleosomal cleavage of DNA induced by CHX, THG and STS, but inhibits that induced by etoposide</td>
</tr>
<tr>
<td>3.12</td>
<td>CHX/TLCK and THG/TLCK induce PARP degradation.</td>
</tr>
<tr>
<td>3.13</td>
<td>Time course of the degradation of DNA and PARP induced by CHX or CHX/TLCK</td>
</tr>
<tr>
<td>3.14</td>
<td>Pre-existing TLCK target</td>
</tr>
<tr>
<td>3.15</td>
<td>A, Inhibition of cell growth by TLCK</td>
</tr>
<tr>
<td></td>
<td>B, TLCK induces no apparent cell cycle changes</td>
</tr>
<tr>
<td>4.1</td>
<td>TPCK causes a concentration-dependent induction of apoptosis</td>
</tr>
<tr>
<td>4.2</td>
<td>TPCK causes a concentration-dependent induction of large kbp DNA fragments</td>
</tr>
<tr>
<td>4.3</td>
<td>TPCK induces concentration-dependent processing of caspase-3 and caspase-7</td>
</tr>
<tr>
<td>4.4</td>
<td>TPCK induces caspase-2 processing</td>
</tr>
<tr>
<td>4.5</td>
<td>TPCK causes concentration-dependent proteolysis of PARP and lamin B1</td>
</tr>
</tbody>
</table>
Fig. 4.6 TPCK inhibits internucleosomal cleavage of DNA induced by apoptotic stimuli 78
Fig. 4.7 Both TLCK and TPCK modify the induction of apoptosis by CHX and etoposide in a similar manner 79
Fig. 5.1 Effect of delayed addition of Z-VAD.FMK on the induction of apoptosis 85
Fig. 5.2 Z-VAD.FMK provides long term protection against cell death 87
Fig. 5.3 Z-VAD.FMK inhibits the cleavage of DNA into large kbp fragments 88
Fig. 5.4 Z-VAD.FMK inhibits PARP cleavage 89
Fig. 5.5 Time course of the biochemical changes induced by TPCK and inhibition of these changes by Z-VAD.FMK 90
Fig. 5.6 Z-VAD.FMK prevents ultrastructural features of apoptosis 92
Fig. 5.7 Z-DEVD.FMK inhibits PARP cleavage 95
Fig. 5.8 Z-DEVD.FMK inhibits the induction of large kbp and internucleosomal cleavage of DNA 96
Fig. 5.9 Both Z-VAD.FMK and Z-DEVD.FMK prevent caspase-3 processing but show different patterns 98
Fig. 5.10 YVAD.CMK has little or no effect on the internucleosomal cleavage of DNA and YVAD.CMK inhibits lamin A/C degradation 99
Fig. 5.11 YVAD.CMK effectively inhibits lamin A/C degradation and less effectively inhibits caspase-3 processing 100
Fig. 6.1 The induction of apoptosis is accompanied by an increase in Z-DEVD-AFC cleavage activity 109
Fig. 6.2 Concentration-dependent induction of the Z-DEVD-AFC cleavage activity by TPCK 110
Fig. 6.3 Time course of the induction of the Z-DEVD-cleavage activity by TPCK 110
Fig. 7.1 Apoptotic pathway in THP.1 cell 122
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>Proteins cleaved by caspases or caspase-like proteases during apoptosis</td>
<td>15</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>Bcl-2 family death repressors and promoters</td>
<td>18</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Z-VAD.FMK inhibits apoptosis induced by various stimuli</td>
<td>84</td>
</tr>
<tr>
<td>Table 5.2</td>
<td>Z-VAD.FMK protects cells from apoptosis but not necrosis</td>
<td>94</td>
</tr>
<tr>
<td>Table 6.1</td>
<td>Effect of protease inhibitors on trypsin-like and chymotrypsin-like protease activities</td>
<td>107</td>
</tr>
<tr>
<td>Table 7.1</td>
<td>Effects of caspase inhibitors on apoptotic cell death</td>
<td>114</td>
</tr>
</tbody>
</table>
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-YVAD.CMK</td>
<td>Acetyl-Tyr-Val-Ala-Asp-CMK</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>CHX</td>
<td>Cycloheximide</td>
</tr>
<tr>
<td>FIGE</td>
<td>Field inversion gel electrophoresis</td>
</tr>
<tr>
<td>FSC</td>
<td>Forward light scatter</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>HO342</td>
</tr>
<tr>
<td>ISEL</td>
<td>In situ end labelling</td>
</tr>
<tr>
<td>kbp</td>
<td>Kilobase pair</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>STS</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>THG</td>
<td>Thapsigargin</td>
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<tr>
<td>TLCK</td>
<td>N-tosyl-L-lysinyl chloromethyl ketone</td>
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<tr>
<td>TPCK</td>
<td>N-tosyl-L-phenylalanyl chloromethyl ketone</td>
</tr>
<tr>
<td>Z-DEVD-AFC</td>
<td>Benzyloxy carbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin</td>
</tr>
<tr>
<td>Z-DEVD.FMK</td>
<td>Benzyloxy carbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone</td>
</tr>
<tr>
<td>Z-VAD.FMK</td>
<td>Benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1 Morphological features of apoptosis

Apoptosis, coined in 1972 by Kerr, Wyllie and Currie, is a descriptive term of a phenomenon associated with physiological cell death (Kerr et al., 1972), which is morphologically distinct from necrosis. Cellular necrosis, an acute pathological form of cell death, is associated with disruption of ionic pumps of the plasma membrane, leading to cytoskeleton disorganisation, cellular and organelle swelling and blebbing of the cell membrane. Finally, disruption of nuclei, organelles, and plasma membrane takes place as well as release of chromatin from disintegrating nuclei. The contents released from dying cells perturb the cellular environment, resulting in inflammatory effects to neighbouring cells (reviewed in Wyllie, 1992; Arends et al., 1990).

In contrast, apoptosis involves a distinct morphological process (Arends and Wyllie, 1991; Wyllie et al., 1980). The loss of cell surface features such as cell/cell junctions, microvilli, and other surface specialisations are early events, and the cell progressively presents a smoother outline with the plasma membrane intact. Apoptotic cells shrink as a result of a reduction in the water content. Cytoplasmic organelles are thus crammed and closer together, although they remain functional and morphologically intact. Much of the water loss is initially into the endoplasmic reticulum of the apoptotic cells so although overall the cell is shrinking, the cisternae of the endoplasmic reticulum dilates. The endoplasmic reticulum develops many outlets to the surface of the plasma membrane, serving to channel water away, and giving the cell a typical vacuolated, pitted surface. Mitochondria shows none of the distension that is characteristic of necrotic cells, and thus remains functional late into the apoptotic process.

The most noticeable changes of apoptotic cells occur in the nucleus. Nuclear events begin with the collapse of the chromatin against the nuclear periphery and into one or a few large clumps within the nucleus. The chromatin becomes progressively more condensed. The appearance of the condensed chromatin is one of the hallmark features in the descriptive morphologic appearance of apoptosis. In many cases, the entire nucleus condenses into a single dense ball, in yet other cases, the chromatin adopts a striking half-moon morphology as it condenses against only one side of the nucleus. The nuclei subsequently break up, and the cell forms surface protuberances termed buds. Buds contain condensed chromatin fragments and rapidly pinch off into apoptotic bodies.
When cells become apoptotic, the changes in cell plasma membrane signal phagocytic neighbouring cells to recognize and engulf them and thus to complete the degradation process. The process of phagocytosis is specific, removing only cells committed to die, and rapid, so that uptake is completed before the integrity of the apoptotic cell membrane is lost. Cells not immediately engulfed break down into smaller membrane-bound fragments, called apoptotic bodies. Apoptosis occurs without leakage of intracellular macromolecules therefore, apoptotic cells can be deleted without inflammation and the broad architecture of the tissue can remain undisturbed.

Apoptosis, an alternative model of cell death to necrosis, is often referred to as programmed cell death, since it commonly occurs during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal cell turnover. However apoptosis and programmed cell death are not synonymous since there are many instances of apoptosis that are clearly not programmed but are the cell’s response to changes in its local environment. Apoptosis can be equated with cell suicide in the sense that the dying cells play an active role in their own demise and removal from the organism.

Although apoptosis occurs in diverse cell types, the morphology and the kinetics of the cell death process are in many cases similar (Stanisic et al., 1978; Cohen and Duke 1984; Martin et al., 1988; Arends and Wyllie, 1991). This suggests that the mechanisms responsible for the programmed cell deaths of different cell types and of cells in different organisms could well be the same. Although the biochemical mechanism underlying the morphological changes of apoptosis is largely unknown, it has been intensively studied in recent years.

1.2 Biochemistry of apoptosis

Apoptosis, in contrast to necrosis, is a genetically programmed process. Like cell proliferation and differentiation, it is controlled by hormone and other receptor-mediated biochemical reactions. Studies of exogenous modulation of apoptosis suggest that this process can be induced by many stimuli capable of initiating signalling events, including cytosolic Ca\(^{2+}\) and cAMP accumulation, protein kinase C and tyrosine kinase activity alterations, oxidative stress and ceramide or nitric oxide generation (McConkey and Orrenius, 1994). These studies suggest that apoptosis can be regulated at the level of signal transduction pathways.
1.2.1 Signalling events associated with apoptosis

Ca\(^{2+}\). Increase in intracellular Ca\(^{2+}\) levels have frequently been associated with apoptosis. Much of the interest in intracellular Ca\(^{2+}\) has arisen from the suggestion that DNA is degraded by a Ca\(^{2+}\)/Mg\(^{2+}\) dependent endonuclease (McConkey et al., 1988; Cohen and Duke, 1984). In some circumstances, it has been possible to protect cells by preventing the increase in intracellular Ca\(^{2+}\) (McConkey et al., 1989; McConkey et al., 1990). However, the fact that DNA digestion does not correlate with the intracellular Ca\(^{2+}\) level (Barry and Eastman, 1993; Reynolds et al., 1996), that DNA digestion occurs in the complete absence of intracellular Ca\(^{2+}\)(Reynolds and Eastman, 1996), and that preventing the increase of intracellular Ca\(^{2+}\) does not protect cells (Li and Eastman, 1995), questions the role of such an endonuclease in apoptosis. Moreover, decreasing levels of intracellular Ca\(^{2+}\) have also been suggested as a cause of apoptosis (Kaneko and Tsukamoto, 1994). This experimental evidence indicates that other endonucleases are involved under different circumstances or Ca\(^{2+}\) may only be involved in some apoptotic signalling pathways, and may not act as a direct mediator of DNA digestion or at any other step in the execution of apoptosis. Increased Ca\(^{2+}\) may activate other latent enzymes, including transglutaminase (Wyllie, 1993) which is found in many apoptotic cells and may be involved in the alteration of the cytoadhesive properties of apoptotic cells (Fesus et al., 1991). Transglutaminase may function to provide a highly cross-linked protein shell in apoptotic cells and bodies, so rendering the apoptotic process ‘silent’ in that there is no leakage of intracellular components which might provoke an inflammatory response. Blocking the induction of the enzyme in dying cells by antisense oligonucleotides results in increased leakage of DNA and protein molecules (Fesus, 1993).

Protein kinase C (PKC). The PKC family consists of at least nine known isoforms, subdivided into the conventional (or Ca\(^{2+}\)-dependent), novel and atypical subfamilies (Hug and Saue, 1993). The different PKC isoforms are targets of important lipid second messengers with diacylglycerol as a major cofactor of the classic and novel isotypes ((Nishizuta, 1992), whereas ceramide activates the atypical types (Lozano et al., 1994; Muller et al., 1995). Most of the evidence supporting a role for PKC in apoptosis comes from studies with phorbol esters, a class of tumour promoters, which stimulates apoptosis in thymocytes (Kozaki et al., 1989). It has been shown that phorbol esters that selectively promote activation of the PKC-\(\beta\) isoform can also stimulate apoptosis in myeloid
leukaemia cell (McConkey and Orrenius 1994). Furthermore, glucocorticoid-induced apoptosis in thymocytes may involve selective activation and translocation of PKCe2 (Iwata et al., 1994). Conversely, there is abundant evidence that PKC is antagonistic to apoptotic events. PKC inhibitors are potent inducers of apoptosis in hematopoietic cells (Bertrand et al., 1994; Jarvis et al., 1994). Phorbol 12-myristate 13-acetate (PMA) antagonizes growth factor deprivation-induced apoptosis in myeloid progenitor cell (Lotem et al., 1991), corticosteroid-mediated apoptosis in lymphoid cells (McConkey et al., 1989), and etoposide-induced apoptosis in HL-60 cells (Solary et al., 1993). PKC activity has been associated with the up-regulation of Bcl-2 mRNA and protein, a repressor of apoptosis (Genestier et al., 1995), leading to the prevention of apoptosis induced by tumour necrosis factor (TNF). Atypical PKC subspecies may also play a role in the control of cell survival and cell growth, since par-4, a gene induced during the induction of apoptosis, selectively interacts with PKC ζ resulting in cell growth inhibition and apoptosis (Diaz-meco et al., 1996). These observations suggest that PKC signalling events are critical for cell survival in selected cellular systems.

**Cyclin AMP (cAMP).** Elevation of intracellular cAMP levels can result in either stimulation or repression of specific gene expression. Most of these genes contain one or more cyclic AMP (cAMP)-responsive elements (CREs). cAMP binds to the regulatory subunit of PKA and releases the active catalytic subunit. This subunit phosphorylates the transactivation domain of CRE-binding protein (CREB), which then induces the expression of genes containing CREs. Pharmacological agonists are known to be cytotoxic to certain lymphoid lines in vitro (Daniel et al., 1973), and the effects of cAMP involve changes in protein phosphorylation that lead to apoptosis (Duprez et al., 1993). Agents that elevate cAMP stimulate DNA fragmentation and apoptosis in thymocytes via activation of cAMP-dependent protein kinase (PKA) (McConkey et al., 1990). However, evidence that cAMP can block apoptosis in other model systems has also been demonstrated. Analogues of cAMP inhibit apoptosis in neurones induced by withdrawal of neuronal growth factor (Edwards et al., 1991) and in T-cell hybridomas following triggering of the T cell receptor (Lee et al., 1993). All these observations are consistent with the divergent effects of cAMP on apoptosis in these models.

**Ceramide.** Ceramide is a newly identified lipid second messenger that is generated through the hydrolysis of sphingomyelin by sphingomyelinases (SMase) (Hannun and
Obeid, 1995; Kolesnick and Goide, 1994). Signals from cell surface receptors such as Fas (CD95/Apo1) (Tepper et al., 1995) and tumor necrosis factor receptor (TNFR) (Kim et al., 1991; Dbaibo et al., 1993) activate SMase and generate ceramide. Free ceramide acts as an intracellular messenger, specifically stimulating both a membrane ceramide-activated serine/threonine protein kinase (Mathias et al., 1991) and a cytosolic ceramide-activated phosphoprotein phosphatase (Dobrowsky and Hanunn, 1992). When applied exogenously, ceramide can cause apoptosis and NF-kB and JNK (c-JUN NH$_2$-terminal protein kinase) activation (Obeid, et al., 1993; Wiegman et al., 1994; Verheij et al., 1996). JNK activation was, in fact, proposed to mediate apoptosis in response to either TNF or exogenous ceramide via a c-jun dependent mechanism (Verheij et al., 1996), however, a recent study dissociated JNK activation from apoptosis (Liu et al., 1996). Although several molecules have been proposed to be the targets for ceramide, including protein phosphatase 2A (PP2A) (Dobrowsky et al., 1993), 97-kD serine/threonine protein kinase (Liu et al., 1994), PKC $\xi$ (Lozano et al., 1994) and c-raf (Huwiiler et al., 1996), the targets for ceramide to induce apoptosis are currently unknown.

**Protein Tyrosine Kinase.** Tyrosine phosphorylation of proteins by protein tyrosine kinases (PTKs) is a primary and important step in the initiation of various mitogenic signalling cascades (Ullrich and Schlessinger, 1990, Cantley et al., 1991). Engagement of receptor-associated PTKs stimulates the ras/raf/map kinase pathway (Johnson and Vaillancourt, 1994), which leads to activation of the c-fos and c-jun proto-oncogenes via phosphorylation of pre-existing DNA-bound factors in their transactivation domain (Karin, 1994; Hill and Treisman, 1995). This is one of the immediate-early responses to cytokines. Another immediate-early response is the induction of c-Myc mRNA, the mechanism of which remains unknown. The available data suggest that signalling through protein tyrosine phosphorylation is required for induction and inhibition of apoptosis. The examples include inhibition of apoptosis in human granulocytes due to increased levels of tyrosine phosphorylation (Yousefi et al., 1994) and requirement of tyrosine phosphorylation for apoptosis induced by ionising radiation in human B-lymphocyte precursors (Uckun et al., 1992). Although involvement of tyrosine phosphorylation in apoptosis has been widely documented, the signalling molecules involved in the regulation of apoptosis remain largely unknown.
Reactive oxygen species. The production of overwhelming levels of reactive oxygen species is thought to be an important mechanism of cytotoxicity in a number of systems (Symonds et al., 1994; Lowe et al., 1994). However some studies have shown that apoptosis could still be induced under hypoxic conditions, in which reactive oxygen species generation is inhibited (Schwartz and Bennet, 1995). The role of reactive oxygen species in apoptosis is not yet clear.

1.2.2 De novo protein synthesis

The ability of macromolecular synthesis inhibitors such as actinomycin D and cycloheximide to prevent cell death in some circumstances has been used as evidence that the cell actively participates in its own demise (Martin et al., 1988; McConkey et al., 1989). However, there are numerous contradictory data whereby cells fail to be protected by actinomycin D or cycloheximide (Lin et al., 1992) or actually undergo apoptosis in response to them (Cotter et al., 1992; Waring, 1990). In most cells induction of apoptosis does not trigger expression of genes, the products of which are involved in killing the cell. The most compelling evidence for the idea that all the proteins required for apoptosis are constitutively expressed in mammalian cells has come from experiments with cells whose nuclei have been removed (Jacobson et al., 1994). The requirement of RNA and protein synthesis for the induction of apoptosis in certain situations may reflect the need to synthesize molecules that activate or repress the existing cell death machinery, rather than making any components required for the basic cell death program itself.

1.2.3 DNA degradation

Irrespective of how apoptosis is caused, the DNA is always degraded to some extent, which appears responsible for the characteristic nuclear morphological changes.

In eukaryotic cells the DNA double helix in each chromosome is folded in a highly ordered fashion: the fundamental packing unit being the nucleosome, a histone octamer consisting of two copies of each of four histones, which are small proteins with a high proportion of positively charged amino acids. A new model has been proposed for the structural organization of chromatin: DNA is envisaged as being ordered into loops of approximately 50 kbp in size with these loops being wound into hexameric structures, termed rosettes, containing approximately 300 kbp of DNA (Alison and Sarraf, 1995).
Chapter 1  Introduction

Fig. 1.1 The structural organization of chromatin. DNA has many orders of packing. The loops are made up of a coiled 30 nm fiber which is itself a contracted solenoid comprising six nucleosomes per turn. In some apoptotic cells, chromatin degradation only appears to proceed as far as the release of rosettes (300 kbp) and loops (50 kbp) from the nuclear protein matrix. Further degradation involves cleavage at linker regions between nucleosomes, yielding fragments of integer multiples of the number of base pairs associated with one nucleosome—hence the 'ladder' pattern of DNA degradation products after agarose gel electrophoresis.

Three types of DNA fragmentation can be distinguished during the induction of apoptosis: internucleosomal DNA cleavage, fragmentation into large 50-300 kbp lengths, and single-strand cleavage events. The earliest study using glucocorticoids and rat thymocytes as a model system showed that DNA was fragmented into 180-200 bp lengths prior to cell death, representing the hallmark of apoptosis (Kerr et al., 1972; Wyllie 1980; Wyllie et al., 1980).
In almost all circumstances of morphologically well-characterized apoptosis, internucleosomal DNA cleavage has been the biochemical event used as the definitive apoptotic marker (Schwartzman and Cidlowski, 1993), suggesting that an endonuclease which cleaves the nuclear DNA at linker sections to fragments of low molecular weight, equivalent to single and multiple nucleosomes, is crucial for the induction of apoptosis. The DNA cleavage products can be separated by conventional agarose gel electrophoresis, which can only resolve DNA fragments of ~20 kbp and below. The DNA from apoptotic cells often separates with stepwise increments in DNA conforming to integer multiples of a subunit which corresponds to the number of base pairs associated with each nucleosome, hence the so-called ladder-pattern of DNA cleavage products (Fig. 1.1) (Alison and Sarraf, 1995). Until recently it was assumed that the activation or induction of the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease was solely responsible for internucleosomal DNA cleavage and collapse of the chromatin structure.

Recent studies have shown that DNA laddering is not a cardinal feature of apoptosis. Rat thymocytes induced to undergo cell death by treatment with dexamethasone demonstrated not only internucleosomal DNA cleavage, but also the presence of large DNA fragments (Brown et al., 1993) and the internucleosomal but not the large kbp DNA cleavage was inhibited in the presence of zinc (Cohen et al., 1992a). Similar large DNA fragments, with or without internucleosomal DNA cleavage were seen in different types of cells treated by different stimuli (Roy et al., 1992; Oberhammer et al., 1993). These large fragments corresponded to >600 kbp, 300 and/or 50 kbp when separated by field inversion gel electrophoresis (FIGE). The formation of the large DNA fragments implies that an enzyme cleaves DNA in apoptotic cells at a very high level of DNA organization. These observations call into the exact role of internucleosomal DNA cleavage and indicate that an additional endonuclease activity may be involved in the degradation of DNA during apoptosis. A Mg-dependent endonuclease activity has been identified that appears responsible for the initial stages of DNA fragmentation in liver, thymocyte and other nuclei (Walker et al., 1994; Sun and Cohen, 1994). The differential activation of the endonucleases depends on cell type and apoptotic stimuli specificity, leading to different patterns of DNA cleavage.

Recently, single-strand cleavage of DNA has also been suggested to occur in the apoptotic process. In thymocytes induced to undergo apoptosis by steroid, numerous
single-strand breaks in the internucleosomal region and in the core-histone-associated DNA were observed (Alnemri and Litwack, 1990). However the role of single-stranded DNA breaks is an open question.

Owing to the prominence of nuclear changes during apoptosis, it has been suggested that cells are killed by the endonucleolytic cleavage of their DNA (Roy et al., 1992; Umansky and Theor, 1982). However cells without a nucleus can be induced to undergo the characteristic cytoplasmic changes of apoptosis, implying that nuclear DNA degradation may be not critical for cell death. Although the exact mechanism of death is unknown progress has been made in identifying key components of the apoptotic machinery. The cellular mechanisms responsible for initiation of apoptosis prior to DNA cleavage by endonuclease are being investigated.

1.2.4 Protein degradation

Some 13 years after the discovery of the apoptosis nuclease (Wyllie, 1980), the second major enzymatic activity specific to apoptosis was revealed. This came with the realization that although apoptosis can be distinguished from necrosis in that it is a regulated process proteolysis nevertheless plays an integral role in apoptotic cell death. In many models of apoptosis a proteolytic step is required prior to DNA cleavage (Bruno et al., 1992; Kaufmann, 1989; MacDonald et al., 1980; Sarin et al., 1993; Squier et al., 1994).

The biochemical events of apoptotic execution begin with cleavage of a specific set of proteins. These include homeostatic cellular substrates: poly (ADP ribose)-polymerase (PARP) (Lazebnik et al., 1994), the 70 kD subunit of the U1 ribonucleoprotein (U1-70 kD) (Casciola-Rosen et al., 1994), topoisomerase I, the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (Casciola-Rosen et al, 1995), sterol regulatory element binding protein (SREBP) (Wang et al., 1996b), and GDP dissociation inhibitor, protein D4-GDI (Na et al., 1996), and several structural proteins: fodrin (Martin et al., 1995b), lamins (Lazebnik et al., 1995), nuclear mitotic apparatus protein (NuMA) (Weaver et al., 1996), the product of a growth arrest specific gene (Gas-2) (Brancolini et al., 1995) and histone (Kaufmann, 1989).

The PARP molecule is composed of three functional domains: an N-terminal DNA-binding domain containing two zinc fingers, a central automodification domain, and a C-
terminal catalytic domain (Desnoyers et al., 1996). It has been suggested that PARP
and/or the polymer it produces is involved in chromatin organization (Poirier and
Moveau, 1992) and DNA synthesis (Anachkova et al., 1989). It also appears that PARP is
indirectly involved in DNA repair (Lindahl et al., 1995). During apoptosis, PARP was
specifically cleaved into a C-terminal polypeptide containing the automodification and
catalytic domains, and an N-terminal polypeptide containing the DNA binding domain
(Kaufmann et al., 1993). This cleavage inhibits most of its DNA repair activity. Although
PARP cleavage appears to be a hallmark of apoptotic cell death, it alone is unlikely to be
sufficient for the mediation of cell death as mice with a targeted disruption in the PARP
gene develop normally (Wang et al., 1995b). The functional consequences of PARP
cleavage during apoptosis are unknown.

DNA-PKcs, a second enzyme implicated in DNA repair, is composed of a 460 kD
catalytic subunit and a DNA binding component Ku which is a heterodimer of 70 and 80
kD subunits (Dvir et al., 1992; Gottlieb and Jackson, 1993). DNA-PKcs is degraded into
240 kD and 150 kD fragments as well as a less abundant 97 kD fragment in different cell
types undergoing apoptosis after exposure to different agents (Song et al., 1996).
Degradation of the DNA-PKcs was paralleled by loss of protein kinase activity. Cleavage
was very specific since the DNA-binding component of the enzyme (Ku) remained intact
under these conditions. As in the case of PARP, a functionally active domain, in this case
the phosphatidylinositol 3-kinase (PI3-kinase) domain, was split off from the molecule
(Brancolini et al., 1995).

U1-70 kD is essential for splicing of precursor mRNA to produce mature mRNA
(Sharp, 1994). Cleavage of U1-70 kD during apoptosis might be an energy saving device
preventing the exit of mRNA to the cytoplasm and ensuing protein synthesis (Casciola-
Rosen et al., 1994). Topoisomerase I plays an important role in modifying the supercoiled
structure of DNA during replication and transcription. Its removal during apoptosis would
enhance the inactivation process (Kaufmann, 1989; Voelkel-Johnson et al., 1995). The
nuclear lamina forms a structural network underlying the nuclear envelope and thought to
act as an attachment site for chromosomes in interphase cells. During apoptosis, lamina is
solubilized, much as it is in mitotic cells (Kumar, 1995), but this solubilization is
accompanied/ caused by specific cleavage of all three lamin subunits rather than their
phosphorylation (Martin and Green, 1995; Ellis et al., 1991). The degradation of lamins is
accompanied by disassembly of the nuclear lamina (Lazebnik et al., 1993). This cleavage could potentially disrupt lamin-lamin interactions as well as interactions of lamins with other nuclear components such as matrix attachment regions on the DNA (Luderus et al., 1992), the retinoblastoma gene product (Ozaki et al., 1994), and core histones (Taniura et al., 1995). Cleavage of the histone proteins might facilitate the opening of chromatin structure for endonucleolytic degradation of DNA or cleavage may be a consequence of other alterations to the chromatin.

The cytoplasmic changes of apoptosis might be explained at least in part, by the proteolytic cleavage of cytoskeletal structural proteins. A partial degradation of the cortical cytoskeleton actin (Kayalar et al., 1996), the actin-associated protein fodrin (Martin et al., 1995), and Gas-2 (Brancolini et al., 1995), a protein involved in actin microfilament recognition, may be responsible for the blebbing of the plasma membrane in apoptosis. A recent study demonstrated that cytoplasts from Jurkat cells behave in similar ways to intact cells with regard to all of the cytoplasmic features of apoptosis (Martin et al., 1996), including proteolysis of non-nuclear proteins. This suggests that although the nucleus is a prominent target for the destructive processes that are set in motion during apoptosis, it does not appear to be required for apoptosis-associated membrane or cytoplasmic events. It should be emphasized, however, that cleavage of nuclear substrates during apoptosis may be important to ensure that DNA repair mechanisms are shut down upon entry of the cell into the irreversible phase of the death program.

It is important to note that apoptotic cells do not undergo wholesale proteolytic digestion (Ellis et al., 1991). That proteolysis is an important mechanism underlying programmed cell death has been enhanced by the discovery of the family of caspase genes, which are homologous to Caenorhabditis.elegants (C. elegans) cell death gene ced-3. The products of the caspase genes have been implicated in the degradation of many proteins in the process of apoptosis.

1.3 Genetic control of apoptosis

Although many different signals are capable of affecting apoptosis, a number of genes seems to regulate the final common apoptotic pathway (Alison and Sarraf, 1995; Steller, 1995). There is increasing evidence that apoptosis occurs by a mechanism that has been at
least partially conserved throughout animal evolution. Much of our knowledge concerning the genetic programmes involved in the regulation of apoptosis has been derived from studies in the nematode *C. elegans* (Arends and Wyllie, 1991; Ellis et al., 1991; Vaux, 1993), in which a genetic pathway of programmed cell death has been established (Ellis and Horvitz, 1986; Hengartner et al., 1992). Programmed cell death in *C. elegans* has been divided into four distinct stages, each controlled by a specific set of genes identified as *ced* (cell death defective). These stages concern decisions as to whether or not the cell will die, cell death execution, engulfment of the dead cell by phagocytes and degradation of the engulfed dead cell. There are 14 genes functioning in programmed cell death, but only *ced-3*, *ced-4* and *ced-9* are involved in regulating the execution of cell death. *ced-3* and *ced-4* are required for all somatic cell deaths, whereas *ced-9* is needed to protect cells that normally survive from undergoing programmed cell death (Hengartner et al., 1992). If either *ced-3* or *ced-4* gene is inactivated, all the cells that normally die during development survive and mutations in *ced-3* or *ced-4* suppress the ectopic cell deaths otherwise seen in animals that have lost *ced-9* function (Hengartner et al., 1992). The extra cell deaths that occur in *ced-9* loss-of-function mutants require the activities of *ced-3* and *ced-4*, indicating that *ced-9* acts by preventing *ced-3* and *ced-4* from causing cell death. The *ced-4* gene encodes a 63-kD protein with no significant similarity to other known polypeptides (Yuan and Horvitz, 1992). In contrast, *ced-3* and *ced-9* have known mammalian counterparts that function in cell death.

1.3.1 *Ced3/ICE* gene family

*Ced-3* encodes a protein with 29% homology to a mammalian cysteine protease, interleukin-1β-converting enzyme (ICE) (Yuan et al., 1993). IL-1β is synthesized as an inactive 33 kD propeptide (March et al., 1985) that must be processed by proteolytic cleavage in order to be secreted (Black and Sleath, 1989). ICE is specifically required to cleave pro-IL-1β between residues Asp116 and Ala117 to generate the 17.5 kD active form cytokine (Thornberry et al., 1992). ICE, the first member of a novel cysteine protease family, which shows no homology with cysteine proteases of the papain superfamily, is unusual in that it cleaves at the carboxy-terminal side of an obligatory aspartate (Asp) residue (Sleath et al., 1990; Howard et al., 1991). ICE is produced as a 45 kD proenzyme that can be proteolytically cleaved at four sites (Asp103, Asp119, Asp297, and Asp316) to yield a large (p20) and small (p10) subunit both of which are required for
enzymatic activity (Cerretti et al., 1992; Thornberry et al., 1992). At least two of these cleavage sites are conserved in Ced-3, indicating that the Ced-3 protein might be processed as well. Ced-3 was identified as a cysteine protease (Hugunin et al., 1996), which strongly argues that Ced3/ICE-related proteases may be a key component in the apoptotic pathway. The question whether ICE has a physiological role comparable to Ced-3 has been tested by overexpression of ICE in mammalian cells resulting in apoptosis. This has also been tested by showing that CrmA, a cowpox virus encoded protein and inhibitor of ICE, blocked programmed cell death in chick dorsal-root ganglion neurons induced by the deprivation of nerve growth factor (Ray et al., 1992). However, given that thymocytes and macrophages from ICE-knockout mice undergo apoptosis normally, except possibly for Fas- induced apoptosis, ICE per se is not required for programmed cell death during development, although it may be involved in apoptosis in some circumstances. The target for CrmA may be another member of the ICE family, which is equivalent to Ced-3 in C. elegans.

In the last 3 years, the family of Ced-3/ICE-related cysteine proteases of human origin has grown to at least ten members. "Caspase", referring to cysteine protease with an ability to cleave after aspartic acid, has been used to name these proteases (Alnemri et al., 1996). Comparative studies of the peptide sequence of these family members have enabled investigators to divide the caspases into three subgroups based on the degree of homology. The human caspase family members are listed below and their original names are included in brackets:

- **ICE subgroup**
  - caspase-1 (ICE)
  - caspase-4 (TX, ICH-2, ICErel-II)
  - caspase-5 (ICErel-III, TY)

- **CPP32 subgroup**
  - caspase-3 (CPP32, Yama, apopain)
  - caspase-6 (Mch2)
  - caspase-7 (Mch3, ICE-LAP3, CMH-1)
  - caspase-8 (MACH, FLICE, Mch5)
  - caspase-9 (ICE-LAP6, Mch6)
  - caspase-10 (Mch4)

- **Ich-1 subgroup**
  - caspase-2 (Ich-1)
All members of the caspase family share the highest degree of homology in the region corresponding to the two subunits of ICE (caspase-1) and carry several Asp residues at which cleavage would generate two subunits similar to the p20 and p10 subunits of ICE. Common features of this family include conservation of the pentapeptide active site containing an essential cysteine (Thornberry et al., 1992; Fernandes-Alnemri et al., 1995b), ability to induce apoptosis when overexpressed in a heterologous system, and requirement for proteolytic cleavage of the proenzymes at conserved Asp cleavage sites for activation. Among the members of the caspase family, caspase-3 exhibits the highest similarity to Ced-3 in both sequence homology and substrate specificity (Fernandes-Alnemri et al., 1994; Tewari et al., 1995a; Nicholson et al., 1995), indicating that caspase-3 may be the mammalian equivalent of Ced-3. Caspase-3 knockout mice display decreased neuronal cell death during early development. By contrast, in caspase-3-deficient mouse thymocytes, the induction of apoptosis is similar to that in the thymocytes of their wild-type littermates (Kuida et al., 1996). The restricted phenotype raises the possibility that other members of the caspase family may also have an important role during apoptosis in other tissues, cell types or in response to other apoptotic stimuli.

Isolation of these mammalian ced-3 family genes allows a better biochemical analysis of the programmed cell death pathways. A growing number of proteins degraded during apoptosis has turned out to be the substrates for the caspases. The substrate specificity of protease is described by use of a model in which the active site of the enzyme is considered to contain a series of subsites that accommodate the side chains of amino acid residues near to the bond that is to be cleaved, the scissile bond. The subsites are numbered S1, S2, and so on towards the N-terminus of the substrate polypeptide, and S1', S2' and so on towards the C-terminus. The corresponding amino acid residues of the substrate are numbered P1, P2, etc., towards the N-terminus, and P1', P2', etc., towards the C-terminus (Barrett, 1992). The feature of the proteolysis by caspases is that the Asp residue is absolutely required in P1 position, although different caspases may recognize different sequence in P2-P4 position (Table 1.1).
Table 1.1. Proteins cleaved by caspases or caspase-like proteases during apoptosis

<table>
<thead>
<tr>
<th>Caspase</th>
<th>Substrate</th>
<th>Sequence around cleavage site</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P4  P3  P2 P1</td>
<td>↓  P1'</td>
<td></td>
</tr>
<tr>
<td>Caspase-1</td>
<td>IL-1β</td>
<td>F   E   A  D (27)   G</td>
<td>Thornberry et al., 1992</td>
</tr>
<tr>
<td></td>
<td>Y    V  H  D (116)  A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>PARP</td>
<td>D    E   V  D (213)  G</td>
<td>Lazebnik et al., 1994</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>SPEBP-1</td>
<td>S    E   P  D (460)  S</td>
<td>Wang et al., 1995</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>SPEBP-2</td>
<td>D    E   P  D (468)  S</td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>DNA-PKcs</td>
<td>D    E   V  D (2712)  N</td>
<td>Han et al., 1996</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>PKCδ</td>
<td>D    M   Q  D (330)  N</td>
<td>Emoto et al., 1995</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Rb</td>
<td>D    E   A  D (886)  G</td>
<td>Janicke et al., 1996</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>D4-GDI</td>
<td>D    E   L  D (19)  S</td>
<td>Na et al., 1996</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Lamin A</td>
<td>V    E   I  D (230)  N</td>
<td>Rao et al., 1996</td>
</tr>
<tr>
<td>Caspase-6</td>
<td>Lamin B</td>
<td>V    E   V  D (230)  S</td>
<td></td>
</tr>
</tbody>
</table>

The caspase family members are synthesized and present in cells as inactive precursors (Cerretti et al., 1992; Thornberry et al., 1992; Thornberry and Molineaux, 1995), therefore the activation of the caspases is a crucial step to the pathway of apoptosis. The caspase members are able to auto-process and process other members of the family. Caspase-8 and caspase-10 can process all known caspases (Fernandes-Alnemri et al., 1996), thus these two caspases may be upstream proteases whose activation initiates the downstream cascade reactions. Caspase-1 has autocatalytic activity and also can act on pro-caspase-2 (Harvey et al., 1996), whereas caspase-4 can process caspase-1 (Kamens et al., 1995) and caspase-3 can process caspase-7, caspase-2, caspase-6, and caspase 9 (Fernandes-Alnemri et al., 1995b). Remaining members of the caspase family probably also interact with each other, indicating a cascade of caspase activities may be involved in the execution of...
apoptosis. So far, caspase-3, caspase-6, caspase-7 and caspase-2 have been shown to be activated by proteolytic cleavage in apoptotic cells (Erhardt et al., 1996; Orth et al., 1996; Harvey et al., 1996; Duan et al., 1996a, 1996b). In addition, the caspases also can be activated by other classes of proteases with specificity for Asp at the P1 position. Granzyme B, a serine protease required for cytotoxic T lymphocytes (CTL)-mediated killing of target cells (reviewed by Berke, 1995; Smyth and Trapani, 1995), has been shown to activate caspase-3 (Darmon et al., 1995) and pro-caspase-2 (Harvey et al., 1996), but not pro-caspase-1 (Darmon et al., 1994) and has been shown to be inhibited by CrmA (Quan et al., 1995). These data suggest that in CTL-mediated killing of target cells, granzyme B is the initial signal required for the activation of a downstream effector caspase. The mechanism for the activation of caspases and the role of each member of the caspase family in apoptosis are under investigation by many researchers.

1.3.2 Ced-9/Bcl-2 gene family

Ced-9 encodes a protein similar in sequence and function to the human proto-oncogene bcl-2 (Hengartner and Horvitz, 1994), which was identified as the proto-oncogene translocated to the immunoglobulin heavy chain locus in follicular B-cell lymphomas (Tsujimoto et al., 1985). As a novel class of oncogene, bcl-2 encodes a 26 kD protein which functions in oncogenic cells by inhibiting apoptotic cell death rather than by causing an increase in cell proliferation (Hockenberry et al., 1990; Nunez et al., 1990).

The anti-apoptotic activity of Bcl-2 has been demonstrated widely in various experimental paradigms (reviewed by Reed, 1994). Human Bcl-2 expressed in transgenic C. elegans can rescue nematode cells that die as a consequence of a ced-9-loss-function mutation and Bcl-2 can inhibit the cell deaths caused by the expression of ced-3, caspase-1 or caspase-2. Thus Bcl-2 and Ced-9 seem to be structurally similar and at least somewhat functionally interchangeable. However, so far there is no report to indicate that Ced-9 can substitute for Bcl-2 and function in vertebrates, suggesting that they are members of the same family but are not equivalent. Bcl-2 markedly reduces cell killing induced by a wide variety of stimuli in divergent cellular systems. These include withdrawal of IL-3 in hemopoietic cells (Hockenberry et al., 1990, Nunez et al., 1990) and withdrawal of neurotrophic factor in neurons (Vaux et al., 1988, Garcia et al., 1992). In addition to growth factor dependency, Bcl-2 has been shown to counter death in cancer
cells induced by chemotherapeutic drugs (Miyashita and Reed, 1992; Newmeyer et al., 1994).

The biochemical mechanisms by which Bcl-2 control cell life and death remain elusive, principally because its predicted amino acid sequences have no significant homology with other proteins. Considerable efforts have been expended on examining the subcellular location of the protein in the hope that such information would provide some clues. It has been proven that Bcl-2 resides in the nuclear envelope, parts of the endoplasmic reticulum, and outer mitochondrial membrane but not in other intracellular membrane compartments including the plasma membrane (Monaghan et al., 1992; Jacobson et al., 1993; Krajewski et al., 1993) and the carboxy terminus functions as a signal anchor sequence responsible for targeting and insertion into the outer mitochondrial membrane.

Bcl-2 can prevent oxidant-induced apoptosis, which correlates well with Bcl-2 being localised in organelles known to participate in redox reactions and the formation of reactive oxygen species (Buttke and Sandstrom, 1994). However Bcl-2 does not have a significant effect on the generation of superoxide, \( \text{O}_2^- \). Subsequent studies of Bcl-2's ability to rescue cells from apoptosis occurring under hypoxic conditions in which the generation of reactive oxygen species is greatly reduced suggest that reactive oxygen species are not essential for the induction of apoptosis and the antioxidant properties are not required for Bcl-2 to inhibit apoptosis.

Bcl-2 prevents mitochondrial membrane potential collapse, both in cells and isolated mitochondria (Zamzami et al., 1996). In a cell-free system, mitochondria is necessary to reproduce the features of nuclear apoptosis (Newmeyer et al., 1994). It has been recently reported that a pre-formed >10 kD protein, possibly a caspase-like protease, is released from mitochondria upon mitochondrial membrane potential disruption which causes isolated nuclei to undergo chromatin condensation and nuclear fragmentation (Zamzami et al., 1996). Bcl-2 hyperexpression in the outer mitochondrial membrane also impedes the release of this protein from mitochondria \textit{in vitro}, suggesting that Bcl-2 prevents apoptosis by favouring the retention of an apoptogenic mitochondrial protease.

Bcl-2 may be involved in the regulation of intracellular calcium homeostasis, suggested by its location to the endoplasmic reticulum. In this regard, many reports have
suggested that elevations in intracellular Ca\(^{2+}\) correlate with apoptosis (McConkey et al., 1988; Jones et al., 1989; Martikainen et al., 1991), in particular thapsigargin-induced apoptosis can be inhibited by Bcl-2. Thapsigargin is a selective inhibitor of the endoplasmic reticulum-associated Ca\(^{2+}\)-ATPase which pumps Ca\(^{2+}\) against a concentration gradient into the endoplasmic reticulum. Inhibition of this pump by thapsigargin induces a transient increase in cytoplasmic free Ca\(^{2+}\), and Bcl-2 prevents depletion of thapsigargin-sensitive Ca\(^{2+}\) stores during apoptosis (Baffy et al., 1993). However, Bcl-2 can even prevent apoptosis induced by intracellular calcium store withdrawal (Reynolds and Eastman, 1996), suggesting that the mechanism of Bcl-2 action in inhibiting apoptosis is independent of intracellular calcium.

Despite numerous positive examples, however the cell survival enhancing activity of Bcl-2 is not universal with bcl-2 loss-of-function mutant mice completing normal embryonic development. In transgenic animals in which Bcl-2 is expressed ectopically in immature cortical thymocytes, Bcl-2 protected these immature thymocytes from glucocorticoid-, radiation-, and anti-CD3-induced apoptosis (Sentman et al., 1991; Strasser et al., 1991), but Bcl-2 does not have a substantial effect on negative selection of thymocytes (Sentman et al., 1991) and does not effectively prevent apoptosis in targets of cytotoxic T-cell killing (Vaux et al., 1992). Thus Bcl-2 functional redundancy or Bcl-2-independent mechanisms clearly do exist.

Indeed, Bcl-2 is only one of a numerous family of recently discovered dimerizing proteins. This family consists of both inhibitors and promoters of programmed cell death (Table 1.2) (Yang and Korsmeyer, 1996).

<table>
<thead>
<tr>
<th>Table 1.2 Bcl-2 family death repressors and promoters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Death Repressors</td>
</tr>
<tr>
<td>Bcl-2</td>
</tr>
<tr>
<td>Bcl-xL</td>
</tr>
<tr>
<td>E1B-19K</td>
</tr>
<tr>
<td>Ced-9</td>
</tr>
</tbody>
</table>

Only molecules with established cell death functions in mammalian cells are shown
Multiple Bcl-2 family members can form homo- or heterodimers (Sedlak et al., 1995; Sato et al., 1994), indicating that the function of Bcl-2 family proteins appears to be dependent at least in part on their ability to interact with each other through a complex network of homo and heterodimers (Zha et al., 1996). The relative ratios of pro- and anti-apoptotic Bcl-2 family proteins determine the ultimate sensitivity or resistance of cells to a wide variety of apoptotic signals (Oltvai et al., 1993). In addition to dimerizing with other homologous proteins, Bcl-2 has been reported to associate with several other nonhomologous proteins, such as the protein kinase raf (Wang et al., 1994a). These observations have opened an avenue of investigation into the signal transduction cascades that can modify the function of Bcl-2 family proteins.

Posttranslational modification of Bcl-2-related proteins may play an important role in regulating their functions, indicated by several recent experiments. Phosphorylation of Bcl-2 is associated with the induction of apoptosis induced by taxol (Haldar, et al., 1995) and only nonphosphorylated Bad heterodimerized with Bcl-xL at membrane sites to promote cell death (Zha et al., 1996).

1.3.3 Fas

Fas, also called APO-1 or CD95, belongs to the tumor necrosis factor/nerve growth factor (NGF) receptor family (Mohler, 1994). The amino acid sequence of the extracellular regions of this family is relatively conserved, whereas the cytoplasmic region is not, except for some similarity between Fas and TNFR1 (TNF receptor 1) (Itoh et al., 1991; Oehm et al., 1989; Yonehara et al., 1989; Trauth et al., 1989). Crosslinking of Fas by Fas ligand (FasL) that belongs to the tumor necrosis factor (TNF) family or Fas monoclonal antibody induces apoptosis in some but not all cells that express the Fas antigen (Itoh et al., 1991; Oehm et al., 1989). This suggests that Fas-mediated apoptosis is regulated not only by interaction of FasL with Fas, but also by permissive Fas signalling pathway. FasL has been observed on T cells but is less conspicuous on B cells, macrophages, or natural killer (NK) cells, whereas Fas is expressed on all these cells (Suda et al., 1995).

Sequence mutational analyses of Fas and TNFR1 indicated that the cytoplasmic domain (about 70 amino acids) conserved between Fas and TNFR1 is necessary and sufficient for transduction of the apoptotic signal (Itoh and Nagata, 1993). This domain
was therefore designated as a death domain. The death domain does not contain domains for kinase or phosphatase activity, suggesting that this region might interact with cytoplasmic enzymes involved in signal transduction. Recently, three proteins have been found to bind to the death domains of Fas or TNFR1, or both. FADD (Fas-associating protein with death domain) binds specifically to Fas (Boldin et al., 1995); TRADD (TNFR1-associated death domain protein) binds to TNFR1 (Hsu et al., 1995) and RIP (receptor-interacting protein) binds to both receptors (Stanger et al., 1995). These proteins are also capable of binding to each other (Varfolomeev et al., 1996; Hsu et al., 1996). It has been elucidated that these nonenzymatic protein-protein interactions that initiate signalling for cell death. Caspase-8 and caspase-10, two adaptors containing FADD-like domain, can bind to FADD and initiate cell death (Munday et al., 1995). It appears that these caspase activities constitute the most upstream enzymatic step in the cascade of signalling for the cytoidal effects of Fas and TNFR1.

1.3.4 Reaper

A step towards understanding how different signals may converge to activate a common cell death program has come from genetic studies on the control of apoptosis in the fruit fly *Drosophila melanogaster*. In *Drosophila*, as in mammals, the onset of apoptosis is influenced by many different intra- and extracellular signals that may either promote or suppress cell death (Steller and Grether, 1994). Nevertheless, it appears that most, if not all programmed cell death in this organism, is mediated by one common mechanism. Genetic analysis has led to the isolation of a gene, *reaper*, which encodes a small polypeptide of 65 amino acids capable of integrating information from different signalling pathways to activate the apoptotic program (White et al., 1994). Deletion of *reaper* suppresses apoptosis in response to every apoptotic stimulus tested to date. Expression of *reaper* is sufficient to induce apoptosis in cells that would normally survive (White et al., 1994). These observations show that multiple signalling pathways for the activation of apoptosis converge onto the *reaper* gene. The Reaper protein shares no significant similarity with other known proteins, so the sequence provides no information about its biochemical function. Although the mechanism by which Reaper induces apoptosis is not yet clear, it will be important to identify downstream targets for *reaper* and also to investigate whether this gene and its function have been conserved during evolution.
1.3.5 *p53, C-myc*

The p53 protein is a nuclear phosphoprotein and a transcription factor capable of both trans-activating and repressing transcription (Ginsberg et al., 1991a; Farmer et al., 1992, Zambetti et al., 1992) thereby mediating either cell growth arrest or apoptosis, depending on the physiological circumstances (Finlay et al., 1989; Diller et al., 1990; Ginsberg et al., 1991b, Yonishrouach et al., 1991; Kastan et al., 1992).

The p53 protein is involved in the cellular response to DNA damage. When DNA damage occurs, the genomeguarding function of p53 is induced, arresting the cells at the G1 stage in the cell cycle to allow DNA repair mechanisms to proceed or alternatively, p53 protein may trigger apoptosis when DNA repair mechanisms fail (Lowe et al., 1993; Kastan et al., 1991; Lane, 1992; Caelles et al., 1994). G1-specific growth arrest by p53 is thought to result, at least in part, from the trans-activation of p21/WAF-1/cip1, the protein product of which inhibits a G1-specific cyclin dependent kinase (El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993), however the mechanisms of the induction of apoptosis by p53 are not clear. Members of the retinoblastoma (*Rb*) gene family and the E2F transcription factor family appear to play an important role in the decision as to whether a cell will respond to wild-type p53 activation by executing a viable G1 arrest or undergoing apoptosis (Haupt et al., 1995a). Rb is thought to regulate passage through the G1 phase of the cell cycle by binding to and inhibiting the activity of members of the E2F family in a cell cycle dependent manner (Hiebert et al., 1992; Helin et al., 1993; Lee et al., 1993), with release of free E2F following phosphorylation of Rb believed to result in the activation of genes required for S phase entry (Means et al., 1992; Lam and Watson, 1993). E2F could induce cell death by stimulating transactivation of p53 target genes involved in apoptosis or could activate p53 indirectly through up-regulation of an activator, such as an S phase kinase (Wang and Orives, 1995).

p53-mediated apoptosis in myeloid leukemia cells correlated with a decrease in the expression of Bcl-2 and an increase in the expression of Bax (Miyashita et al., 1994), and p53 is a direct transcriptional activator of the *bax* gene (Miyashita and Reed, 1994). p53-mediated cell death in certain cell types may therefore result from either the trans-activation of cell death genes or the transcriptional repression of cell survival factors. Alternatively, p53 may be capable of mediating apoptosis through transcriptionally independent mechanisms (Caelles et al., 1994) as it has been demonstrated that
transcriptionally defective mutants can induce apoptosis in Hela cells (Haupt et al., 1995b). It has also been observed that DNA-damaging agents cause apoptotic cell death in p53 deficient cells, and the cell death is associated with a $G_2$ arrest rather than $G_1$ arrest (Haupt et al., 1995a). Thus, there are p53-dependent and p53-independent mechanisms of cell death.

The identity of other proteins that might help to regulate the induction of genes required for apoptosis in the absence of p53 are at present unknown, but p62-c-Myc is a potential candidate. The phosphoprotein c-Myc, in partnership with Max (Myc-associated factor X), is a sequence-specific transcription factor that is essential for G1-to-S-phase progression in the cell cycle but is oncogenic when overexpressed (Evan and Littlewood, 1993; Marcu et al., 1992). c-Myc, usually implicated in cell transformation, differentiation and cell cycle progression, has also been consistently found to mediate apoptosis following c-Myc overexpression, growth arrest, or withdrawal of certain growth stimuli (Evan et al., 1995). Given that cell death needs no RNA and protein synthesis c-Myc may be not essential for apoptosis (Ucker et al., 1992), although it has been demonstrated that inhibition of c-Myc expression is associated with the induction of apoptosis (Wu et al., 1996).

Although a variety of genes involved in the regulation of apoptosis has been identified the major challenge remains in placing these molecules in the pathway of apoptosis. The identification, interaction, and sequence of the molecular and cellular events that characterize the apoptotic process continue to be a major research focal point.

1.4 Physiological and pathological roles of apoptosis

Programmed cell death is a major and apparently universal aspect of metazoan development and tissue homeostasis. It serves as a prominent force in sculpting the developing organism, as a major mechanism for the precise regulation of cell numbers in tissue homeostasis in essentially all organs where cell division occurs (Raff et al., 1993; Raff, 1992), and as a defence mechanism to remove unwanted and potentially dangerous cells, such as self-reactive lymphocytes (Cohen, 1991), cells that have been infected by viruses (Vaux et al., 1994; Debbas and White, 1993), and tumor cells (William et al., 1991). The importance for the organism of maintaining tight control over which cells will live and which cells die is underscored by the observation that breakdown in the regulation
of programmed cell death is associated with several types of cancer, with autoimmunity, and possibly with neurodegenerative disease.

1.4.1 Apoptosis in development

Apoptosis is a physiological process of selective cell deletion probably occurring in all animal tissues at least at some stages of their development. In the embryo, morphogenesis is in part through the elimination of cells that are only necessary for a limited time span. One of the most characteristic situations where apoptosis figures prominently during embryogenesis is in limb development with separation of the digits from the limb bud (Hammar and Mottet, 1971).

Neuronal death is an important phenomenon in the developing nervous system and during development many types of neurons are produced in excess. The survival of the neurons is dependent on specific neurotrophic factors secreted by target cells innervated by the neurons and only those which get enough neurotrophic support from their target cells survive (Bosman et al., 1996). The excess neurons are eliminated by programmed cell death to match the innervation density with the target size. Thus it is thought that programmed cell death plays a key role in forming the neuronal architecture and the neuron network organization of the developing nervous system.

Extracellular survival signals act by suppressing an intrinsic cell suicide program which is constitutively expressed in cells and operates by default when a cell is deprived of such signals. A neurotrophic-like mechanism may operate in many types of vertebrate cells. Many of the other normal cell deaths in developing animals may occur because the cells fail to receive sufficient survival signals. This can be due to either low levels of the signals available or the cells are insensitive to the survival signals.

1.4.2 Apoptosis in the immune system

Apoptosis is a critical mechanism by which the immune system maintains tolerance to self-antigens by the clonal deletion of autoreactive T and B cells (Golstein et al., 1991; Cohen et al., 1992b). In the life of lymphocytes, both T cells and B cells normally die at various stages of their development. The T cells that can interact with self-major histocompatibility complex (MHC) expressed in thymus are positively selected (positive selection). On the other hand, T cells that strongly react with self antigen complexed with self-MHC are induced to undergo apoptosis (negative selection) (von Boehmer, 1994).
More than 95% of the T cells that migrate into the thymus die there; the remaining 5% then migrate to the peripheral lymphoid organs as mature T lymphocytes (Egerton, et al., 1990) where the mature T cells again undergo an additional selection process. Those T cells which interact with the self antigen expressed only in peripheral tissues would die (peripheral clonal deletion) (Webb et al., 1994). In the bone marrow, the B cells that strongly react to self components are deleted with surviving B cells then migrating to peripheral lymphoid organs where they can be activated by antigens.

Apoptosis is also a mechanism for cytotoxic T-cell-mediated targets such as virus-infected or tumor cell death. Apoptosis after FasL binding to Fas has been proposed to be the main mechanism involved in activation-induced apoptosis of mature T cells.

The spontaneous mutations of lpr and gld in the mouse are loss-of-function mutations of Fas and FasL, respectively. The positive and negative selections in the thymus are apparently normal in lpr mice (Sidman et al., 1992), while the peripheral clonal deletion and elimination of activated T cells are impaired in lpr and gld mice. This indicates that a Fas-mediated mechanism is unlikely to be involved in the positive and negative selection but may be normally involved in both the clonal deletion of autoreactive T cells in peripheral lymphoid organs and the elimination of activated T cells after they have responded to a foreign antigen. Although mature T cells constitutively express Fas, activation by antigen up-regulates this expression and renders the T cells sensitive to Fas-mediated apoptosis. At the same time, activation by antigens induces FasL expression on cytotoxic T cells (Owen-Schaub et al., 1992; Klas et al., 1993) thus Fas is not only a major pathway used by cytotoxic T cells to kill target cells (Raff et al., 1993), but is also a key element in the elimination of activated T cells during the down regulation of the immune response.

1.4.3 Apoptosis and autoimmune disease

In addition to the beneficial effects of programmed cell death, dysregulation of apoptosis has important implications in the pathogenesis of a number of diseases (Kerr et al., 1972; Barr and Tomei, 1994).

Failure to remove autoimmune cells that arise during development or that develop as a result of somatic mutation during an immune response can result in autoimmune disease, characterized by the proliferative expansion of lymphocytes reactive to self-antigens. In
the absence of a properly functioning Fas-FasL system activated lymphocytes accumulate
and because these cells are not efficiently eliminated autoimmune disease is enhanced.
The lpr or gld mice produce large amounts of immunoglobulin, including autoantibodies,
and develop an autoimmune disease that resembles human systemic lupus erythematosus
(SLE). A lupus-like autoimmune disease has also been reported in transgenic mice
constitutively overexpressing Bcl-2 in their B cells (Strasser et al., 1991). Investigations
into the role of apoptosis in the development of autoimmune disease in human are just
beginning with alterations in the susceptibility of lymphocytes to die by apoptosis in vitro
being reported in several diseases.

1.4.4 Apoptosis and AIDS

Infection by the human immunodeficiency virus (HIV) leads to acquired
immunodeficiency syndrome (AIDS) which is characterized by progressive cell loss from
several organs. These include loss of CD4+ T cells from the peripheral circulation and
lymphoid organs, haematopoietic progenitors from the bone marrow, and neurons from the
brain (Everall et al., 1991; Fauci et al., 1993; Levy et al., 1993). Most immunological and
nonimmunological defects in HIV infected persons may be attributed to the inappropriate
induction of apoptosis in various cell populations. It has been shown that CD4 acts as a
receptor for viral attachment, thus facilitating HIV infection of CD4+ T cells and
stimulation of the CD4 receptor, by its binding to a soluble viral product gp120, results in
the enhanced susceptibility of uninfected T cells to undergo apoptosis (Ameisen and
Capron, 1991). Thus interference of HIV with the intracellular signalling pathways that
regulate apoptosis could induce cell death in both uninfected and infected cell populations.

1.4.5 Apoptosis and neurodegenerative disease

A wide variety of neurological diseases is characterized by the gradual loss of specific
sets of neurons (Isacson et al., 1993). Such disorders include Alzheimer’s disease,
Parkinson’s disease, amyotrophic lateral sclerosis (ALS), retinitis pigmentosa, spinal
muscular atrophy, and various forms of cerebellar cell degeneration. Oxidative stress,
calcium toxicity, and deficiency of survival factors have all been postulated to contribute
to the pathogenesis of these diseases (Zhong et al., 1993) with each of these pathways
predisposing neurons to apoptosis, either in vitro or in vivo. The cell loss in these diseases
does not induce an inflammatory response and apoptosis appears to be the mechanism of
cell death as overexpression of Bcl-2 decreases the neurotoxicity of each of these potential inducers of cell death.

1.4.6 Apoptosis and neoplastic diseases

For a long time the dysregulation of growth which leads to cancer was explained largely in terms of increased cell proliferation. It has become clear now, apoptosis is also an important mechanism in controlling cancer. The observation that all normal mammalian nucleated cell types studied to date, with the exception of embryonic blastomeres, undergo apoptosis when deprived of exogenous signals and/or cellular contacts (Raff et al., 1992), has led to the concept that the fate of each cell may be dependent on the presence and activity of other cells and may require an active and constant repression of apoptosis induction. The dependence of normal cells on environment-specific factors to maintain their viability may serve to prevent normal cells from surviving in nonphysiological sites. Cells from a wide variety of human malignancies have a decreased ability to undergo apoptosis in response to some physiological stimuli (Hoffman and Liebermenn, 1994), this being most apparent in metastatic tumors as these cells can survive at sites distinct from the tissue in which they arose. Enhancing cell survival through inhibition of apoptosis may be one of the mechanisms through which tumor promoters, such as phorbol esters exert their effects.

The involvement of cancer genes in the regulation of apoptosis is indicative of the role apoptosis plays in the pathogenesis of cancer with bcl-2 production being a common feature of many carcinomas and lymphomas. Although the precise biochemical activity of Bcl-2 remains uncertain, genetic studies of Bcl-2 family members have established the importance of these genes in the normal development and maintenance of the organism. Transgenic mice carrying bcl-2-Ig minigenes, with the molecular features of the t(14;18), displayed polyclonal lymphoid hyperplasia due to increased lymphocyte survival (McDonnell et al., 1993) and although Bcl-2 itself has relatively weak transforming activity, it cooperates with c-Myc (Vaux et al., 1988; Nunez et al., 1989; Fanidi et al., 1992) and members of the Ras family (Reed et al., 1990) to transform cells. Recently, it has been shown that Bcl-2 protects cells from c-Myc and p53-induced apoptosis (Fanidi et al., 1992; Bissonnette et al., 1992; Chiou et al., 1994; Ryan et al., 1994), suggesting that Bcl-2 suppresses apoptotic signals that occur with transformation. Bcl-xL can be highly expressed in both primary tumors and tumor cell lines (Dole et al., 1995; Schlaifer et al., 2000).
1995) and overexpression of Bcl-2 or Bcl-xL can protect tumor cells from a wide variety of apoptotic stimuli and confer a multidrug resistance phenotype (Miyashita and Reed, 1993; Dole et al., 1994). Bcl-2 expression has been investigated in nonlymphoid tumors and it is well established that some breast carcinomas, prostate cancers, and non-small-cell lung cancers express Bcl-2 (reviewed by Yang and Korsmeyer, 1996). In a cell line model, cells selected for acquired resistance to cytotoxic drugs associated with overexpression of \textit{MDR1} gene showed induction of Bcl-xL and were also resistant to \( \gamma \)-irradiation-induced apoptosis (Datta et al., 1995). Thus induction of \textit{bcl-xL} may play a role in the etiology of chemotherapy and radiation-resistant tumors and may also prove to have prognostic significance.

Inactivation of the p53 tumour suppressor gene function represents the most common genetic abnormality known in human cancer, occurring in >50% of all tumours (Hollstein et al., 1991). In an \textit{in vivo} model of choroid plexus tumor progression comparing \textit{p53}+/- with \textit{p53}+- mice, it was found that the aggressive tumor progression that occurred in the absence of p53 function was attributable to decreased apoptosis (Symonds et al., 1994). The function of \textit{p53} as a tumor suppressor gene may be largely explained by its role in promoting cell death. In breast cancer, two of the most common abnormalities are deregulated expression of \textit{c-myc} and inactivation or mutation of the \textit{p53} tumor suppressor gene (Mazars et al., 1992). Expression of \textit{p53} and \textit{c-myc} can induce some cancer cells to undergo apoptosis (Yonish-Rouach 1991; Ryan et al., 1993; Evan et al., 1992; Tanaka et al., 1994), which suggests that the cell death programme is a critical defence mechanism against malignancy.

Immune escape of tumor cells and successful tumor outgrowth may be due to the inability of the immune system to react to the tumor as partial or complete loss of Fas have been found in a variety of different tumors that had originated from tissues normally expressing Fas. Loss of Fas may result in reduced sensitivity of the tumor cells towards the cytotoxic action of T lymphocytes. Although FasL expression was initially thought to be restricted to activated T lymphocytes (Suda et al., 1993), the ligand is also expressed in nonlymphoid cells such as certain epithelial cells, sertoli cells, and neurons (French et al., 1996; Bellgrau et al., 1995; Griffith et al., 1995). As FasL is upregulated during tumorigenesis (Hahne et al., 1996) and killing of lymphocytes has been demonstrated as
the result of FasL expression in nonlymphocytic cells, it is suggested that FasL expression may be a strategy used by tumor cells to escape immune response.

It is important that anti-cancer drugs mediate their therapeutic response by triggering apoptosis and a variety of anti-cancer drugs, such as topoisomerase inhibitors, DNA binding drugs, anti-metabolites and others, can induce apoptosis in cancer cells. However in neuronal cell degenerative disease, apoptotic pathways may be inappropriately activated, demonstrating that identification of the molecular pathway of apoptosis is of importance in both prevention and treatment of human diseases.

1.5. Detection of apoptosis

Recent advances in research on the molecular determinants of cell death have been approached using a combination of morphological, biochemical, physiological and molecular biological methods. Currently, no simple markers or criteria are available to unequivocally discriminate necrosis from apoptosis. Toxic injury may cause necrosis or apoptosis depending on the extent of damage to their membrane, adenosine triphosphate synthesis, gene expression, or to other vital functions. In damaged cells, signs of both apoptosis and necrosis are frequently found at the same time. As exemplified by the study of the effect of different doses of tamoxifen on MCF 7 cells (Bursch et al., 1996). At a high dose (10^{-5} M), tamoxifen caused rapid lytic cell death (necrosis) whereas lower concentrations of tamoxifen induced a more gradual appearance of apoptotic cell death, indicating there may exist a threshold of injury beyond which necrosis occurs in a given cell. On yet another occasion, single doses of thioacetamide or dimethylnitrosamine first induced apoptosis and then necrosis in rat liver (Ledda-Columbano et al., 1991; Pritchard and Butler et al., 1989). Even individual cells may show indications of both if they enter a pathway to apoptosis but then collapse and end in necrosis, so-called secondary necrosis. The patterns of cellular reaction to injury leading to cell death are important in the understanding and recognition of toxic and other types of injury. The factors that determine which process occurs remain to be identified.

The identification and quantification of apoptosis depend on the available markers. Morphological methods are still required to identify cells undergoing apoptosis and electron microscopy may be necessary for unequivocal identification. DNA fragmentation during apoptosis has been intensively studied and may be a crucial part in the ordered
process of apoptosis (Arends et al., 1990; Walker et al., 1994b). Electrophoresis may yield different patterns of DNA fragments. However, the lack of DNA laddering is not per se diagnostic of the absence of apoptosis. Even the ladder pattern of DNA fragmentation, which is regarded as a hallmark of apoptosis, is not necessarily specific for apoptosis and can occur in lytic necrosis as well (Collins et al., 1992). It is clearly not possible to distinguish apoptosis from necrosis using only one or two endpoints, and multiple criteria are necessary.

The morphological and biochemical differences between apoptotic and viable cells have been utilized to develop a number of flow cytometric assays for precise analysis and reliable quantification. Light scatter and fluorescence are two types of measurement made in most flow cytometers. Due to the condensation and nuclear fragmentation, apoptotic cells usually produce lower forward scatter (FSC) and higher side scatter (SSC) signals than viable cells (Darzynkiewicz et al., 1992). Transport of the benzimidazole dye Hoechst 33342 (HO342) across the plasma membrane is altered in apoptotic cells (Ormerod et al., 1992). HO342 bind preferentially to AT-rich regions in the small groove of double-stranded DNA and fluorescent blue when excited by UV light. Differential staining of DNA with this dye, thus discriminates between live and apoptotic cells (Dive et al., 1992; Ormerod et al., 1992). The charged dye PI intercalates into double-stranded nucleic acids leading to intense fluorescence of this dye, which otherwise, unbound and in aqueous solution, shows weak fluorescence and is excluded by live cells. Supravital uptake of HO342 combined with exclusion of PI and analysis of the cell’s light scatter properties, provides an useful assay of apoptosis (Dive et al., 1992; Ormerod et al., 1992), but this method can not distinguish terminal apoptosis from necrosis.

As a consequence of activation of the apoptosis-associated endonuclease, massive numbers of DNA strand breaks occur in apoptotic cells. The presence of 3'-OH termini in DNA breaks can be labelled with biotin- or digoxygenin-conjugated nucleotides, in a reaction catalysed by exogenous terminal deoxynucleotydyl transferase (TdT) (Gorczyca et al., 1992; Gorczyca et al., 1993) or DNA polymerase (Gold et al., 1993). The labelled strand breaks can be detected using fluoresceinated avidin or fluoresceinated antibody to digoxygenin. Fixation with formaldehyde prevents the extraction of degraded, low molecular-weight DNA, thus this method can be used to analyze cell cycle-related apoptosis in situ. It should be noted, however, that when the cells are more advanced in
apoptosis, their DNA is apparently degraded more extensively so that even in this procedure, a portion of the DNA becomes extracted; apoptotic cells are then characterized by a significantly lower DNA content and thus their cell cycle distribution can not be estimated.

There are still some newly developed flow cytometric methods to detect different changes of apoptosis. The single stranded DNA in apoptotic cells could be detected when stained by the metachromatic fluorochrome acridine orange (Bruno et al., 1992), alternatively stained by an antisingle stranded DNA antibody (Frankfurt et al., 1994). Loss of cell surface structures such as pseudopodia and microvilli can result in the reduction of F-actin staining (Endresen et al., 1995). Apoptosis induced by a variety of agents, was accompanied by dramatic changes in phosphatidylserine distribution, as assessed by the increase annexin V-binding properties of these cells. AnnexinV, a member of newly discovered family of proteins, has been found to have high affinity for aminophospholipids in the presence of Ca$^{2+}$ ions (Martin and Reutelinsperger, 1995). Notably, changes in phosphatidyserine asymmetry were detected before the morphological changes.

The advantages of flow cytometry over other methods, such as microscopy include the rapid analysis of large numbers of cells and the identification and quantitation of subpopulations in heterogeneous cell systems. Flow cytometry allows one to rapidly and accurately measure individual cells in large cell populations and cell subpopulations can be identified and sorted before use in function assays.

1.6 Aims of the study

The present study was to investigate the involvement of proteases in the regulation and execution of apoptosis using a human monocytic THP.1 cell line, which was established from a patient with acute monocytic leukemia (Tsuchiya et al., 1980). Like many other tumor cells, THP.1 cells express inactive $p53$ (Sugimoto et al., 1992) and more importantly, THP.1 cells express caspase-1, caspase-2, caspase-3 and other caspas (Wang et al., 1994b), and caspase-1 and caspase-3 were initially purified from these cells (Thornberry 1994; Nicholson et al., 1995). Thus it is an ideal cellular model in which to investigate the role of caspases and noncaspase-like proteases in the regulation and the execution of apoptosis.
A number of methods was established to characterize the induction of apoptosis, including fluorescence and electron microscopic assessment of morphological changes, biochemical analysis of nuclear DNA fragmentation, and flow cytometric study of cellular membrane alterations. The putative protease involved in apoptosis were assessed by using different groups of peptide-based protease inhibitors, with TLCK and TPCK for serine protease and cystein protease, Z-VAD.FMK, Z-DEVD.FMK and YVAD.CMK for caspases. The participation of individual caspases in the execution of apoptosis was determined by their activation/processing during the induction of apoptosis, measured by western blotting. The research programme was outlined in Figure 1.2.
Fig 1.2. Research programme

The morphological changes of cell death were studied by fluorescence and electron microscopy. Different types of DNA degradation were detected using the methods of in situ end labelling (ISEL), field inversion gel electrophoresis (FIGE) and conventional gel electrophoresis. Apoptosis was also quantitatively analysed using a Hoechst/PI staining flow cytometric method. The activation of caspases was indicated by the formation of active subunits, detected by western blotting. The increased caspase activity during the induction of apoptosis was confirmed by fluorimetric analysis using peptide based fluorogenic substrates.
CHAPTER 2

MATERIALS AND METHODS
2.1 Materials

Media, sera and terminal deoxynucleotidyl transferase (TdT), Lambda DNA Hind III fragments and 123 base pair DNA ladders were from Gibco (Paisley, UK). Digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (dig-11-dUTP), antidigoxigenin-fluorescein Fab fragments (anti-Dig), Boc-Val-Leu-Lys-7-amino-4-methylcoumarin (Boc-Val-Leu-Lys-AMC), Suc-Ala-Ala-Pro-Phe-AMC, Pronase, TLCK and TPCK were all purchased from Boehringer Mannheim (Lewes, East Sussex, UK). Benzyloxy carbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (Z-VAD.FMK), benzyloxy carbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone (Z-DEVD.FMK) and benzyloxy carbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Z-DEVD-AFC) were from Enzyme Systems Inc. (Dublin, CA, USA). Acetyl-Tyr-Val-Ala-Asp-chloromethyl ketone (AC-YVAD.CMK) was from Bachem (Bubendorf, Switzerland). Agarose was from BDH Chemicals Ltd (Poole, UK). Agarose NA was from Pharmacia LKB, Biotechnology AB (Uppsala, Sweden). 

Saccharomyces cerevisiae chromosomes (243 - 2200 kbp) were from Clontech (Cambridge, UK). Other chemicals were obtained from Sigma Chemical Company (Poole, UK), unless otherwise indicated.

2.2 Cell culture

Human monocytic THP.1 cells were obtained from ECACC (Wiltshire, U.K.) and were grown as a suspension in RPMI 1640 medium supplemented with 10% FCS and 2 mM glutamine at 37°C in an atmosphere of 5% CO₂ in air. Cells were split every third day and the density was kept at 0.25-1 x 10⁶ /ml. Logarithmically growing cultures of THP.1 cells were used for all experiments and were seeded at a density of 0.5-0.7 x 10⁶ /ml one day prior to the experiment. Cell growth was assessed by counting cell numbers using a SCHARFE CASY II cell counter.

2.3 Microscopy

2.3.1 Fluorescence microscopy

Fluorescence microscopy was used to examine apoptotic morphological changes. 1 ml cell suspensions (~1x10⁶ cells) were stained with 30 µl of 50 µg/ml HO342 for 5 min at 37°C. After centrifugation at 200g for 3 min at 4°C, cells were resuspended in 300 µl ice cold PBS containing 30 µl of 50 µg/ml PI. Cells were examined by fluorescence.
microscopy. Cells which took up HO342 and stained blue were considered viable, whereas cells that included PI and stained red were non-viable. Apoptotic changes assessed include cytoplasmic and nuclear shrinkage, chromatin condensation and nuclear fragmentation.

2.3.2 Electron microscopy

Cell pellets (1 mm in thickness) were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) overnight at 4°C and postfixed with 1% osmium tetroxide and 1.7% potassium ferrocyanide for 90 min. After fixation, cells were stained en bloc for 1 h with 2% aqueous uranyl acetate, dehydrated, and embedded in Araldite. Sections were stained with lead citrate and examined by electron microscopy (Cohen et al., 1992a).

2.4 Flow cytometry

2.4.1 HO342/PI flow cytometry

A flow cytometric method previously used in thymocytes to separate and quantify apoptotic and non-apoptotic cell populations was modified and applied to the THP.1 cells. Cells were stained with HO342 (1.5 μg/ml) for 1 min. Cells were resuspended in 700 μl of PBS containing 5 μg/ml of PI. Analysis was carried out using a Becton Dickinson flow cytometer with Lysis II software. Flow rate was 200 cells/second. The HO342 and PI were excited using the 352 nm ultraviolet line of a krypton laser and 488 nm of argon laser respectively, and the resultant blue (420-480 nm) with linear amplification vs red (560-600 nm) fluorescence with logarithmic amplification was recorded. Non-viable, red fluorescent cells were gated out. The remaining cells were displayed as a cytogram of blue fluorescence vs light forward scatter (FSC) (indicative of cell size). Cell sorting was carried out at a flow rate of 4000 cells/sec on the basis of the above parameters. Sorted cells were collected for the assessment of morphology.

2.4.2 In situ terminal deoxynucleotidyl transferase assay

Apoptotic DNA breaks were analysed by the in situ end labelling (ISEL) method described by Gold et al (1993). Control cells and cells treated with different agents were fixed in 1% buffered formaldehyde (pH 7.4) for 15 min on ice. After washing with PBS, cells were resuspended in 100 μl of PBS and further fixed with 70% cold ethanol at 4°C overnight. Fixed cells were centrifuged at 600g for 6 min at 4°C and rehydrated in TBS.
(50 mM Tris, 150 mM NaCl). Cells were resuspended in 100 µl 1 x TdT buffer (50 mM sodium cacodylate, 1 mM cobalt chloride, 0.1 mM dithiothreitol) containing 10 units of terminal deoxynucleotidyl transferase (TdT) and 2 µM digoxigenin-11-dUTP and incubated at 37°C for 1h. The reaction was terminated by placing the suspensions on ice for 3 min. Cells were resuspended in 0.5 ml of antibody labelling buffer (5% non-fat dried milk, 0.1% Triton X-100, 4 x standard saline citrate, pH 7.2) for 15 min to block non-specific binding sites. After centrifugation, cells were incubated with 0.25 ml of staining buffer containing 0.1 µg of anti-digoxigenin-FITC antibody at room temperature for 1 h. Cells were resuspended in 1 ml PBS containing 5 µg/ml PI.

A FACScan flow cytometer (Becton Dickinson, USA) was used to measure the red (PI) and green (fluorescein ; anti-Dig) fluorescence of labelled cells. Both PI and FITC were excited at 488 nm using an argon laser and the resultant red (564-606 nm) and green (515-545 nm) fluorescence with linear and logarithmic amplification, respectively, were recorded. The data was initially displayed as a cytogram of SSC vs FSC. Having gated out the debris, the intact cells were displayed as a second cytogram of red fluorescence signal area against red fluorescence width. This is a program called doublet discrimination and allows single cells to be distinguished from doublets and clumps. 5000 single cells were analysed and displayed as bivariate cytograms of red vs green fluorescence.

2.4.3 Detection of cell cycle

THP-1 cells were washed in PBS and fixed with ice-cold 70% ethanol for at least 30 min on ice. Cells were harvested after centrifugation at 600g for 6 min at 4°C. After washing with PBS, cells were resuspended in 800 µl of PBS, to which 100 µl of RNase (1 mg/ml) and 100 µl of PI (50 µg/ml) were added. This cell suspension was then incubated at 37°C for 30 min. The cellular DNA content was determined using a FACScan flow cytometer (Becton Dickinson, USA). Data from 5000 single cells were acquired and displayed as a histogram of red fluorescence which indicates the cell cycle distribution.

2. 5. Agarose gel electrophoresis

2.5.1 Conventional agarose gel electrophoresis

Conventional agarose gel electrophoresis was used to detect internucleosomal cleavage of DNA, as described by Sorenson et al. (1990). 1 x 10⁶ cells were pelleted, suspended in
20 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) to which 7 µl of RNase (50 mg/ml) was added. The cells were digested for 20 min at room temperature and mixed with loading buffer. The running gel was made of 1.8 % agarose in 0.5 x TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). The digestion gel was made of 0.8 % agarose in 1 x TBE containing 2.5% SDS and 1.25 mg/ml of proteinase K, which was added when the gel had cooled below 50° C. 1 x 10^6 cells were loaded per lane. Lambda DNA Hind III fragments and 123 base pair DNA ladders were used as standards.

Electrophoresis was carried out in 1 x TBE buffer at 20 v for 1 h then at 100 v for 4 h. The gel was washed in water and stained with ethidium bromide (1 µg/ml) for 30 min. After being destained for 4 h in water, the DNA was visualised under UV light.

2.5 2 Field inversion gel electrophoresis

Field inversion gel electrophoresis (FIGE) was employed to detect the large kbp DNA fragments (Brown et al., 1993). DNA plugs (1 mm³) were made of 0.5% agarose L containing 0.5x10^6 cells and were processed at 50° C in NDS solution (1% N-lauryl sarcosine, 0.5 M EDTA, 10 mM Tris, pH 9.5) containing Pronase (1 mg/ml) for 48 h.

The running gel was made of 1% Agarose NA in 0.5 x TBE buffer. The plugs were washed 3 times with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) before loading. Two sets of standards were used: Saccharomyces cerevisiae chromosomes (243 - 2200 kbp) and pulse markers (0.1 - 200 kbp). The sample wells were cemented with molten Agarose NA.

Electrophoresis was carried out vertically at 200 v with a 15 min forward pulse followed by a 2.4 sec forward to 0.8 sec reverse constant pulse generated by a PC 750 pulse controller (Hoefer Scientific Instruments, San Francisco, CA, USA) for 1 h. After this step, a ramp factor of 1.5 was applied to increase the forward pulse interval to 24 sec and reverse interval to 8 sec. The gel was run for a further 6 h. The gel was stained, destained, and the DNA was visualised in the same way as described for the detection of internucleosomal cleavage of DNA by conventional agarose gel electrophoresis.

2.6 Analysis of protease activity

2.6.1 Preparation of cell lysates
Chapter 2 Materials and Methods

Cytosol lysates were prepared from control or cells treated with different agents. Cells were lysed by using 0.25% Nonidet P-40 (50 x 10^6 cells/100 µl) for 5 min on ice. The cells were centrifuged at 2,000g for 10 min and the supernatant was collected and stored at -80°C until used for measurement of protease activity. The protein concentration in the supernatant fraction (the lysate) was determined by the Bradford assay (Bio-Rad).

2.6.2 Fluorimetric measurement of protease activity

Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC were used as substrates for measuring trypsin-like and chymotrypsin-like serine protease, respectively. Ac-YVAD-AMC and Z-DEVD-AFC were used as caspase-1- and caspase-3-like substrates, respectively. The substrate (20 µM) was added to a cuvette containing 1.25 ml of 50 mM Tris buffer, pH 7.4, maintained at 37°C. Lysate (10 µl) was added to initiate the reaction. The increase in fluorescence versus time was monitored using a Perkin Elmer fluorimeter at excitation and emission wavelengths of 380 and 460 nm for AMC, 400 and 505 nm for AFC, respectively. Calibration was carried out with standard solutions of AMC (0-1 µM) and AFC (0-1.6 µM). The protease activity was expressed as pmol AMC (or AFC) / mg protein / min.

2.7 SDS/PAGE and Western blotting

Cells were prepared for SDS-PAGE as described (Harlow and Lane, 1988). An aliquots of 10^6 cells were lysed in 100 µl of sample buffer (0.125 Tris-HCl, pH 6.8, containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.004% bromphenol blue). Proteins were denatured at 95°C for 5 min, resolved on a 7% (PARP), 10% (lamin B1), 12% (lamin A/C) or 15% (caspase-3, caspase-7 and caspase-2) SDS polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane (Hybond-C extra; Amersham, Little Chalfont, UK) overnight at 30 v. A western blotting method was used to detect PARP and lamin degradation, as well as caspase-3, caspase-7 and caspase-2 processing. PARP was detected using rabbit antiserum (318) (diluted 1:10,000), provided by Dr G. Poirier (Quebec, Canada). Intact lamin B1 and a proteolytic fragment were detected using a monoclonal antibody (diluted 1:50) (Serotec Ltd., Oxford, UK). Intact lamin A/C and its proteolytic fragments were detected using a rabbit antibody to the carboxy terminus of lamin A/C (diluted 1:2000) provided by Dr J. Lord (Birmingham, UK). Pro-caspase-3 and its catalytically active p17 subunit were detected using a rabbit...
polyclonal antibody directed to the p17 subunit (Nicholson et al., 1995). Pro-caspase-7 and its active p20 subunit was detected using rabbit antiserum (MacFarlane et al., 1997). Caspase-2 was detected using a rabbit polyclonal antibody to the carboxy terminus of caspase-2L (Ich-1L) (diluted 1:500) (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody was incubated with the membrane for 1 h at room temperature in TBST (20 mM, 137 mM NaCl, 0.1 % Tween 20, pH 7.6, containing 5% skim milk). The membrane was washed three times in TBST for 15 min before the incubation for 1 h with the secondary antibody, which was conjugated to horseradish peroxidase (diluted 1:2000 in TBST). Immune complexes were detected by enhanced chemiluminescence (ECL) by treating the membrane with ECL detection system according to the manufacturer’s protocol (Amersham Life Science, UK) and then exposed to x-ray film (Kodak Scientific imaging systems, Eastman Kodak company, Rochester, New York).

2.8 Statistics

In Table 6.1, one way analysis of variance was used to study the effects of different treatments on the incidence of protease activity. Analyses was carried out using the MINITAB statistical package (version 10.2). All experiments were repeated at least for three times and the results were expressed as the mean ± s.e. m, unless otherwise indicated.
Chapter 3 Effects of TLCK on Apoptosis

3.1 Introduction

N-tosyl-L-lysyl chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), two structurally related compounds (Fig. 3.1), were among the first affinity-labelling reagents to be developed (Shaw 1970). They irreversibly and uniquely alkylate the active centre histidine in serine proteases on only one of the two nitrogen atoms of the imidazole ring, representing an unusually selective protein chemical modification (Schoellmann and Shaw, 1963; Ong et al., 1965; Shaw et al., 1965). TLCK requires a basic amino acid in the P1 position whereas TPCK requires an aromatic amino acid in this position (Powers and Harper, 1986). These reagents react with trypsin or chymotrypsin at the chloromethyl group, which is activated by the presence of the carbonyl group in a manner that is subject to competitive inhibition of the reaction by substrates. TPCK is found to alkylate His-57 of chymotrypsin and cause complete loss of enzymatic activity, whilst TLCK alkylates His-46 of trypsin and inactivates the enzyme (Shaw, 1970).

Following the impressive selectivity and discrimination achieved in the affinity labelling of functionally similar enzymes (e.g., the classical trypsin/chymotrypsin case), TLCK and TPCK have been used to study complex biological processes such as fertilization, cell growth, protein synthesis, and virus maturation and also diseases such as cancer (reviewed by Powers, 1977). Recently these two compounds and many other natural or synthetic protease inhibitors have been widely used to investigate the role of proteases in apoptosis. As derivatives of chloromethyl ketones, TLCK and TPCK have
CHAPTER 3

EFFECTS OF N-TOSYL-L-LYSINYL CHLOROMETHYL KETONE (TLCK) ON APOPTOSIS
been subsequently found to non-specifically alkylate cysteine proteases and other intracellular components, leading to cytotoxicity (Shaw, 1990). Both can also inhibit functionally unrelated enzymes such as cAMP-dependent protein kinase and PKC (Kupfer et al., 1979; Solomon et al., 1985).

Whilst most proteins maintain their integrity in apoptotic cells, specific proteolytic events have been found to occur at early stages in the process of apoptosis. These include proteolysis of nuclear lamins (Kaufmann, 1989), fodrin (Martin et al., 1995b), histone H1 (Gaziev et al., 1992) and PARP (Kaufmann et al., 1993). Caspase family members as well as proteases present in cytotoxic granules (Shi et al., 1992a; Shi et al., 1992b), when introduced and/or activated in the cell, are able to induce apoptosis. Moreover, serine protease inhibitors prevented the apoptosis-associated DNA strand breaks in HL-60 cells treated with antitumor drugs and in rat thymocytes treated with glucocorticoids, respectively (Gorczyca et al., 1992; Bruno et al., 1992); calpain inhibitors prevent apoptosis in U937 cells exposed to TNF (Vanags et al., 1996). These studies imply that proteolysis is involved in the execution of apoptosis. Some of the proteases involved in apoptosis have been identified, including caspases responsible for cleavage of PARP and lamins (Takahashi et al., 1996; Lazebnik et al., 1994), a Ca^{2+}-dependent cysteine protease involved in cytoskeletal alterations (i.e. blebbing) (Squier et al., 1994) and a novel 24 kD serine protease involved in the activation of nuclease-dependent DNA digestion (Wright et al., 1994). However, it was also observed that although internucleosomal DNA degradation in thymocytes treated with dexamethasone or teniposide was prevented by serine protease inhibitors including TPCK, the initial step of DNA cleavage, generating ≥ 50 kbp fragments, was not affected (Weaver et al., 1994). Moreover, there is accumulating evidence that protease inhibitors can either potentiate apoptosis induced by other stimuli or induce apoptosis in some circumstances in the absence of other stimuli. TLCK potentiated apoptosis induced by cycloheximide (Gong et al., 1993); both calpain inhibitors and serine protease inhibitors can induce apoptosis in HL-60 cells (Lu and Mellgren, 1996), although the latter inhibit the generation of low molecular weight DNA fragments. Taken together, these observations imply that proteases may not only be involved in the execution of apoptosis but also participate in the regulation of apoptosis at multiple stages. It also appears that the role of protease in apoptosis is cell type dependent. The identification of the role of proteases involved in the control of apoptosis is still at an early stage.
In thymocytes, TLCK inhibits apoptosis induced by diverse compounds (Fearnhead et al., 1995a), indicating that a TLCK inhibitable target may be a common effector of apoptosis. To test whether the TLCK target exists and plays a similar role in the induction of apoptosis in THP.1 cells, a range of agents with different mechanisms of action was studied for their ability to induce apoptosis in the presence or absence of TLCK. These agents include staurosporine (STS), an inhibitor of protein kinases; thapsigargin (THG), an inhibitor of the endoplasmic reticulum-associated Ca\(^{2+}\) ATPase; cycloheximide (CHX), an inhibitor of protein synthesis, and etoposide, an inhibitor of topoisomerase II.

3.2 Flow cytometric study of apoptosis

3.2.1 TLCK potentiates apoptosis induced by CHX and THG but inhibits that induced by etoposide

The HO342/PI staining flow cytometric method has been successfully used to separate normal and apoptotic cells in thymocytes in this laboratory. In the present study this method has been adapted to detect apoptosis in THP.1 cells. Cells stained with HO342/PI were subject to flow cytometric detection. The data was first displayed as a cytogram of side scatter (SSC) vs forward light scatter (FSC) (Fig. 3.2 A and C). Having gated out the debris, indicated by the low SSC and FSC, the remaining cells were displayed as a cytogram of blue fluorescence vs red fluorescence (Fig. 3.2 B and D). Analysis of THP.1 cells for the intensity of HO342 vs PI staining revealed that the majority of untreated cells (~ 97%) were low red (PI) and low blue (HO342) fluorescent (Fig. 3.2 B). In contrast, after treatment for 4 h with CHX (25 \(\mu\)M), a population of cells showed apparently increased HO342 fluorescence (Fig. 3.2 D). Treatment with THG (100 nM) and etoposide (25 \(\mu\)M) also induced this population of cells. In some experiments, a small number of cells with increased PI staining (indicative of necrosis) was observed with these treatments (Fig. 3.2 D). The induction of high blue fluorescent cells by CHX and THG was markedly increased in the presence of TLCK. However the induction of the high blue fluorescent cells by etoposide was abolished when TLCK was present. Control THP.1 cells and those treated with different stimuli were also examined by fluorescence microscopy. In control THP.1 cells (Fig. 3.3 A upper panel), nuclei and cytoplasm...
Fig. 3.2 HO342/PI Flow cytometric analysis of apoptosis. Cells untreated (A, B) or treated for 4 h with CHX (25 μM) (C, D) were stained with HO342/PI. Cells were first displayed as a cytogram of SSC vs FSC (A, C). The debris, indicated by the low SSC and FSC, was gated out and the remaining cells were displayed as a cytogram of blue fluorescence vs red fluorescence (B, D). Three populations of cells were separated. Nor, normal population; Apo, apoptosis; Nec, Necrosis (PI including cells).

were stained blue. Cells treated with TLCK for 4 h showed a similar staining pattern to control cells (Fig. 3.3 A lower panel). CHX, THG and etoposide all caused an increase in the number of cells with morphology distinct from control cells (Fig. 3.3 B, C and D upper panels). These cells contained condensed or fragmented nucleus with enhanced blue staining. TLCK enhanced the induction of these cells by CHX and THG (Fig. 3.3 B and C lower panels) but diminished those induced by etoposide (Fig. 3.3 D lower panel). In some experiments, a number of PI including cells was observed with these treatments,
Fig 3.3 Fluorescence microscopic analysis of apoptosis. Cells were untreated or treated for 4 h with different stimuli, stained with HO432/PI and examined by fluorescence microscopy. A, control THP.1 cells (Upper) and TLCK (100 μM) treated cells (Lower). B, cells were treated with CHX (25μM) alone or in the presence of TLCK (CHX/TLCK). C, cells treated with THG (100 nM) alone or in the presence of TLCK (THG/TLCK). D, cells treated with etoposide alone or in the presence of TLCK (ETOP/TLCK). Magnification: x 100.
Control

TLCK

Fig. 3.3 A
Fig. 3.3 B
Chapter 3 Effects of TLCK on Apoptosis

ETOP

ETOP/TLCK

Fig. 3.3 D
consistent with the measurement by the HO342/PI flow cytometric method (Fig. 3.2). These cells showed apoptotic changes, including nuclei condensation and fragmentation. Thus these cells may represent secondary necrosis. It appears that the induction of the apoptotic morphological changes correlates with the appearance of high blue fluorescent cells. To characterize the different populations, cells treated for 4 h with CHX (25 μM) in the presence of TLCK (100 μM) were sorted. Debris and non-viable cells were gated out and the remaining viable cells were displayed as a cytogram of blue fluorescence vs FSC (Fig. 3.4). Two populations with either low or high blue fluorescence arbitrarily designated as R1 and R2, were sorted for microscopic analysis. As showed in figure 3.4, most cells from R2 were intensely stained with HO342 and displayed an apoptotic morphology, whereas almost all cells from R1 were less stained with HO342 and exhibited a morphology similar to untreated cells. These results revealed an association between the overall increase in HO342 fluorescence staining and the detection of apoptotic alterations in nuclei, confirming the validity of this flow cytometric method for the separation and quantitation of normal and apoptotic THP.1 cells. The induction of apoptosis by different stimuli in the absence or presence of TLCK was quantitatively studied using this HO342/PI flow cytometry method (Table 5.1).

TLCK enhanced the induction of apoptosis induced by CHX and THG but inhibited that induced by etoposide, suggesting that different mechanisms are involved in the modulation of apoptosis by TLCK. To study further the nature of the TLCK target responsible for the modulation of apoptosis, concentration related effects of TLCK on apoptosis induced by CHX and etoposide were studied. Cells were incubated with CHX or etoposide for 4 h either alone or in the presence of a range of concentrations of TLCK. Analysis of apoptosis by HO342/PI flow cytometry showed that TLCK enhanced CHX-induced apoptosis and inhibited etoposide-induced apoptosis at a similar concentration range (Fig. 3.5), suggesting that either the same TLCK target plays a distinct role in different apoptotic pathways or different targets with similar sensitivity are responsible for the TLCK effects.
**Fig. 3.4 Separation of apoptotic and normal cells by flow cytometry.** THP.1 cells were incubated for 4 h with CHX (25 μM) in the presence of TLCK (100 μM), stained with HO342/PI and sorted by flow cytometry. Non-viable cells, which included PI, were gated out and the remaining cells were displayed as a cytogram of blue fluorescence vs FSC. Two populations were sorted and examined by fluorescence microscopy. Cells exhibiting low blue fluorescence (R1) with morphology similar to untreated cells and those exhibiting high blue fluorescence (R2) with distinct apoptotic morphology.
3.2.2 CHX/TLCK induces classical apoptotic morphology

Untreated THP.1 cells were large (12-14 μm) and irregular in outline with many irregular microvilli and a multi-lobed nucleus (Fig. 3.6 A). The diffuse perinuclear and perinucleolar heterochromatin were weakly differentiated from the euchromatin but the nucleolus was clearly visible in most sections. Electron-dense cytoplasmic granules (0.2 μm), characteristic of monocytic cells, were present in many of the cells. Mitochondria, present throughout the cytoplasm, were more numerous than in cells from either bone marrow or circulating blood. Several electron-lucent vacuoles were associated with a well-developed Golgi apparatus. Exposure to cycloheximide (25 μM) in the presence of TLCK (100 μM) resulted in the formation of numerous condensed cells with a densely staining cytoplasm and all the characteristic features of fully apoptotic cells (Fig. 3.6 B). The Golgi vesicles within this cytoplasm contained discrete accumulations of electron-dense material and the matrix of many mitochondria was condensed, although the intracristal compartment remained electron lucent. Many cisternae of the endoplasmic reticulum were dilated to produce electron-lucent vacuoles (Fig. 3.6 B). This was often most pronounced towards one pole of the cell and fusion of these vacuoles with the cell membrane resulted in a “bubbling” appearance. Small clusters of 50-80 clear vesicles (50-
Fig. 3.6 Ultrastructures of normal and apoptotic THP.1 cells. A, untreated THP.1 cells showing the multi-lobed nucleus with distinct nucleolus (n). The cytoplasm contains a well-developed Golgi apparatus (g). Mitochondria is present throughout the cytoplasm (m). B, cells exposed to cycloheximide (25 μM) in the presence of TLCK (100 μM) showing dense cytoplasm. Golgi vesicles (arrow heads) contain discrete accumulations of electron-dense material. The endoplasmic reticulum are dilated, particularly towards one pole of the cell (solid arrows). A small cluster of clear vesicles (open curved arrow) is also present, together with several clumps of fine granular material (solid curved arrows). The heterochromatin is condensed (open arrows) whereas the nucleolus is dispersed to reveal the dense fibrillar component (*). C, for comparison with the fully apoptotic changes induced by CHX/TLCK, the intermediate stage of apoptotic changes induced by TPCK was displayed here (see chapter 4 for TPCK details). Cell treated with TPCK (75 μM) showing dilation of the endoplasmic reticulum (solid arrows) and the accumulation of fine granular material (solid curved arrows). Numerous small clumps of partially condensed chromatin are distributed throughout the nucleoplasm (open arrows) and partial nucleolar disintegration has resulted in dispersal of the dense fibrillar component (*).

All bars = 1 μM
Chapter 3 Effects of TLCK on Apoptosis

**CHX/TLCK**

**TPCK**
250 nm) were also present, together with several clumps of fine granular material (<1 μm) which were not membrane delimited. The heterochromatin in the nuclei of these cells was condensed and largely restricted to crescentic accumulations which were closely associated with the inner nuclear membrane. The edges of these clumps were clearly distinguishable from the electron-lucent euchromatin of the remaining nucleoplasm. Nucleolar disintegration resulting in irregular clusters of the dense fibrillar component was evident in the nuclei of most of the affected cells.

Cells treated THG/TLCK displayed similar morphological changes as those treated with CHX/TLCK (see chapter 5, Fig. 5.6). Treatment with TLCK (100 μM) alone did not cause any signs of apoptosis but resulted in a slight dilation of the endoplasmic reticulum and nuclear envelope of some cells (results not shown).

Exposure of THP-1 cells to etoposide (25 μM) alone resulted in the formation of many apoptotic cells characterised by cytoplasmic and nuclear changes similar to those found after treatment with cycloheximide and TLCK (Fig 3.6 B). In marked contrast to the potentiation of cycloheximide-induced apoptosis by TLCK, this protease inhibitor prevented all the ultrastructural changes induced by etoposide (data not shown). Thus, depending on the stimulus used, a single protease inhibitor exerted diametrically opposed effects on apoptosis in one cell type.

### 3.2.3 STS induces both necrosis and apoptosis and TLCK prevents necrosis but enhances apoptosis

STS, a broad protein kinase inhibitor, has been reported to be a common inducer of apoptosis (Bertrand et al., 1994). However, incubation of THP.1 cells for 4 h with STS (0.5-1 μM) induced a large proportion of necrotic cells (PI including cells) and a small number of apoptotic cells (blue fluorescent cells). To test further the effects of TLCK on the induction of apoptosis via different pathways, cells were cotreated with STS and TLCK. Intriguingly, the induction of necrosis was largely diminished, and instead a large number of apoptotic cells was detected. Cell death induced by STS and STS/TLCK was also examined by fluorescence microscopy. After a 4 h incubation, STS induced a range of cell death morphologies. Some PI staining cells showed necrotic features, such as enlargement (indicative of swelling), yet some others showed apoptotic nuclear changes, such as shrinkage and fragmentation (Fig 3.7 upper panel). A small number of cells,
Fig 3.7 Morphological examination of STS induced-cell death. Cells were treated for 4 h with STS (0.5 μM) alone (upper figure) or in the presence of TLCK (100 μM) (lower figure). These cells were stained with HO342/PI and examined by fluorescence microscopy. Magnification: x 100
which stained blue with condensed and fragmented nuclei, was also observed. By contrast, most cells treated with STS/TLCK exhibited homogeneous apoptotic changes, including nuclear condensation and fragmentation, and enhanced nuclear staining (Fig. 3.7 lower). The nature of cell death induced by STS in the absence or presence of TLCK was further characterized by a kinetic study using the flow cytometric method. STS induced a time dependent induction of necrosis and apoptosis, with the necrosis occurring as early as 1 h and continuing to increase over the next 3 h (Fig. 3.8 a). Apoptotic cells could only be detected at 4 h (Fig 3.8 b). STS/TLCK induced a low level of necrosis at early times (Fig. 3.8 a). After 4 h treatment, only apoptosis was detected. (Fig. 3.8 b). Thus it appeared that in THP.1 cells, STS induced two independent types of cell death, necrosis and apoptosis, and TLCK has an ability to prevent necrosis and to enhance apoptosis.

![Graph](image)

**Fig. 3.8 Kinetics of the induction of necrotic and apoptotic cells by STS or by STS/TLCK.** Cell were treated with STS (0.5 μM) in the absence or presence of TLCK (100 μM) for the indicated time periods. Cells were analysed using the HO342/PI flow cytometric method. a), percentage of necrosis; b), percentage of apoptosis.

### 3.2.4 Cell-cycle-related analysis of apoptosis: in situ end labelling (ISEL)

The detection of apoptotic cells by in situ end labelling (ISEL) was based on the intense labelling with marked nucleotides, such as digoxygenin-nucleotidyl, inserted by exogenous terminal transferase, due to the presence of a large number of free 3’OH termini in the DNA strand breaks (Gorczyca et al., 1992; Gorczyca et al., 1993). The labelled strand breaks can be assessed by FITC- conjugated antidigoxygenin antibody and the cell
cycle distribution can be assessed by PI staining. Fixation in formaldehyde prevented extraction of the degraded DNA from apoptotic cells and thus the cell cycle distribution of both apoptotic and non-apoptotic cells can be estimated by this method.

TLCK had a broad effect on apoptosis induced by multiple stimuli, implying that the TLCK target(s) is an important regulator of apoptosis. To investigate the mechanism by which TLCK modulates apoptosis, cell cycle-related apoptosis was measured by the ISEL method.

**TLCK enhances CHX- and THG-induced apoptosis without cell cycle specificity but inhibits etoposide-induced apoptosis in S phase.** As shown in Fig. 3.9, DNA content (PI, red fluorescence) is plotted on the x-axis, which gives an indication of the position in the cell cycle, and DNA strand breakage (anti-dig-FITC, green fluorescence) is plotted on the y-axis, which in these cells corresponds to the development of apoptosis. Control THP.1 cells exhibited low green fluorescence (Fig. 3.9 A) in agreement with the low level of spontaneous apoptosis under these culture conditions. A small increase in this labelling was observed when cells were incubated for 4 h with CHX (Fig. 3.9 C) and THG (Fig. 3.9 G), consistent with the small percentage of apoptosis as assessed using the HO342/PI flow cytometric method (Table. 5.2). In cells treated with these agents in the presence of TLCK, when a marked potentiation of apoptosis was induced (Table. 5.2), a commensurate increase was observed in cells exhibiting green fluorescence (Fig. 3.9 D and H). This increase occurred in cells from all phases of the cell cycle. In contrast, etoposide induced apoptosis primarily in cells in S phase (Fig. 3.9 E), which was inhibited by TLCK (Fig. 3.9 F). These studies suggest that the targets of TLCK involved in the modulation of apoptosis exist in all phases of the cell cycle and the effect of TLCK on apoptosis is dependent on apoptotic stimulus.

**TLCK partially prevents DNA strand breaks induced by STS.** Treatment of cells for 4 h with STS, which induced a large number of necrotic cells and a small number of apoptotic cells (Fig. 3.7), caused a massive induction of green fluorescence labelling cells (Fig. 3.10 C). TLCK reduced the induction of these cells in a concentration-dependent manner (Fig. 3.10 E and G). However, at the highest concentration (100 µM), which completely prevented the induction of necrotic cells, as assessed by HO342/PI flow cytometry (Fig. 3.10 H), TLCK only partially inhibited cells with high green fluorescence.
Fig. 3.9 Analysis of cell cycle-related apoptosis. Cells untreated (A) or treated for 4 h with CHX (C) (25 μM), ETOP (E) (25 μM) or THG (G) (100 nM) either alone or in the presence of TLCK (100 μM) (B, D, F, H). Data was displayed as cytogram of green (fluorescein) vs red (PI) fluorescence.
Chapter 3 Effects of TLCK on Apoptosis

Fig. 3.10 Analysis of STS- and STS/TLCK-induced cell death by flow cytometry using ISEL and HO342/PI methods. Cells untreated (A) or treated for 4 h with STS (0.5 μM) either alone (C, D) or in the presence of the indicated concentrations of TLCK (E, F, G, H). Cells were detected for DNA strand breaks by ISEL method (A, C, E, G) and for apoptotic and necrotic cell death by HO342/PI method (B, D, F, H). The attached table gives the percentage of anti-dig-FITC labelled cells as well as apoptotic and necrotic cells.

<table>
<thead>
<tr>
<th></th>
<th>ISEL Labelled cells</th>
<th>HO342/PI Apo</th>
<th>Necro</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>0.5</td>
<td>3.5</td>
<td>2.0</td>
</tr>
<tr>
<td>STS (0.5μM)</td>
<td>47.8</td>
<td>14</td>
<td>62</td>
</tr>
<tr>
<td>STS/TLCK (50μM)</td>
<td>35.6</td>
<td>32</td>
<td>3.9</td>
</tr>
<tr>
<td>STS/TLCK (100μM)</td>
<td>29</td>
<td>30</td>
<td>3.3</td>
</tr>
</tbody>
</table>
Chapter 3 Effects of TLCK on Apoptosis

(Fig. 3.10 G). These results demonstrated that DNA strand breaks induced by STS were coincident with the induction of both necrosis and apoptosis, whilst those induced by STS in the presence of TLCK were coincident only with apoptosis. This study provides evidence that necrosis and apoptosis both give rise to DNA strand breaks, which can be detected by the ISEL method. The cells with increased green fluorescence induced by STS alone or in the presence of TLCK were detected in all phases of the cell cycle.

3.3 Effects of TLCK on biochemical changes of apoptosis

The induction of apoptosis by different stimuli and the effects of TLCK on apoptosis were studied biochemically using two apoptotic markers, DNA and PARP degradation.

3.3.1 TLCK enhances apoptotic DNA degradation induced by CHX, THG and STS but inhibits that induced by etoposide

Little or no DNA cleavage products were detected in control cells (Fig. 3.11, lane 1) and those treated with TLCK alone (100 μM) (Fig. 3.11, lane 10). Incubation of THP.1 cells for 4 h with CHX (25 μM) or THG (100 nM) did not induce apparent DNA cleavage (Fig. 3.11 lanes 4 and 6), possibly due to the low level of apoptosis. However, the internucleosomal cleavage of DNA was detected when TLCK (100 μM) was present (Fig. 3.11, lanes 5 and 7). Treatment of cells with etoposide (25 μM) for 4 h also induced this pattern of DNA cleavage (Fig. 3.11 lane 8), which was decreased when TLCK (100 μM) was present (Fig. 3.11 lane 9). Treatment of THP.1 cells for 4 h with STS (0.5 μM) appeared to cause a smear rather than internucleosomal pattern of DNA cleavage (Fig. 3.11, lane 2). In the presence of TLCK, however, nucleosomal DNA fragments become more apparent (Fig. 3.11, lane 3). TLCK also enhanced the induction of large kbp DNA fragmentation induced by CHX and THG, but inhibited that induced by etoposide (See chapter 5 Fig. 5.3). These data were in good agreement with the flow cytometric study, which showed that TLCK enhanced the induction of apoptosis induced by CHX and THG but inhibited that induced by etoposide. STS alone caused an apparent smear pattern of DNA degradation, consistent with the flow cytometric assessment, where STS induced a large proportion of necrotic cells (PI including cells) and only a small number of apoptotic cells (high HO342 fluorescent cells) (Fig. 3.10 D). TLCK completely prevented necrotic cells but increase the number of apoptotic cells (Fig. 3.10 H). Thus the cell deaths induced by STS either alone, or in the presence of TLCK, were distinct.
Fig 3.11 TLCK enhances internucleosomal cleavage of DNA induced by CHX, THG and STS, but inhibits that induced by etoposide. Cells incubated either alone (lane 1) or with TLCK (100 μM) (lane 10) for 4 h were used as controls. Cells were treated for 4 h with STS (0.5 μM), CHX (25 μM), THG (100 nM) or ETOP (25 μM) either alone (lanes 2, 4, 6, 8) or in the presence of TLCK (lanes 3, 5, 7, 9).

3.3.2 CHX/TLCK and THG/TLCK induce PARP degradation

Degradation of PARP to yield an 85 kD fragment is a common feature of apoptosis in many forms of apoptosis. To ascertain the induction of apoptosis by CHX/TLCK and THG/TLCK, PARP degradation was studied. CHX and THG induced a small amount of the 85 kD fragments (not shown), whilst TLCK alone did not induce PARP degradation (Fig. 3.12 lane 5). Clearly, CHX/TLCK and THG/TLCK caused an almost complete loss of the intact PARP, accompanied by the appearance of a large amount of 85 kD proteolytic fragments (Fig. 3.12, lanes 3 and 5). In contrast with the effects of serine protease inhibitor TLCK, Z-VAD.FMK, a caspase inhibitor, completely inhibited the induction of apoptosis and the PARP degradation induced by these treatments (Fig. 3.12, lanes 4 and 7 also see chapter 5).
Fig. 3.12 CHX/TLCK and THG/TLCK induce PARP degradation. THP.1 cells were incubated for 4 h alone (lane 1). Cells were also incubated for 4 h with TLCK (100 μM) either alone (Lane 5) or in the presence of CHX (25 μM) (lane 3) or THG (100 nM) (lane 6). Cells were preincubated for 1 h with Z-VAD.FMK (50 μM) followed by an incubation for 4 h either alone (lane 2) or with CHX/TLCK (lane 4) or THG/TLCK (lane 7). The arrows indicate intact PARP (116 kD) and the 85 kD fragments.

The PARP cleavage was also caused when cells were treated for 4 h with etoposide (see Fig. 5.4, lane 6), whereas TLCK and Z-VAD.FMK both prevented this event (Fig. 5.4, lanes 7 and 8) (see chapter 5 for Z-VAD.FMK details).

3.3.3 Time course of large kbp DNA degradation and PARP proteolysis induced by CHX in the absence or presence of TLCK

To further study the nature of the effects of TLCK on apoptosis, a time course of two early apoptotic events, large kbp DNA degradation and PARP cleavage, was analysed. Cells incubated either alone or with TLCK for 4 h did not show any DNA cleavage (Fig 3.13 upper panel, lanes 1 and 2). CHX caused a slight increase in the 50 kbp DNA fragments (Fig. 3.13 upper panel, lanes 3, 5 and 7), which were markedly increased in the presence of TLCK (Fig. 3.13 upper panel, lanes 4, 6 and 8). This formation of 50 kbp DNA fragments
Fig. 3.13 upper panel, Time course of the degradation of DNA induced by CHX in the absence and presence of TLCK. Cells were untreated (lane 1) or treated with TLCK (100 μM) for 4 h (lane 2). Cells were treated for 1, 2, 3 and 4 h with cycloheximide alone (25 μM) (lanes 3, 5, 7 and 9) or in the presence of TLCK (lanes 4, 6, 8 and 10). Lower panel, time course of PARP degradation. The same samples were also analysed for PARP degradation. The lines in the figure indicate intact PARP and the 85 kD proteolytic fragment.
DNA fragments induced by CHX/TLCK was detected in a significant amount at 1 h (Fig. 3.13 upper panel, lane 4) and reached maximum at 2 h (Fig. 3.13 upper panel, lane 6) then decreased over the next 2 hours (Fig. 3.13 upper panel, lanes 8 and 10). The decrease of these large DNA fragments is due to a further degradation, since a large amount of internucleosomal DNA cleavage was detected at 4 h (Fig. 3.11).

In control THP.1 cells only intact 116 kD PARP was detected without its degradation products (Fig. 3.13 lower panel). Treatment of cells with CHX caused a low level of proteolysis of PARP to an ~85 kD fragment, which was detected at 3 h and 4 h but not at 1 h and 2 h (Fig. 3.13 lower panel). However, in the presence of TLCK, the cleavage of PARP induced by CHX was largely enhanced and was detected as early as 2 h, reaching a maximum over the next 2 hours (Fig. 3.13 lower panel). Thus it was demonstrated that under these experimental conditions, when apoptosis was induced by CHX/TLCK, the degradation of DNA into large kbp fragments appeared to precede the cleavage of PARP. TLCK modified the induction of apoptosis at early stages before the degradation of DNA into large kbp fragments and the proteolysis of PARP.

3.4 Putative TLCK target

3.4.1 Pre-existing TLCK target

The effects of TLCK on apoptosis induced by CHX, an inhibitor of protein synthesis, indicate that the TLCK target(s) is not newly synthesized in response to the apoptotic signal. TLCK is unstable at pH above 6 (Beynon and Salvesen, 1994). In order to understand better the nature of the TLCK target(s) involved in the modulation of apoptosis, cells were preincubated with TLCK (100 μM) for a variety of time periods before the addition of CHX or etoposide. Incubation of cells for 4-24 h with TLCK had little or no effect on cell viability. The effects of TLCK on apoptosis induced by both CHX and etoposide were observed even when cells were preincubated with TLCK for up to 24 h before adding these stimuli (Fig. 3.14). These data suggested that the TLCK target(s) responsible for its effects on apoptosis is pre-expressed in THP.1 cells and is resynthesized slowly in the presence of TLCK.
Chapter 3 Effects of TLCK on Apoptosis

Fig. 3.14 Pre-existing TLCK target. Cells were untreated or treated for 4 h with CHX (25 μM) or etoposide (25 μM) either alone (open boxes) or in the presence of TLCK (100 μM) (filled boxes). Alternatively, cells were preincubated with TLCK for 24 h (hatched boxes) prior to incubation either alone or with CHX or etoposide for a further 4 h. Apoptosis was assessed by HO342/PI flow cytometry.

3.4.2 Effect of TLCK on cell growth

To test whether the modulation of apoptosis by TLCK is associated with its effects on other important cellular functions, the ability of TLCK to affect cell growth was assessed. Incubation of cells for up to 72 h with 100 μM TLCK, a concentration which modulates apoptosis, caused a profound cell growth inhibition (Fig. 3.15 A), as assessed by cell counting, while cell viability was not altered by TLCK.

Treatment of cells with TLCK for 4 h caused no apparent cell cycle blocking (Fig. 3.15 B.a), as assessed by measuring DNA content. After 24 h, a slight increase of cells in G2/M (Fig. 3.15 B.b) was observed. The effect of TLCK on cell cycle was coincident with its effect on the induction of apoptosis, showing no cell cycle specificity.
Chapter 3 Effects of TLCK on Apoptosis

Fig. 3.15 A, Inhibition of cell growth by TLCK. Cells were cultured for 0-72 h either alone (■-■) or in the presence of TLCK (100 μM) (□-□). Cell number was counted at the indicated times using a SCHARFE CASY II cell counter. B, TLCK induces no apparent cell cycle changes. Cells were incubated with either alone (Filled area) or with TLCK (100 μM) (open area) for 4 h (a) and 24 h (b). Cells were fixed with 70% ethanol and stained with PI. DNA content was measured by a FACScan. The x-axis displays the DNA content in individual cells and the y-axis displays the number of events.
Chapter 3 Effects of TLCK on Apoptosis

3.5 Discussion

3.5.1 Mechanisms of the modulation of apoptosis by TLCK

Diverse stimuli were able to induce apoptosis in THP.1 cells. Previous studies described the possible mechanisms of the agents used in this study for the induction of apoptosis. CHX, at concentrations which were shown to be inhibitory to protein synthesis, induced apoptosis in a number of cell types (Martin et al., 1990). Etoposide, an agent which has been used to treat acute lymphoblastic leukemia and a variety of solid tumors (Hainsworth and, 1995), interacts covalently with topoisomerase II and DNA, inducing DNA strand breaks and apoptosis in many systems (Kataoka et al., 1994; Sinha et al., 1995). Thapsigargin is a non-phorbol ester type tumor promoter (Tsukamoto et al., 1993). Because this reagent was thought to specifically inhibit endoplasmic reticulum Ca^{2+}-ATPase but not plasma membrane Ca^{2+}-ATPase (Thastrup et al., 1990), THG-induced elevation of \([\text{Ca}^{2+}]_i\) reflects the calcium release from this intracellular pool (Thastrup et al, 1990; Tsukamoto et al., 1993). The depletion of intracellular Ca^{2+} pool has been hypothesized to induce apoptosis (Kaneko and Tsukamoto, 1994). Staurosporine, an alkaloid isolated from Streptomyces culture (Okasaki et al., 1988), is a potential protein kinase inhibitor with a broad spectrum of activity (Tamaoki et al., 1986; Kiyoto et al., 1987; Nakano et al., 1987). It has been shown to induce apoptosis in almost all types of cells tested. Although the mechanisms of the action of these stimuli differ, TLCK enhanced the induction of apoptosis by all these agents except etoposide, where the induction of apoptosis was prevented.

TLCK modulated the induction of apoptosis by CHX and THG in a similar manner. TLCK enhanced biochemical changes, including the degradation of DNA and PARP (Fig. 3.11 and 3.12). Apoptosis induced by CHX/TLCK or THG/TLCK showed similar morphology without cell cycle specificity (Fig. 3.3 and 3.9). A similar effect of TLCK on CHX-induced apoptosis was observed in HL-60 cells (Gong et al., 1993). These results suggest that TLCK interferes with a general regulator of apoptosis at an early phase in the apoptotic pathway.

Caspase-3 is a member of the caspase family. Its activity is associated with the induction of apoptosis in a number of model systems (Nicholson et al., 1995; Tewari et al., 1995a; Lazebnik et al., 1994). Caspase-3 or a related caspase activity is involved in the induction of apoptosis by many stimuli in THP.1 cells, indicated by the cleavage of...
PARP (Fig. 3.12; Fig. 5.4, lane 6). TLCK alone is incapable of inducing apoptosis, consistent with its inability to activate caspase activity. However, TLCK enhanced the activation of caspase by CHX and THG. It seems that the TLCK target(s) is not an effector of apoptosis but rather it is involved in the control of caspase activities.

TLCK enhanced CHX-induced apoptosis, however it inhibited morphological and biochemical changes of apoptosis induced by etoposide at a similar concentration (Fig. 3.3 and 3.11), implying that either a single TLCK target plays a distinct role in different apoptotic pathways or two structurally related targets were involved. It was reported that TLCK enhanced apoptosis induced by CHX but inhibited that induced by camptothecin, a DNA topoisomerase I inhibitor, in HL-60 cells (Gong et al., 1993). It also inhibits apoptosis induced by inhibitors of topoisomerase I and topoisomerase II in thymocytes (Bruno et al., 1992). The reason why TLCK inhibits apoptosis induced by these DNA damaging agents is unclear but may be due to TLCK blocking a very early step or inhibiting a pre-apoptotic signalling event in response to the direct DNA cleavage induced by these treatments.

The present study was in marked contrast to the studies with thymocytes where apoptosis induced by diverse stimuli was inhibited by TLCK (Fearnhead et al., 1995a). These contrasting results suggest that the TLCK target(s) is not part of the effector mechanism but rather that it has an important function either positively or negatively regulating apoptosis depending on the cell type. Moreover TPCK but not TLCK prevented apoptosis, induced via p53-dependent and -independent pathways in myeloid leukemic cells (Lotem and Sachs, 1996), suggesting that there are cell type differences in the proteases involved in apoptosis.

**3.5.2 The role of TLCK target in cell death and cell growth**

The modulation of apoptosis by TLCK was associated with its ability to inhibit cell growth (Fig. 3.15 A). Cell cycle analysis revealed that the potentiation of apoptosis and the inhibition of cell growth by TLCK showed no cell cycle specificity (Fig. 3.9 and 3.15 B). Both effects of TLCK were observed at the same concentration. It seems that a TLCK inhibitable target has a function in both cell growth and cell death.

There is evidence that cell survival signals can prevent apoptosis. Growth factors that promote T lymphocyte proliferation prevent these cells from undergoing apoptosis (Gillis et al., 1978; Duke, 1991). Ligation of the type I surface glycoprotein CD40, which
delivers a survival signal in B cell types, prevents lymphoma B cells from calcium ionophore- and antigen receptor-triggered apoptosis by inhibiting activation of caspase-3 (An and Knox, 1996). It is likely that TLCK potentiated the induction of apoptosis by blocking a survival signal.

The mechanism of cell growth inhibition by TLCK was not investigated in this study. The NF-κB family of transcription factors plays a major role in the control of the expression of *c-myc* gene (Lee et al., 1995), which is an oncogene product regulating cell cycle from G1 phase to S phase (Cole, 1986, Luscher and Eisenman, 1990). Therefore the decreased *c-myc* expression will lead to cell growth arrest. In addition, NF-κB has been reported to be a cell death inhibitor (Wang et al., 1996a; Antwerp et al., 1996). These studies suggest an important role of NF-κB in the control of both cell death and cell growth. The constitutive activation of NF-κB and the expression of c-Myc have been detected in THP.1 cells, and TLCK inhibited the activation of NF-κB in these cells (unpublished data). Recently, it has been reported that TLCK and TPCK inhibit the activation of pp70(s6k), a mitogen-regulated serine/threonine kinase involved in the G1 to S phase transition of the cell cycle (Grammer et al., 1996). In Hela cells, TLCK effectively inhibited the induction of DNA synthesis in isolated nuclei by cytoplasmic factors (Wong et al., 1987). In addition, TLCK can directly interact with PKC and inhibit it activity, whereas TPCK is less effective (Solomon et al., 1985). The inhibition property of TLCK on cell growth in THP.1 cells did not support that the effect of TLCK on cell growth is due to the inhibition of a single cell cycle specific regulator. A number of important targets is presumably involved and the inhibitory effects occurred at multiple cell-cycle stages. In Hela cells three proteases functioning at G1, S, and G2 phases have been suggested to be trypsin-like serine proteases with different specificity (Kozaki et al., 1994). It is unknown whether these proteases are existing in THP.1 cells. The modulation of apoptosis by TLCK was observed even when cells were pre-incubated with TLCK for 24 h, suggesting that the responsible TLCK target(s) is preexisting in the cells and may have an important function in preventing cell death under physiological conditions. Many early studies have clearly documented anticarcinogenic effects of protease inhibitors directed toward chymotrypsin-like or trypsin-like proteases (Kennedy, 1994; Kennedy and Little 1978), although the effects of relevant protease inhibitors on apoptosis were not investigated simultaneously. Taken together, serine proteases may be involved in the control of multiple complex cellular functions. The ability of TLCK to enhance the

Chapter 3 Effects of TLCK on Apoptosis
induction of apoptosis may be related to its ability to prevent cell growth. However the final effect on the induction of apoptosis is dependent on an early signal transducing pathway.

### 3.5.3 The role of TLCK target in necrosis and apoptosis

In THP.1 cells, STS induced a more rapid, nonapoptotic cell death and delayed apoptotic cell death assessed by HO342/PI flow cytometric assay (Fig. 3.8). Morphological studies revealed that some PI including cells induced by STS contained enlarged and intact nuclei, signs of necrosis, whilst others contained condensed and fragmented nuclei, signs of apoptosis (Fig. 3.7 upper panel). These PI including cells with apoptotic nuclei may initially undergo apoptosis then progress into secondary necrosis. STS induced a smear pattern of DNA cleavage, consistent with necrotic form of cell death (Fig. 3.11). Kinetic study of the two forms of cell death revealed that necrosis appeared earlier than apoptosis (Fig. 3.8), excluding the possibility that the necrotic cell death was solely developed from apoptosis. This study demonstrated that STS activates the necrotic pathway concomitant with the apoptotic pathway. In the presence of TLCK, necrotic changes were prevented and apoptotic cells were predominant, assessed by morphological and flow cytometric studies (Fig. 3.7 lower panel and Fig. 3.10). TLCK enhanced the induction of apoptosis by STS but did not alter the kinetics of the induction (Fig. 3.8). A similar time course of the induction of apoptosis by STS was observed in GM701 fibroblasts cell line (Jacobson et al., 1996). It is not clear what happens in the lag period, prior to the induction of apoptosis. It presumably involves the inhibition of one or more protein kinases. It seems that the TLCK confers cells resistant to STS-induced acute membrane and DNA damage, therefore allowing more cells to undergo apoptosis. TLCK may also prevent the loss of membrane integrity at a later stage of apoptosis, therefore preventing secondary necrosis. These results suggest that TLCK target(s) is also involved in the maintenance of membrane integrity. Although the ISEL method has been widely used to study apoptosis, the present study demonstrated that this method can also detect necrotic DNA breaks, suggesting that the type of cell death can not be characterized by using this single measurement. The differences between apoptosis and necrosis are clearly fruitful areas and the identification of the mechanisms of apoptosis and necrosis will lead to better methods in diagnostic and research toxicological pathology.
3.6 Summary

1. In THP.1 cells, TLCK enhanced the induction of apoptosis by a number of stimuli with different mechanisms of action, suggesting that TLCK may react with an important regulator of apoptosis, which is upstream of a key effector of apoptosis.

2. TLCK inhibited etoposide-induced apoptosis, indicating that the TLCK target(s) has a distinct role in different apoptotic pathways.

3. TLCK also inhibited cell growth and necrotic cell death.

4. This study demonstrated that a single protease inhibitor had effects on both cell death and cell growth at the same concentration, and therefore it is important to investigate whether these fundamental cellular functions are regulated by related mechanisms.

(Part of the work presented in this chapter has been published (Zhu et al., 1995)
CHAPTER 4

EFFECTS OF N-TOSYL-L-PHENYLALANYL CHLOROMETHYL KETONE (TPCK) ON APOPTOSIS
4.1 TPCK induces apoptosis

In order to distinguish the role of structurally related proteases in apoptosis, the effects of TPCK on apoptosis were also studied. To determine whether TPCK has any intrinsic effect on apoptosis, cells were incubated with TPCK and analysed for apoptosis. Incubation of THP.1 cells for 4 h with TPCK (25-75 μM) caused a concentration-dependent induction of apoptosis as assessed by flow cytometry (Fig. 4.1). As TPCK has been described as an inhibitor of apoptosis in several cellular systems (Ghibelli et al., 1995; Suffys et al., 1988; Ruggiero et al., 1987; Weaver et al., 1994), TPCK treated cells were also examined biochemically and by electron microscopy.

![Graph showing TPCK concentration-dependent induction of apoptosis](image)

**Fig. 4.1** TPCK causes a concentration-dependent induction of apoptosis. THP.1 cells were incubated for 4 h with the indicated concentrations of TPCK. Apoptosis was assessed using the HO342/PI flow cytometric analysis. Data are mean ± S.D. of three different determinations.

4.2 Ultrastructural and biochemical study of TPCK-induced apoptosis

4.2.1 TPCK induces intermediate phase of apoptotic morphology

Treatment with low concentrations of TPCK (2 - 6 μM), which did not induce apoptosis, had no detectable effect on the ultrastructure of THP.1 cells (data not shown).
In contrast, exposure to higher concentrations (50 - 75 µM) resulted in cytoplasmic condensation accompanied by dilation of the endoplasmic reticulum and the formation of small fine granular material (0.5-1.5µm diam.) (Fig. 3.6 C), similar to that observed after other apoptotic stimuli. Partial nucleolar disintegration was indicated by the dispersal of the dense fibrillar component which, like numerous small clumps of partially condensed chromatin, was distributed throughout the nucleoplasm (Fig. 3.6 C). The degree of condensation was intermediate between control cells and those treated with cycloheximide and TLCK (100 µM) (Fig. 3.6 B). Thus in THP.1 cells, TPCK alone induced ultrastructural signs of apoptosis intermediate between normal cells (Fig. 3.6 A) and those with the full apoptotic phenotype (Fig. 3.6 B).

4.2.2 TPCK induces formation of large kbp DNA fragments

Incubation of cells with TPCK at concentrations of 50-75 µM, which induced apoptosis assessed by flow cytometry, caused formation of large kbp DNA fragments ranging from 30-50 to 700 kbp (Fig. 4.2) without internucleosomal DNA cleavage (data not shown). A low concentration of TPCK (25 µM) did not induce the formation of any large fragments, consistent with its inability to induce apoptosis.

The large kbp DNA cleavage in the presence or absence of internucleosomal DNA cleavage has been associated with the induction of apoptosis in many cases, including in thymocytes treated with glucocorticoids (Walker et al., 1991), in CTLL cells following IL-2 depletion (Walker et al., 1993), and other cells following serum withdrawal (Oberhammer et al., 1993). These data suggest that an endonucleolytic activity cleaving DNA at specific, long-range intervals is involved in apoptosis. To further confirm that TPCK induced apoptosis in THP.1 cells, other biochemical features of apoptosis were examined.

4.2.3 TPCK induces processing/activation of caspases and proteolysis of PARP and laminas

Processing of caspase-3 and caspase-7. The caspase family plays a central role in the execution of apoptosis, although the mechanisms of their activation and regulation are unknown. Caspase-3, a 32 kD protein, is a member of this family known to be responsible for a number of key proteolytic events of apoptosis (Table 1.1), while caspase-7, a 35 kD
Fig. 4.2 TPCK causes a concentration-dependent induction of large kbp DNA fragments. Samples were analysed by field inversion agarose gel electrophoresis. THP.1 cells were incubated for 4 h either alone (lane 1) or with indicated concentrations of TPCK (Lanes 2-4). Cells were also incubated with Z-VAD.FMK either alone (lane 6) or in the presence of TPCK (75 μM) (lane 5) (see chapter 5 for Z-VAD.FMK details).
protein, is a close homologue of caspase-3 (Duan et al., 1996a). Like other proteases in this family, both procaspase-3 and procaspase-7 have to be proteolytically processed to become active. Active caspase-3 comprises two subunits about 17 kD and 12 kD in size (Nicholson et al., 1995) and active caspase-7 comprises ~20 kD and ~ 12 kD subunits (Duan et al., 1996a). Both procaspase-3 and procaspase-7 but not their active subunits were detected in control THP.1 cells and cells treated for 4 h with a low concentration of TPCK (25 μM) (Fig 4.3). However, higher concentrations of TPCK (50-75 μM), which induced large kbp DNA fragments (Fig. 4.2 lanes 3 and 4), also induced a concentration-dependent decrease in proform of caspase-3 accompanied by the appearance of two protein bands, possibly being the p17 subunit and an intermediate product p19 (Fig. 4.3 upper panel). Similarly, the decrease in proform of caspase-7 and the appearance of the p20 subunit was also induced by TPCK at higher concentrations (50-75 μM) but not at a low concentration (25 μM) (Fig. 4.3 lower panel). These results indicate that caspase-3 and caspase-7 are activated by TPCK.

Fig. 4.3 TPCK induces concentration-dependent processing of caspase-3 and caspase-7. Cells were incubated either alone or with indicated concentrations of TPCK. Caspase-3 and caspase-7 were detected by western blotting. The lines in the figure indicate procaspase-3 and procaspase-7 and their processing products.
**Processing of caspase-2.** Caspase-2 is a member of caspase family about 51 kD in size (Kumar et al., 1994). In vitro it can be processed into the p19 and p12 subunits of its active form by many other members of the family with caspase-3 being most efficient (Harvey et al., 1996). Some apoptotic cells, such as apoptotic NIH-3T3 cells and CTL-mediated apoptotic cells, contain caspase-2 processing activity (Harvey et al., 1996), although the substrate of the enzyme has not been identified. To test the possible involvement of caspase-2 in the induction of apoptosis induced by TPCK, caspase-2 processing was examined by western blotting. In control THP.1 cells, procaspase-2 is expressed (Fig. 4.4 lane 1). TPCK caused a concentration-dependent decrease of procaspase-2 and the appearance of p12, a small subunit of active caspase-2 (Fig. 4.4). It was noticed that, a low concentration of TPCK (25 μM), which was incapable of inducing the processing of caspase-3 and caspase-7, caused an apparent decrease in procaspase-2 (Fig. 4.4). These data suggest that the loss of proform of caspase-2 is not correlated with the induction of apoptosis.

<table>
<thead>
<tr>
<th>TPCK (μM)</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
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<tr>
<td>Pro-caspase-2</td>
<td>-</td>
<td>-</td>
<td>51</td>
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<td>PARP</td>
<td>-</td>
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<td>12</td>
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*Fig. 4.4 TPCK induces caspase-2 processing.* Cells were incubated for 4 h either alone or in the presence of indicated concentrations of TPCK. The lines in the figure indicate the procaspase-2 and its processing product.

**PARP and Lamin cleavage.** *In vitro,* caspase-3 and caspase-7 can efficiently cleave intact PARP into fragments indistinguishable from those observed in apoptotic cells.
Chapter 4 Effects of TPCK on Apoptosis

(Tewari et al., 1995a, Fernandes-Alnemri et al., 1995b), although it is likely that all caspases have the capacity to cleave PARP but with markedly different efficiencies (reviewed in Kumar and Harvey, 1995). To confirm the activation of caspase-3 and caspase-7 by TPCK, PARP cleavage was analysed. A low concentration of TPCK (25 μM), which failed to induce caspase-3 and caspase-7 processing, did not increase the proteolysis of PARP above that observed in control cells (Fig. 4.5). Higher concentrations of TPCK (50-75 μM) induced a concentration-dependent proteolysis of PARP to yield a fragment of ~85 kD (Fig. 4.5), a similar size to that observed when PARP was cleaved by caspase-3 and caspase-7 in vitro (Tewari et al., 1995a; Fernandes-Alnemri et al., 1995b).

Lamin degradation is a common feature of apoptosis in a variety of systems. It has been suggested that caspase-6 is responsible for lamin degradation during the apoptosis (Takahashi et al., 1996a). To investigate whether lamin degradation is induced by TPCK, control cells and cells treated for 4 h with TPCK were examined for intact and degraded lamins. It was demonstrated that TPCK at concentrations which induce apoptosis caused a degradation of lamin B1 into p46 fragments indistinguishable from those produced by caspase-6 in vitro (Fig. 4.5) (Orth et al., 1996), indicating that the laminase, possibly caspase-6, was activated by TPCK.

Fig. 4.5 TPCK causes concentration-dependent proteolysis of PARP and lamin B1. Cells were untreated or treated for 4 h with TPCK at indicated concentrations. PARP and lamin B1 were detected by western blotting. The lines in the upper figure indicate intact PARP (116 kD) and its 85 kD proteolytic product. The lines in the lower figure indicate intact lamin B1 (67 kD) and its ~46 kD proteolytic fragment.
4.3 TPCK inhibits internucleosomal cleavage of DNA

Degradation of chromatin into oligonucleosome-sized fragments is a well documented biochemical change in apoptosis (Wyllie, 1980). TPCK induced many common biochemical features of apoptosis except for the internucleosomal cleavage of DNA in THP.1 cells. TPCK has been reported as an inhibitor of apoptosis in a number of different systems (Ghibelli et al., 1995; Suffys et al., 1988; Ruggiero et al., 1987; Neamati et al., 1995; Weaver et al., 1994), while many of these studies have utilised an increase in internucleosomal cleavage as a marker of apoptosis. To further understand the role of the TPCK target in apoptosis, the effects of TPCK on apoptosis induced by other stimuli were studied. Incubation of THP.1 cells for 4 h with etoposide (25 μM) alone (Fig. 4.6, lane 3) or CHX (25 μM) in the presence of TLCK (Fig. 4.6 lane 5) caused an increase in internucleosomal cleavage of DNA compared with control cells (Fig. 4.6 lane 1). TPCK at 10 μM (Fig. 4.6, lane 2), which is insufficient to induce apoptotic changes, effectively inhibited etoposide- and CHX/TLCK-induced internucleosomal cleavage of DNA (Fig. 4.6 lanes 4 and 6). These data suggest that a TPCK sensitive protease activity is associated with the endonuclease responsible for the internucleosomal cleavage of DNA. The paradoxical effects of TPCK on apoptosis can be explained by an apoptosis-inducing effect which requires higher concentrations, and an anti-DNA degradation effect which requires low concentrations. Thus it seems that TPCK can target different intracellular components depending on the concentration used to either initiate apoptosis or to inhibit internucleosomal cleavage of DNA induced by other stimuli.

4.4 TPCK modifies the induction of apoptosis by CHX and etoposide in a similar manner to TLCK

Low concentrations of TPCK (10 μM), which inhibited the induction of internucleosomal cleavage of DNA by etoposide or by CHX/TLCK, was also tested for its ability to modify the induction of apoptosis using flow cytometric analysis. Low concentrations of TPCK (2-25 μM) didn't induce apoptosis compared with control cells (Fig. 4.7). However, it potentiated CHX-induced apoptosis but inhibited etoposide-induced apoptosis (Fig. 4.7), a similar effect observed with TLCK. These results suggest that TLCK and TPCK have a common target in the pathway of apoptosis.
Fig. 4.6 TPCK inhibits internucleosomal cleavage of DNA induced by apoptotic stimuli.
Cells incubated for 4 h either alone (lane 1) or with TPCK (10 µM) (lane 2) were used as controls. Cells were treated for 4 h with etoposide (25 µM) either alone (lane 3) or in the presence of TPCK (10 µM) (lane 4). Cells were also treated for 4 h with CHX (25 µM)/TLCK (100 µM) in the absence (lane 5) or presence of TPCK (10 µM) (lane 6).
4.5 Discussion

4.5.1 Implication of multiple targets of TPCK in the regulation of apoptosis

In THP.1 cells, TPCK (50-75 μM) appeared to initiate several features characteristic of early apoptotic changes (Kerr et al., 1987; Walker et al., 1988). The endoplasmic reticulum was dilated to produce electron-lucent vacuoles and the chromatin was partially condensed. The apparent difference between the morphology induced by TPCK and by CHX/TLCK is that apoptosis induced by TPCK did not exhibit full cytoplasm and nuclear condensation, consistent with the different biochemical changes induced by these two treatments. CHX/TLCK induced internucleosomal cleavage of DNA, but TPCK did not. TPCK also inhibited internucleosomal cleavage of DNA induced by other apoptotic stimuli. Thus, apoptotic morphological changes induced by TPCK may be arrested at the stage before the internucleosomal cleavage of DNA. The induction of apoptosis by TPCK was associated with the activation of caspase-3 and caspase-7, possibly caspase-6 and caspase-2, suggesting that these caspases may have a role in execution of apoptosis induced by TPCK. Other studies have implicated a number of different caspases in the execution phase of apoptosis (reviewed in Kumar 1995; Martin and Green, 1995). It has been shown that
high concentrations of TLCK and TPCK prevent the cleavage of PARP and nuclear lamins mediated by caspases and inhibit the processing of caspases into their catalytic active subunits in vitro (Kumar and Harvey, 1995, Harvey et al., 1996; Wilson et al., 1994; Mishima et al., 1995). Thus in some cellular systems, TLCK and TPCK may prevent apoptosis by inhibition of caspases. For example in Jurkat cells, TLCK (1 mM) and TPCK (200 μM) prevent Fas-induced α-fodrin and PARP cleavage, as well as the DNA fragmentation and membrane blebbing. However both TLCK and TPCK at the same concentrations induce cell death (Cryns et al., 1996). The difference between in vitro and in intact cell study may be due either to the high concentration of TPCK and TLCK required to inhibit recombinant caspase-3 and caspase-7 (Fernandes-Alnemri et al., 1994 and 1995b) or to an inability to achieve adequate concentrations of TPCK and TLCK within cells. Thus in intact cells, they may target different proteases. It is likely that in THP.1 cells one of the intracellular targets of TPCK is upstream of caspase-3/7, the inhibition of which results in activation of the cell death program.

TPCK also induces apoptosis in murine B cells possibly by inhibition of NF-κB/Rel activation and therefore c-Myc expression (Wu et al., 1996). However, in THP.1 cells, the concentration of TPCK required to decrease c-Myc expression was lower than that to induce apoptosis (data not shown), indicating that decrease of c-Myc may not be sufficient to induce cell death. Due to multiple functions of chloromethyl ketone groups, TPCK may affect apoptosis by different mechanisms in different cell types.

Low concentrations of TPCK (5-10 μM) inhibited the internucleosomal cleavage of DNA induced by different apoptotic stimuli (Fig. 4.6) in agreement with its effects in several different systems (Bruno et al., 1992; Chow et al., 1995; Fearnhead et al., 1995a; Hara et al 1996; Neamati et al., 1995). Several previous studies suggest that the degradation of chromatin to DNA fragments of < 50 kbp was catalyzed by an endogenous Ca²⁺/Mg²⁺-dependent endonucleolytic activity and proteolysis was a prerequisite for either the activation of this enzyme or for its accessibility to DNA (Hewish and Burgoyne, 1973; Walker et al., 1994b; Bruno et al., 1992). In a cell free system, serine protease inhibitors inhibited apoptotic cytosol-induced DNA fragmentation in isolated nuclei, suggesting that an endonuclease appears to be activated by a serine protease (Shimizu et al., 1996c). The observation that the induction of the internucleosomal cleavage of DNA was inhibited by TPCK suggests that the 24 kD protease with chymotrypsin-like activity...
Chapter 4 Effects of TPCK on Apoptosis

described by Wright et al (1994) may be also responsible for initiating internucleosomal DNA cleavage in THP.1 cells. Thus one of the TPCK targets in THP.1 cells may be involved in the control of an endonuclease responsible for internucleosomal cleavage of DNA. TPCK inhibits TNF α induced DNA fragmentation and the formation of apoptotic bodies in ML-1a cell line. TPCK also blocks TNF-induced activation of NF-κB. Study of the concentration-related effect and the time course of the inhibitor, however, indicated that the site of action of TPCK for NF-κB activation and for DNA fragmentation are quite distinct. It was concluded that TNF activates two distinct TPCK-sensitive pathways, one leading to apoptosis and the other to NF-κB activation (Higuchi et al., 1995).

TLCK potentiated apoptosis induced by some apoptotic stimuli, including CHX, but inhibited apoptosis induced by etoposide. A similar effect was observed with TPCK (5-10 μM) (Fig. 4.7), suggesting that they may react with a common cellular target.

The observation that TPCK has three distinct effects on apoptosis dependent on concentration and apoptotic stimuli used suggests that different intracellular components may be responsible for these effects. It is possible that the same protease may play a distinct role in different signalling pathways and a single protease inhibitor may also have more than one target, which plays opposing roles in regulating apoptosis at different stages. In the present study, TLCK and TPCK were used at relatively low concentrations and they showed distinct effects, arguing that they act through specific inhibition of a small subset of cellular proteases, but not through non-specific alterations in total cellular protease activity. It is critical to identify and to distinguish the intracellular targets responsible for the variable effects of these protease inhibitors on apoptosis.

4.5.2 Different patterns of DNA degradation in apoptosis

The execution of apoptosis involves a chain of events which leads to the full development of the apoptotic features. The progressive digestion of the cell genome is carried out by several endonucleases (Walker et al., 1995). They appear to act in a hierarchical order, producing at first well defined classes of large DNA fragments followed by internucleosomal DNA cleavage. Apoptosis induced by CHX/TLCK showed classical internucleosomal cleavage of DNA (Fig. 3.11) preceded by large kbp fragmentation (Fig. 3.13), representing a multiple step process of DNA degradation. Recent studies suggest that the internucleosomal cleavage of DNA is not required for the
induction of apoptosis. TPCK induced almost all the characteristic morphological and biochemical changes of apoptosis except for the internucleosomal cleavage and the full condensation of the chromatin. The large kbp DNA fragments (50-700 kbp) represented the only form of DNA degradation induced by TPCK (Fig. 4.2), in accordance with previous studies in thymocytes treated with either TPCK or dexamethasone in the presence of zinc. In these studies, an early apoptotic morphology was observed in the absence of internucleosomal cleavage of DNA but was associated with similar sized large kbp fragments of DNA (Brown et al., 1993; Cohen et al., 1992a; Fearnhead et al., 1995a). TPCK and another serine protease inhibitor, dichloroisocoumarin (DCI), prevented late stages of DNA degradation but not cell death induced by dexamethasone in thymocytes (Walker et al., 1994). It is possible that DNA cleavage at specific long-range sites is sufficient to cause collapse of chromatin structure. It appears that the induction of apoptosis by different means showed different patterns of DNA degradation.

4.6 Summary

1. TPCK may interact with different intracellular proteases to induce apoptosis, to modulate the induction of apoptosis by other stimuli, or to inhibit internucleosomal cleavage of DNA.

2. TPCK induced an intermediate phase of apoptotic morphological changes, providing a good model for study of the morphological features in the absence of internucleosomal cleavage of DNA.

3. This study provides a framework for further differentiation of the intracellular targets of TPCK involved in the regulation of apoptosis.

(Part of the work presented in this chapter has been accepted for publication (Zhu et al., 1997).
CHAPTER 5

EFFECTS OF CASPASE INHIBITORS ON APOPTOSIS
5.1 Introduction

Following the identification of multiple caspases, specific peptide inhibitors have been developed to explore the physiological roles of these proteases. These inhibitors include derivatives of chloromethyl ketone (CMK), fluoromethyl ketone (FMK) and aldehyde (CHO). Study of the caspase-1 substrate specificity showed that the peptide must contain at least four amino acids to the left of the cleavage site. Regarding substitutions in these positions, hydrophobic residues are favoured in the P4 position, Val is preferred in P3, liberal substitutions are tolerated in P2, and Asp is absolutely required in P1 (Thornberry 1994). Not surprisingly, the optimal sequence to the left of the cleavage site, Ac-Tyr-Val-Ala-Asp, closely matches the corresponding sequence of Tyr^{113}-Val^{114}-Cys^{115}-Asp^{116} in the natural human substrate IL-1β. Caspase-3 has a stringent requirement for Asp in P1 and P4 (Casciola-Rosen et al., 1996).

As an alkylating agent, chloromethyl ketone (CMK) and fluoromethyl ketone (FMK) can irreversibly inhibit cysteine protease by alkylating the active centre -SH group (Shaw 1990). The peptide-(OMe)-fluoromethyl ketones were designed to enter live cells as the O-methyl ester (of the aspartyl carboxy side-chain) and to be converted to the active inhibitor by intracellular esterases. They are relatively poor non-specific alkylating agents and have low reactivity toward serine proteases, but they retain a high degree of reactivity towards cysteine proteases (Shaw, 1990). It is thought that the fluoromethyl ketones, despite a more gradual onset of action, are more potent inhibitors of cysteine proteases than the chloromethyl ketones (Shaw, 1990). Acetyl-Tyr-Val-Ala-Asp-CMK (Ac-YVAD.CMK) is more selective for caspase-1 (Thornberry et al., 1992), whereas benzyloxy carbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-FMK (Z-DEVD.FMK) inhibits caspase-3 more selectively (Cain et al., 1996). Benzyloxy carbonyl-Val-Ala-Asp(OMe)-FMK (Z-VAD.FMK), which lacks an amino acid in the P4 position, is a relatively nonselective inhibitor. It blocks caspase-1 more effectively than caspase-3 (Armstrong et al., 1996), although the inhibition profile of Z-VAD.FMK has not yet been fully determined. Peptidyl aldehydes are competitive and reversible inhibitors of serine and cysteine proteases (Shaw, 1990). Ac-YVAD.CHO and Ac-DEVD.CHO are two well characterised inhibitors of caspases (Margolin et al., 1997). Considering their relative poor cellular permeability, they were not used in this present study. The combined profile
of inhibition obtained with these reagents is a useful approach with which to define the various caspase activities during different phases of apoptosis.

5.2 Z-VAD.FMK Inhibits apoptosis

5.2.1 Flow cytometric assessment

To determine whether caspase-1 or a related caspase plays an essential role in apoptosis, the induction of apoptosis by different stimuli in the presence of Z-VAD.FMK was studied. Analysis of apoptosis by HO342/PI flow cytometry showed that Z-VAD.FMK at concentrations of 5-50 μM remarkably inhibited apoptosis regardless of the stimulus (Table 5.1).

<table>
<thead>
<tr>
<th>Stimuli</th>
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<tr>
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</tr>
<tr>
<td>Control</td>
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<tr>
<td>TLCK</td>
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<td>CHX</td>
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<td>CHX/TLCK</td>
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<tr>
<td>ETOP</td>
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</tr>
<tr>
<td>ETOP/TLCK</td>
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</tr>
<tr>
<td>STS</td>
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</tr>
<tr>
<td>STS/TLCK</td>
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</tr>
<tr>
<td>THG</td>
<td>9.6±1.3</td>
</tr>
<tr>
<td>THG/TLCK</td>
<td>59.3±8.2</td>
</tr>
<tr>
<td>TPCK</td>
<td>25.0±2.5</td>
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</table>

THP.1 cells were incubated for 1 h either alone or in the presence of Z-VAD.FMK (50 μM). They were further incubated for 4 h in the presence or absence of TLCK (100 μM) either alone or with CHX (25 μM), etoposide (ETOP) (25 μM), STS (0.5 μM) or THG (100 nM).

a The percentage of apoptotic cells was assessed by flow cytometry. Results are expressed as mean ± s.e.m. of at least 3 separate experiments.

b As TLCK and Z-VAD.FMK alone inhibited etoposide-induced apoptosis, the combination was studied in one experiment only.

c With STS alone, necrosis was also induced.

To further investigate the relative position of the Z-VAD.FMK target within the apoptotic pathway, the addition of Z-VAD.FMK was delayed up to 2 h after the induction...
of apoptosis by CHX/TLCK. The results revealed that coincubation of cells with Z-VAD.FMK and CHX/TLCK for 4 h completely prevented apoptosis. However, when Z-VAD.FMK was added 1 h later, it only offered about 60% of inhibition of apoptosis (Fig. 5.1). If the addition of Z-VAD.FMK was further delayed up to 2 h, a time point, when maximum degradation DNA to large kbp fragments was observed (Fig. 3.11), the inhibition was only 10% (Fig. 5.1). These results suggest that the Z-VAD.FMK target is involved at an early stage in the apoptotic pathway and is activated shortly after the treatment with CHX and TLCK.

![Graph](image_url)

**Fig. 5.1. Effect of delayed addition of Z-VAD.FMK on the induction of apoptosis.** Cells were treated with CHX/TLCK. At 0 h, 1 h and 2 h after treatment Z-VAD.FMK (50 μM) was added. Apoptosis was detected by HO342/PI flow cytometry at 4 h.

To study whether the protection of apoptosis by Z-VAD.FMK is a long term effect, cell culture was prolonged in the presence of apoptotic stimuli and Z-VAD.FMK and cell death was detected at 24 h using the HO342/PI flow cytometric method. Treatment of
cells for 4 h with either etoposide or CHX/TLCK induced an increased number of apoptotic cells compared with control, while the number of necrotic cells (PI including cells) was only slightly increased (Fig. 5.2, top panel, compare b and d with a). Z-VAD.FMK abolished the induction of cell death induced by etoposide and by CHX/TLCK. After 24 h, Z-VAD.FMK still protected a significant number of cells from death induced by etoposide or CHX/TLCK (Fig. 5.2, lower panel, compare c with b and e with d).

5.2.2 Z-VAD.FMK inhibits biochemical changes of apoptosis

Z-VAD.FMK was also studied for its effects on the endonucleolytic and proteolytic events during the induction of apoptosis. As with CHX, STS (0.5 μM), THG (100 nM) and etoposide (25 μM) all caused the induction of 50 kbp DNA fragments when incubated with cells for 4 h (Fig. 5.3, lanes 4, 8, 12 and 16). TLCK (100 μM) enhanced the induction of the DNA degradation caused by THG and CHX (Fig. 5.3, lanes 10 and 14) but inhibited that caused by etoposide (Fig. 5.3, lane 18). The production of 50 kbp DNA fragments induced by STS was not affected by TLCK (Fig. 5.3, lane 6). The 50 kbp DNA fragments induced by these treatments were completely abrogated in the presence of Z-VAD.FMK (50 μM) (Fig. 5.3, lanes 7, 9, 11, 13, 15 and 17), except in the case of STS alone (Fig. 5.3, lane 5), where the decrease of 50 kbp fragments was accompanied by the appearance of some large DNA fragments above 50 kbp in size.

Z-VAD.FMK was also tested for its ability to inhibit the cleavage of PARP, a hallmark of apoptosis. Control cells and TLCK-treated cells contain intact PARP (Fig. 5.4, lanes 1, 2 and 8). Treatment of cells for 4 h with CHX or etoposide caused a loss of intact PARP (Fig. 5.4, lanes 3 and 6). TLCK enhanced the loss of intact PARP induced by CHX (Fig. 5.4, lane 4), but inhibited that induced by etoposide (Fig. 5.4, lane 7) which is consistent with its effects on apoptosis induced by these two stimuli (Table 5.1). The loss of intact PARP induced by CHX/TLCK and by etoposide was accompanied by the appearance of the 85 kD cleavage product (Fig. 5.4, lanes 4 and 6) which was not detected in the cells treated with CHX (Fig. 5.4, lane 3), possibly due to the low level of apoptosis (Table 5.1).
Fig 5.2. Z-VAD.FMK has a long term effect against cell death. Cells were incubated alone (a); with etoposide (25 μM) (b); with etoposide in the presence of Z-VAD.FMK (50 μM) (c); with CHX (25 μM)/TLCK (100 μM) (d); with CHX/TLCK in the presence of Z-VAD.FMK (e), or with Z-VAD.FMK alone (f). Cell death was assessed by HO342/PI flow cytometry at 4 h and 24 h. Cells with increased HO342 staining were considered as apoptotic, with increased PI staining as necrotic. The differences between the treatments with etoposide in the absence and presence of Z-VAD.FMK were significant at 24 h (p < 0.05, by Student’s t test). Similarly, the differences between the treatments with CHX/TLCK in the absence and presence of Z-VAD.FMK were significant at 24 h (p < 0.01, by Student’s t test).
Fig. 5. Z-VAD.FMK inhibits the cleavage of DNA into large kbp. As controls (CON), cells were either untreated (lane 1) or incubated with TLCK (100 μM) (lane 2) or Z-VAD.FMK (50 μM) (lane 3). Cells were incubated for 4 h with STS (0.5 μM) (lanes 4-7), THG (100 nM) (lanes 8-11), CHX (25 μM) (lanes 12-15), and etoposide (25 μM) (lanes 16-18) in the absence (lanes 4, 8, 12, 16) or presence (lanes 6, 10, 14, 18) of TLCK (100 μM). Cells were also treated with these stimuli in the presence of Z-VAD.FMK (50 μM) (lanes 5, 7, 9, 11, 13, 15, 17). The arrows in the figure indicate the size of the DNA fragments.
Chapter 5 Effects of Caspase Inhibitors on Apoptosis

The induction of PARP degradation by CHX/TLCK and by etoposide was completely prevented by Z-VAD.FMK at the same concentration for the inhibition of apoptosis (Fig. 5.4, lanes 5 and 8) (Table 5.1). These results further demonstrated the ability of Z-VAD.FMK to inhibit apoptosis.

![Graph showing PARP cleavage](image)

Fig. 5.4. Z-VAD.FMK inhibits PARP cleavage. As controls, cells were incubated either alone (lane 1) or with TLCK (100 μM) (lane 2). Cells were treated for 4 h with CHX (25 μM) (lane 3) or etoposide (25 μM) (lane 6) either alone or in the presence of TLCK (lanes 4, 7). Cells were also coincubated with CHX/TLCK or with etoposide in the presence of Z-VAD.FMK (50 μM) (lanes 5 and 8). Positions of intact PARP was indicated alone with its cleavage product (arrows).

Previous study showed that TPCK caused a concentration dependent induction of apoptosis. A time course of TPCK-induced biochemical changes of apoptosis was also studied here. It was interesting to note that procaspase-2 was decreased as early as 1 h after treatment with TPCK (75 μM) (Fig. 5.5 upper panel, lane 2) and maintained at almost the same level over the next 3 h (Fig. 5.5, lanes 3, 4 and 5). Only a very small amount of the p12 subunit was observed after 4 h treatment (Fig. 5.5, lane 5). The processing of caspase-3 and the degradation of PARP were detected at 3 h and were increased at 4 h (Fig. 5.5, lanes 4 and 5). Lamin B1 degradation was only detected at 4 h (Fig. 5.5, lane 5). Z-VAD.FMK effectively inhibited all caspase processing and cleavage.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CHX</th>
<th>ETOP</th>
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<tbody>
<tr>
<td>TLCK</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Z-VAD.FMK</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

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89
intracellular caspase substrates as well as large DNA fragments (Fig. 5.5 lane 6) (Fig. 4.2 lane 6). Presumably a Z-VAD.FMK inhibitable target is involved at an early stage in the induction of apoptosis, suggested by that Z-VAD.FMK inhibits all apoptotic changes, as

![Fig. 5.5 Time course of the biochemical changes induced by TPCK and the inhibition of these changes by Z-VAD.FMK. Cells were untreated (lane 1) or treated with TPCK (75 μM) for 1, 2, 3, and 4 h (lanes 2-5). Cells were also treated for 4 h with the same concentration of TPCK in the presence of Z-VAD.FMK (20 μM) (lane 6). Processing of caspase-2 and caspase-3 and the degradation of lamin B1 and PARP were detected by western blotting. The lines in the figure indicate the procaspases and their processing products or intact proteins and their degradation fragments.](image-url)
assessed by flow cytometry and by biochemical study. Z-VAD.FMK appeared to prevent the appearance of the p12 subunit more effectively than the decrease of procaspase-2 (Fig. 5.5, lane 6), indicating that may be two proteases were involved in the processing of caspase-2: one is responsible for the initial processing of caspase-2, which is less sensitive to Z-VAD.FMK; another is involved in the production of p12, which is Z-VAD.FMK inhibitable. The significance of caspase-2 activation in apoptosis in these cells is unknown.

5.2.3 Z-VAD.FMK inhibits apoptosis assessed ultrastructurally

Z-VAD.FMK inhibited all the biochemical changes tested associated with the induction of apoptosis. To further demonstrate the critical role of caspases in apoptosis, Z-VAD.FMK was studied for its ability to inhibit ultrastructural features of apoptosis induced with a number of treatments. As described in chapter 3, THG/TLCK induced a large number of apoptotic cells (Fig. 3.3 C, lower panel), these cells were examined ultrastructurally. Cells treated with THG/TLCK showed ultrastructural changes typical of apoptosis (Fig. 5.6 B). However, in the presence of Z-VAD.FMK cells exhibited normal morphology (Fig. 5.6 compare A and C), their membrane and organelle structure were well preserved. More remarkably, nuclei of Z-VAD.FMK treated cells resembled those of control cells, with reticular chromatin bounded by intact nuclear lamina and membrane (Fig. 5.6 compare A with C). Z-VAD.FMK also inhibited all the ultrastructural changes of apoptosis induced by CHX/TLCK, etoposide and TPCK (data not shown). These data provided evidence that caspases are required for the induction of apoptotic morphological changes in THP.1 cells.

5.3 Z-VAD.FMK failed to prevent cell death induced by STS

There is evidence that caspase activity is not involved in necrotic cell death (Hogquist et al., 1991), however it has been noticed that in THP.1 cells, STS appeared to induce features of necrotic cell death. It was therefore interesting to study the effects of caspase inhibitors on these forms of cell death. Cell death induced by STS (0.5 μM) or STS/TLCK in the presence and absence of Z-VAD.FMK was measured by HO342/PI flow cytometry.

As shown in Table 5.2, STS induced a large number of necrotic cells and a small number of apoptotic cells. Z-VAD.FMK only partially inhibited STS-induced necrosis, however it completely inhibited apoptosis induced by STS/TLCK. Based on the sensitivity of the cell
Fig. 5.6 Z-VAD.FMK prevents ultrastructural features of apoptosis. A, control cells; B, cells were treated for 4 h with THG (100 nM)/TLCK (100 μM); C, cells were treated with THG/TLCK in the presence of Z-VAD.FMK (50 μM). n, nucleolus; g, Golgi apparatus; m, mitochondrial. Magnification: x 3000.
Chapter 5 Effects of Caspase Inhibitors on Apoptosis

Fig. 5.6 B THG/TLCK

Fig. 5.6 C THG/TLCK/Z-VAD.FMK
death to the inhibition by Z-VAD.FMK, it is likely that some of the necrotic cells induced by STS may develop from terminal apoptosis where caspase activity was involved, and the remaining necrotic cells that did not contain caspase activity were therefore resistant to the inhibition by Z-VAD.FMK.

Table 5.2 Z-VAD.FMK protects cells from apoptosis but not necrosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Apoptosis</th>
<th>% Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.2 ± 1.1</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>STS</td>
<td>9.3 ± 6.8</td>
<td>62 ± 9.4</td>
</tr>
<tr>
<td>STS/Z-VAD.FMK</td>
<td>4.1 ± 0.8</td>
<td>48 ± 11.4</td>
</tr>
<tr>
<td>STS/TLCK</td>
<td>53.8 ± 9.8</td>
<td>4.9 ± 1.3</td>
</tr>
<tr>
<td>STS/TLCK/Z-VAD.FMK</td>
<td>3.0 ± 1.6</td>
<td>11.0 ± 1.7</td>
</tr>
</tbody>
</table>

Cells were incubated for 4 h with STS (0.5 μM) alone or in the presence of Z-VAD.FMK (50 μM). Cells were also cotreated for 4 h with STS (0.5 μM) and TLCK (100 μM) in the absence or presence of Z-VAD.FMK (50 μM). Apoptosis was measured using the HO342/PI flow cytometric method. Results are expressed as mean ± s.e.m.

5.4 Z-DEVD.FMK inhibits apoptosis

5.4.1 Inhibition of PARP degradation

To determine whether caspase-3 or a related protease is the key effector of apoptosis, a potent caspase-3 inhibitor, Z-DEVD.FMK was also studied for its ability to inhibit apoptosis. As expected, Z-DEVD.FMK inhibited the activity of caspase-3, indicated by the observation that the cleavage of PARP induced by CHX/TLCK was abolished in the presence of Z-DEVD-FMK (5-25 μM) (Fig. 5.7).
Chapter 5 Effects of Caspase Inhibitors on Apoptosis

<table>
<thead>
<tr>
<th>Con</th>
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<td></td>
</tr>
<tr>
<td>5</td>
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<tr>
<td>10</td>
<td></td>
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<tr>
<td>25</td>
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</table>

PARP – Frag.

Fig. 5. 7. Z-DEVD.FMK inhibits PARP cleavage. Cells were untreated (Con) or treated for 4 h with CHX (25 μM)/TLCK (100 μM) in the absence or presence of the indicated concentrations of Z-DEVD.FMK. The lines in the figure display the intact PARP and its apoptotic fragment.

5.4.2 Inhibition of DNA degradation

To investigate whether caspase-3 or a related family member is required for the induction of DNA degradation during apoptosis, cells treated with CHX/TLCK were assessed for the formation of the large kbp fragments (Fig. 5.8, lanes 1-6) and oligonucleosomal DNA fragments (Fig. 5.8, lanes 7-12) in the presence of Z-DEVD.FMK. A low concentration of Z-DEVD.FMK (5 μM) appeared to increase the formation of large kbp DNA fragments, but had no detectable effect on internucleosomal cleavage of DNA induced by CHX/TLCK (Fig. 5.8 compare lane 2 with lane 3, lane 8 with lane 9). A higher concentration of Z-DEVD.FMK (10 μM) caused an apparent reduction of the internucleosomal-sized but not the large kbp DNA cleavage (Fig. 5.8, lanes 4 and 10). The highest concentration of Z-DEVD.FMK (25 μM) completely inhibited the formation of both large kbp and oligonucleosomal DNA fragments (Fig. 5.8, lanes 5 and 11). Z-VAD.FMK (5 μM) had similar effects to the highest concentration of Z-DEVD.FMK. It appears that two caspases are involved in DNA degradation: one is involved in internucleosomal cleavage of DNA, which is sensitive to Z-DEVD.FMK, another is involved in large kbp DNA cleavage, which is less sensitive to the inhibitor.
Fig. 5.8 Z-DEVD.FMK inhibits the induction of large kbp and internucleosomal cleavage of DNA. Cells were untreated (lanes 1 and 7) or treated for 4 h with CHX/TLCK in the absence (lanes 2 and 8) or presence of either indicated concentrations of Z-DEVD.FMK (lanes 3-5 and 9-11) or Z-VAD.FMK (5 μM) (lanes 6 and 12).
5.5 **Z-VAD.FMK and Z-DEVD.FMK inhibit caspase-3 processing in a different manner**

Although Z-VAD.FMK and Z-DEVD.FMK both prevented the induction of apoptosis, they may inhibit different caspases, as suggested by their different structures. In an attempt to dissociate the intracellular targets of Z-VAD.FMK and Z-DEVD.FMK, their effects on the processing of caspase-3 were studied. Treatment of THP.1 cells with CHX/TLCK caused activation of caspase-3, as indicated by the formation of p17 subunit (Fig. 5.9 upper panel), and PARP degradation (Fig. 5.7). It has been suggested that caspase-3 is first cleaved into two fragments, N-terminal p20 and C-terminal p12, and the p20 is further cleaved into a p19 or a p17 fragments (Fernandes-Alnemri et al., 1996; Nicholson et al., 1995). The additional processing of the p20 is most likely due to the autocatalytic activity of caspase-3 (Fernandes-Alnemri et al., 1996). In the presence of Z-VAD.FMK (10 μM), the p19 and p17 fragments induced by CHX/TLCK (Fig. 5.9 upper panel, lane 2) were completely prevented with a slight accumulation in the p20 band (Fig. 5.9 upper panel, lane 3). At low concentrations (1 - 5 μM), Z-DEVD.FMK caused an accumulation of the p19 band (Fig. 5.9 upper panel, lanes 4 and 5), whereas at higher concentrations (10-25 μM), it decreased the p19 band and increased the p20 band (Fig. 5.9 upper panel, lanes 6 and 7). Thus it seemed that Z-VAD.FMK prevented the initial processing of procaspase-3 to p20 fragment, while Z-DEVD.FMK suppressed the further processing of the p20. Both Z-VAD.FMK and Z-DEVD.FMK also inhibited caspase-7 processing (Fig. 5.9 Lower panel) with the former more effective than the latter. These results imply that Z-VAD.FMK and Z-DEVD.FMK inhibit apoptosis by suppressing different subset of caspases. The target of Z-VAD.FMK appears to be upstream of that for Z-DEVD.FMK. The inhibition of apoptosis by a potent caspase-3 inhibitor strongly suggest that caspase-3 or a related family member(s) plays a pivotal role in the induction of apoptosis.

5.6 **YVAD.CMK is a potent inhibitor of lamin degradation**

During the induction of apoptosis lamin degradation occurs (Ucker et al., 1992; Kaufmann, 1989; Neamati et al., 1995) which may be mediated by a caspase sensitive to YVAD.CMK (Lazebnik et al., 1995), possibly caspase-6 (Takahashi et al., 1996b). In order to understand the importance of lamin degradation in apoptosis and the responsible
proteases in THP.1 cells the effects of YVAD.CMK was studied. Treatment of cells with YVAD.CMK (100 µM) failed to inhibit apoptosis induced by CHX/TLCK and by THG/TLCK, as assessed by HO342/PI (Fig. 5.10, upper panel).

YVAD.CMK (100 µM) showed less inhibition on internucleosomal DNA cleavage induced by THG/TLCK compared with the inhibition by Z-VAD.FMK (50 µM) and TPCK (10 µM) (Fig. 5.10 upper panel, compare lane 7 with lanes 6 and 8). YVAD.CMK showed no detectable inhibition on CHX/TLCK induced-internucleosomal cleavage of DNA, which was significantly inhibited by Z-VAD.FMK and TPCK (Fig. 5.10, upper panel, compare lane 11 with lanes 10 and 12). However it effectively prevented the induction of lamin A/C cleavage by CHX/TLCK at concentrations of 25-100 µM (Fig. 5.10 lower panel). Although YVAD.CMK (25 -100 µM) was incapable of inhibiting TPCK induced- apoptosis, as measured by flow cytometry (data not shown), it inhibited lamin A/C cleavage as effectively as Z-VAD.FMK (Fig. 5.11. upper panel, compare

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**Fig. 5.9** Upper panel, both Z-VAD.FMK and Z-DEVD.FMK prevent caspase-3 processing but show different patterns. Cells were untreated (lane 1) or treated for 4 h with CHX together with TLCK (lane 2). Cells were also treated with these agents in the presence of Z-VAD.FMK (10 µM) (lane 3) or Z-DEVD.FMK at concentrations of 1, 2.5, 5, 10 µM (lanes 4-7). The lines indicate the size of the protein bands. Lower, Z-VAD.FMK and Z-DEVD.FMK prevent caspase-7 processing.

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98
Chapter 5 Effects of Caspase Inhibitors on Apoptosis

<table>
<thead>
<tr>
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<th>CHX+TLCK</th>
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<tbody>
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<td>51</td>
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<td>TPCK</td>
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<td>Z-VAD.FMK</td>
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<td>YVAD.CMK</td>
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Fig. 5.10 upper panel: YVAD.CMK has little or no effect on the internucleosomal cleavage of DNA. As controls, cells were incubated for 4 h alone (lane 1) or in the presence of TPCK (10 μM) (lane 2), Z-VAD.FMK (50 μM) (lane 3) or YVAD.CMK (100 μM) (lane 4). Cells were treated for 4 h with the indicated agents in the absence or presence of different protease inhibitors. The concentrations for these agents are: THG 100 nM, CHX 25 μM. Percentage of apoptosis was displayed in the figure. Lower panel: YVAD.CMK inhibits lamin A/C degradation. Cells were untreated (CON) or treated with cycloheximide (25 μM) together with TLCK (100 μM) and the indicated concentrations of YVAD.CMK.
Fig. 5.11. YVAD.CMK effectively inhibits lamin A/C degradation and less effectively inhibits caspase-3 processing. Control cells (lane 1) or cells treated for 4 h with TPCK (75 μM) either alone (lane 2) or in the presence of Z-VAD.FMK (20 μM) (lane 3) or YVAD.CMK (25 and 100 μM) (lanes 4 and 5). Samples from these treatments were assessed for the lamin A/C degradation (upper panel) and caspase-3 processing (lower panel).

lane 3 with lanes 4 and 5). However, it can only inhibit caspase-3 processing to a similar extent as Z-VAD.FMK (20 μM) at a high concentration (100 μM) (Fig. 5.11 lower panel, compare lane 3 with lanes 4 and 5). These results support that in THP.1 cells the YVAD.CMK sensitive protease, possibly caspase-6 may be responsible for the lamin degradation, but it may not be required for caspase-3 processing and DNA degradation during apoptosis.

5.7 Discussion

5.7.1 Multiple caspases are active in apoptotic cells, while Z-VAD.FMK and Z-DEVD.FMK prevent apoptosis by inhibiting different caspases
In order to ascertain the involvement and the individual role of caspases in apoptosis in THP.1 cells, three distinct caspase inhibitors were studied for their ability to inhibit different aspects of apoptosis.

In THP.1 cells the induction of apoptosis by diverse stimuli involved multiple caspase activities (Fig. 5.5 and Fig. 5.9). Z-VAD.FMK completely prevented the cells from undergoing apoptotic morphological and biochemical changes regardless of the stimuli (Fig. 5.6 and 5.3), and it provided long term protection of cells against the loss of viability (Fig. 5.2). Z-VAD.FMK also inhibits apoptosis by blocking DNA fragmentation, proteolysis of PARP and processing of caspase-3 in different systems including Jurkat cells, thymocytes, hepatocytes (Cain et al., 1996; Chow et al., 1995; Fearnhead et al., 1995b; Slee et al., 1996). Moreover, Z-VAD.FMK inhibits both interdigital programmed cell death and the removal of the interdigital web during digit formation (Jacobson, 1996) and also blocks Reaper-induced cell death (Pronk et al., 1996). These studies imply that the Z-VAD.FMK inhibitable apoptotic pathway is conserved between animals and different cell types. Z-VAD.FMK inhibits recombinant caspase-1 and caspase-3 ~ 10,000-fold and 500-fold faster, respectively, than it dose the cysteine protease calpain I. It also has no observable inhibitory activity against the Asp-X-directed serine protease granzyme B (Armstrong et al., 1996). Thus Z-VAD.FMK appears to be a specific inhibitor of caspases. Z-VAD.FMK was about 25-50 times more potent than Ac-YVAD-CMK as an inhibitor of purified ICE in in vitro assays using YVAD-AFC or proIL-1β as substrates (Stefanis et al., 1996), and also inhibits other caspases effectively (Armstrong et al., 1996; Fraser and Evan 1996). These data strongly suggest that the superior efficacy of Z-VAD.FMK as a inhibitor of apoptosis is due to its superior potency as a general inhibitor of caspases.

The inhibition of apoptosis by Z-DEVD.FMK (Fig. 5.8), a caspase-3 specific inhibitor, indicated that caspase-3 plays a major role in the control of apoptosis in THP.1 cells. The mechanism which triggers the activation of the caspase-3 is presently unknown. Partially purified active caspase-3 from Hela cell extracts was able to cleave the caspase-3 precursor in vitro and this reaction was partially, but not completely, inhibited by a caspase-3 specific tetrapeptide inhibitor. This supports a role for autocatalytic activation as well as the existence of another activating enzymes (Wang et al., 1996b).
Although in THP.1 cells Z-VAD.FMK and Z-DEVD.FMK inhibited apoptosis, they showed a different pattern in inhibition of caspase-3 processing (Fig 5.9). Z-VAD.FMK prevented the initial processing of caspase-3 to p20, whereas Z-DEVD.FMK blocked the further processing of p20. This also indicated that at least two enzymes were involved in the processing of caspase-3, one is Z-VAD.FMK sensitive and another is Z-DEVD FMK sensitive. In vitro, Z-VAD.FMK inhibits caspase-3 processing more effectively than its ability to cleave PARP (Slee et al., 1996). Based on these observations it is likely that the intracellular target of Z-VAD.FMK is upstream of caspase-3. This study adds to the accumulating evidence that points to caspase-3 and/or its close relatives as crucial components of the death machinery in mammalian cells.

5.7.2 The YVAD.CMK target is responsible for lamin cleavage

Lamin A and C are alternatively spliced products of the same gene and differ only in their carboxy terminus, whereas the B-type lamins are distinct but structurally related proteins (Nigg, 1992). Lamin A contains two putative cleavage consensus sites for caspases, the Asp residues at position 230 and 446 in the context of EVDNG and EIDSG, respectively (Rao et al., 1996). The Asp 230 is conserved among lamins from human, mouse, chicken, Xenopus, and Drosophila. The appearance of ~ p46 band using COOH-terminal antibody indicated the presence of a single cleavage site consistent with that at Asp230. Lamin cleavage is essential for the completion of nuclear disassembly in apoptosis (Neamati et al., 1994; Lazebnik et al., 1995; Fernandes-Alnemri et al., 1995a).

To date, caspase-6 which is sensitive to YVAD.CMK, is the only one protease in the caspase family found to cleave lamins (Takahashi et al., 1996b). In THP.1 cells, YVAD.CMK inhibited lamin cleavage and yet had little effect on apoptosis (Fig. 5.10 and fig. 5.11), indicating that caspase-6 is responsible for lamin cleavage, but not required for the induction of apoptosis in these cells.

5.7.3 Ordering the apoptotic pathway.

Evidence is accumulating for the existence of a cascade of caspases that can activate each other, thereby amplifying the death signals (Orth et al., 1996; Liu et al., 1996a). Caspase-3-like protease appears to be an important central intermediary in the cell death pathway. This is supported by the fact that granzyme B and upstream FADD-like proteases caspase-8 and caspase-10 can active caspase-3 (Munday et al., 1995), which in
turn can cleave the well characterized cell death substrate PARP (Faucheu et al., 1995; Kamens et al., 1995) and induce the morphological changes associated with apoptosis in isolated nuclei (Trapani et al., 1994). Caspase-1, caspase-2, caspase-4, caspase-6, and caspase-7 are all able to cleave PARP (Fernandes-Alnemri et al., 1995a, 1995b; Lippke et al., 1996; Gu et al., 1995) but it appears that the affinity of these caspases for PARP is considerably lower than in the cases of caspase-3 and caspase-7. Caspase-3 and caspase-7 have been demonstrated to have similar substrate specificity (Fernandes-Alnemri et al., 1995). Recombinant caspase-3 and caspase-7 exhibited a $k_m$ of 13 and 51 μM respectively, for cleaving the PARP-like substrate DEVD-AMC (Fernandes-Alnemri et al., 1995b) ($k_m$: the concentration of substrate required to achieve half the maximum velocity of the enzyme). Thus both caspase-3 and 7 are probably responsible for PARP cleavage in cells undergoing apoptosis. Although these two enzymes may have important roles in the execution of apoptosis, they are unable to cleave lamins (Takahashi et al., 1996). Thus far the only known caspase that can cleave lamins is caspase-6 (Takahashi et al., 1996a).

In THP.1 cells caspase-2, caspase-3, caspase-6 and caspase-7 all have been observed to be activated during apoptosis, possibly activated in a cascade fashion. Thus the activation of an upstream protease appears to be a key step to the induction of apoptosis. A time course study of apoptosis induced by TPCK revealed that the loss of intact caspase-2 was an early event, occurring 1 h after TPCK treatment (Fig. 5.5). This is consistent with other studies, which showed that caspase-2 processing appeared at an early stage (Srinivasan et al., 1996, MacFarlane et al., 1997), whereas the processing of caspase-3 into p17 was observed at 3 h after treatment, and was accompanied by the degradation of PARP (Fig. 5.5). *In vitro*, caspase-2 can not autoprocess but can be processed by caspase-1 and caspase-3 (Harvey et al., 1996). However, during the induction of apoptosis, the processing of caspase-3 occurred later than the processing of caspase-2, making caspase-3 unlikely to be upstream of caspase-2. Caspase-2 can interact with the adaptor protein, CRADD (caspase and RIP adaptor with death domain) (Ahmad et al., 1997), which has an NH$_2$-terminal caspase homology domain and a COOH-terminal death domain. Based on these data, it is likely that the activation of caspase-2 is due to its interaction with CRADD or by another caspase-1-related protease. Caspase-2 can not process a number of caspases, indicating that caspase-2 is not directly upstream of caspase-3 (Harvey et al.,
The significance of the processing of caspase-2 and the mechanism of the caspase-3 activation are presently unknown.

The TPCK induced-lamin cleavage was a late event, consistent with observations in other systems (Greidinger et al., 1996; Lazebnik et al., 1995). In vitro caspase-6 can be activated by caspase-3 (Takahashi et al., 1996b). In cells undergoing apoptosis or in nuclei incubated with apoptotic extracts, lamin cleavage occurs after PARP cleavage and is much slower than PARP cleavage (Enari et al., 1996, 31, Kaegi et al., 1994). These studies suggest that activation of caspase-3 may lead to the activation of caspase-6. It appears that in THP.1 cells, the induction of apoptosis by TPCK involved sequential activation of a number of caspases, with the order being caspase-2, caspase-3 and caspase-6. Whether these caspase act in a cascade fashion needs further investigation.

5.7.4 Z-VAD.FMK-inhibitable caspase may not be involved in necrotic cell death

Z-VAD.FMK failed to inhibit STS-induced cell death (Table 5.2). Since STS appears to induce two forms of cell death, necrosis and apoptosis, the inability of Z-VAD.FMK to inhibit STS-induced cell death may be due to the lack of role of caspases in necrosis. This observation was consistent with the report that Z-VAD.FMK did not inhibit necrotic death of cerebellar neurones induced by glutamate (Armstrong et al., 1997). Since caspase-3 was not active during the induction of this form of necrosis, it was thought that caspase-3 activation is a useful marker for distinguishing apoptosis and necrosis. Thus, in addition to the kinetic and morphological differences, necrosis and apoptosis may be distinguished by their sensitivity to the inhibition by a selective inhibitor of caspases. Z-VAD.FMK, at concentrations which inhibited apoptosis, had no effect on cell growth, and it failed to prevent TLCK-induced cell growth inhibition (data not shown). A similar observation was made in SKW 6.4 cells, where Z-VAD.FMK had no apparent effect on the growth rate for 48 h (Jacobson et al., 1996). These data support a specific role of caspases in apoptosis.

5.8 Summary

1. In THP.1 cells, multiple caspases, including caspase-2, caspase-3, caspase-7, and possibly caspase-6, are active in CHX/TLCK- and TPCK-induced apoptosis.
2. Cell death can be prevented by a relatively non-specific caspase inhibitor Z-VAD.FMK and a caspase-3 inhibitor Z-DEVD.FMK, suggesting that caspases play a key role in the activation of apoptosis and caspase-3 may be a key effector.

3. This study demonstrated the usefulness of cell permeable inhibitors of the caspase family for studying apoptosis in intact cells. More specific inhibitors are needed to characterize the individual role of distinct members of the caspase family in apoptosis.
CHAPTER 6

ALTERATION OF PROTEASE ACTIVITIES
IN THE INDUCTION OF APOPTOSIS
6.1 Introduction

The fact that protease inhibitors modify the induction of apoptosis suggests that proteases are involved in the regulation of apoptosis. Thus to identify the intracellular targets responsible for the effects of protease inhibitors will prove to be a key step towards the discovery of the mechanisms by which proteases regulate cell death. TPCK and TLCK are two peptide chloromethyl ketone derivatives widely used as active-site-directed-inhibitors for chymotrypsin and trypsin proteases, respectively. The inhibition by peptide chloromethyl ketones results from the inhibitor binding the protease in a substrate-like manner followed by alkylation of the active-site histidine by the chloromethyl moiety (Shaw 1970). These two compounds function in THP.1 cells to either induce apoptosis or to modify the induction of apoptosis by other apoptotic stimuli, suggesting that they may have distinct targets. Since a number of caspases is activated during the induction of apoptosis, it is of importance to investigate which particular caspase is crucial to apoptosis.

Peptide-7-amino-4-methylcoumarin (AMC) substrates have been developed for fluorimetric assay of specific enzymes (Zimmerman et al., 1976). This assay basically makes use of peptide substrates which have a fluorogenic coumarin derivative linked to the C-terminal end via a peptide bond. These substrates are cleaved by the enzyme to release the fluorescent leaving group. Rates of hydrolysis of AMC substrates are measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. At these wavelengths, the free 7-amino-4-methylcoumarin group is approximately 500-700 times more fluorescent than the conjugated forms (Zimmerman et al., 1976). In this study the Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC were used as trypsin-like and chymotrypsin-like substrates (Kunugi et al., 1985; Sawada et al., 1984), respectively, to detect the serine protease activities in cell lysates, whilst Ac-YVAD.AMC was used to detect caspase-1 like protease activities. Z-DEVD-7-amino-4-trifluoromethylcoumarin (AFC) was used to detect caspase-3 like activity (Stefanis et al., 1996). The rate of hydrolysis of the AFC substrate was measured at an excitation wavelength of 400 and an emission wavelength of 505 nm.
6.2 Boc-Val-Leu-Lys- but not Suc-Ala-Ala-Pro-Phe- cleavage activity is inhibited by TPCK

In order to investigate the nature of the putative protease(s) which TPCK and TLCK interfere with to induce or to modulate apoptosis in THP.1 cells, cells were exposed to TPCK and TLCK at 37°C for 1 h. Lysates from these cells were assayed for their activity to cleave Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC.

Table 6.1. Effect of protease inhibitors on trypsin-like and chymotrypsin-like protease activities

<table>
<thead>
<tr>
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<th>Boc-Val-Leu-Lys-AMC</th>
<th>Suc-Ala-Ala-Pro-Phe-AMC</th>
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<tr>
<td>Control</td>
<td>167 ± 30</td>
<td>1416 ± 487</td>
</tr>
<tr>
<td>TPCK 10 µM</td>
<td>149 ± 15</td>
<td>989 ± 45</td>
</tr>
<tr>
<td>TPCK 25 µM</td>
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<td>844 ± 64</td>
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</tr>
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</tr>
<tr>
<td>TLCK 100 µM</td>
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<td>1089 ± 460</td>
</tr>
<tr>
<td>PMSF 1 mM</td>
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<td>339 ± 94</td>
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THP.1 cells were treated with TLCK or TPCK for 1 h. Lysates from control cells and those treated with these protease inhibitors were measured for Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC cleavage activities. The results are presented as pmol. AMC/mg. protein/min and are the mean value ± s.e.m of at least three experiments.

a For the Boc-Val-Leu-Lys-AMC cleavage activity, the differences between control and TPCK at concentrations of 50-75 µM were significant (P< 0.05), whilst the differences between control and other treatments were not significant.

b For the Suc-Ala-Ala-Pro-Phe-AMC cleavage activity, the difference between control and the treatment with PMSF was significant (P< 0.05), while the differences between control and other treatments were not significant.

Lysates from cells incubated with low concentrations of TPCK (10-25 µM) showed no apparent difference in the Boc-Val-Leu-Lys-AMC cleavage activity (Table 6.1) compared
with the lysates from untreated cells. Apoptosis-inducing concentrations of TPCK (50-75 μM) caused a significant decrease in this activity (Table 6.1). TLCK at a concentration of 100 μM, which modulated the induction of apoptosis (Table 5.1), caused no significant inhibition of this cleavage activity (Table 6.1). Both TLCK and TPCK at concentrations known to have effects on apoptosis showed little or no effect on the Suc-Ala-Ala-Pro-Phe- cleavage activity (Table 6.1). To further study the nature of the proteases capable of cleaving Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC, PMSF (phenylmethylsulfonyl fluoride), a common serine protease inhibitor, was tested for its ability to inhibit these protease activities. Incubation of cells with PMSF (1 mM) for 1h caused an inhibition of the Suc-Ala-Ala-Pro-Phe-AMC but not the Boc-Val-Leu-Lys-AMC cleavage activity, indicating that the intracellular protease capable of cleaving Suc-Ala-Ala-Pro-Phe-AMC may not be a serine protease.

6.3 DEVD- but not YVAD- cleavage activity is associated with the induction of apoptosis

*In vitro* studies have demonstrated that several caspases, including caspase-3, caspase-2, caspase-6 and caspase-7, all are able to cleave PARP specifically into similar fragments (Harvey et al., 1996; Duan et al., 1996a; Lazebnik et al., 1995). Also caspase-3 has the ability to cleave DNA-PKcs, SREBP1 and SREBP2 (Han et al., 1996; Wang et al., 1995), indicating the importance of caspase-3-like or PARP cleavage activity in the execution of apoptosis. Having established a role for caspase-3 related proteases in apoptosis in THP.1 cells, the process of the protease activation was analyzed. The protease activity was measured in lysates using the fluorogenic substrate Z-DEVD-AFC, which mimics the PARP cleavage site of caspase-3. Lysates from control cells exhibited a small amount of Z-DEVD- AFC cleavage activity, whilst this activity was increased in lysates exposed to CHX alone (Fig. 6. 1), and was markedly potentiated in the presence of TLCK (Fig. 6.1 CHX/TLCK). Etoposide also induced the Z-DEVD-AFC cleavage activity at a apoptosis-inducing concentration (Fig. 6.1).

Similarly, TPCK but not TLCK alone caused an increase in the DEVD-AFC cleavage activity (Fig. 6. 1). Study of the concentration-related effect of TPCK on this DEVD-AFC cleavage activity showed that a low concentration (25 μM), which did not induce apoptosis, had a little effect (Fig. 6. 2). At apoptosis-inducing concentrations (50-75 μM),
TPCK induced an increase in the DEVD-AFC cleavage activity (Fig. 6.2). A time course study revealed that TPCK (75 μM) induced a significant increase in the DEVD-AFC cleavage activity 3 h after treatment. A further increase of this activity was detected at 4 h (Fig. 6.3), whereas this DEVD-AFC cleavage activity in control cells was not changed over the period of the 4 h incubation. The time course of the increase in the DEVD-AFC cleavage activity caused by TPCK was similar to that of the processing of caspase-3 and the degradation of PARP (Fig. 5.5), suggesting that the increase of DEVD-AFC cleavage activity may result from the processing of caspase-3. The present study demonstrated that the induction of apoptosis induced by all stimuli tested is associated with an elevation in the DEVD-AFC cleavage activity, indicating that the activation of caspase-3-like protease is a common event during the induction of apoptosis. In contrast, the cleavage YVAD-AMC, which mimics the cleavage site of caspase-1 on proIL-1β, was not detected when

Fig. 6.1 The induction of apoptosis is accompanied by an increase in Z-DEVD-AFC cleavage activity. THP.1 cells were incubated alone (Con) or treated for 4 h with different stimuli, including etoposide (25 μM), CHX (25 μM) in the absence or presence of TLCK (100 μM), TPCK (75 μM) in the absence or presence of Z-VAD.FMK (50 μM), and TLCK (100 μM) alone. Lysates from these cells were assayed for the Z-DEVD-AFC cleavage activity. The protease activity was expressed as pmol. AFC/mg. protein/min. The results were representative of three independent experiments.
Fig. 6.2. Concentration-dependent induction of the Z-DEVD-AFC cleavage activity by TPCK. Lysates from THP.1 cells treated for 4 h with the indicated concentrations of TPCK were assayed for DEVD-AFC cleavage activity. The results were representative of three independent experiments.

Fig. 6.3. Time course of the induction of the Z-DEVD-AFC cleavage activity by TPCK. Lysates from control cells or from cells treated with TPCK (75 μM) for the indicated period of times were assayed for DEVD-AFC cleavage activity.
apoptosis was induced in THP.1 cells (MacFarlane et al., 1997). These observations suggest that the caspase-3-like but not the caspase-1-like protease activity is a key effector of apoptosis.

6.4 Discussion

TPCK showed ability to either induce apoptosis or to modulate apoptosis induced by other stimuli dependent on the concentration used. To test whether the TPCK effects on apoptosis are related to its specific inhibition of chymotrypsin-like protease activity, fluorogenic substrates designed for trypsin or chymotrypsin were used to measure the protease activities in THP.1 cell lysates. Lysates from control cells contain Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC cleavage activities. It was somewhat surprising that TPCK at concentrations which induce apoptosis caused a decrease in the trypsin-like substrate Boc-Val-Leu-Lys-AMC but not the chymotrypsin-like substrate Suc-Ala-Ala-Pro-Phe-AMC cleavage activity (Table 6.1). The nature of the Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC cleavage activities in the lysates was examined using a common serine protease inhibitor PMSF. PMSF significantly inhibited the Suc-Ala-Ala-Pro-Phe-AMC but not the Boc-Val-Leu-Lys-AMC cleavage activity (Table 6.1), suggesting that this intracellular Boc-Val-Leu-Lys-AMC cleavage activity may be derived from nonserine proteases. Whether this protease is responsible for the induction of apoptosis by TPCK needs further investigation. A low concentration of TPCK (10 μM), which inhibited internucleosomal DNA cleavage (Fig. 4.6) and had differential effects on CHX- and etoposide-induced apoptosis (Fig. 4.7), showed no significant effect on this substrate cleavage activity (Table 6.1). TLCK, at concentrations which modulated apoptosis (Table 5.1), also showed no significant inhibition on the Boc-Val-Leu-Lys-AMC cleavage activity (Table 6.1). Both TLCK and TPCK had little or no effect on the Suc-Ala-Ala-Pro-Phe-AMC cleavage activity (Table 6.1). These results suggest that the modulation of apoptosis by TLCK and TPCK and the inhibition of internucleosomal DNA cleavage by TPCK are not due to the inactivation of these trypsin-like and chymotrypsin-like proteases activities. TPCK inhibits the apoptotic DNA fragmentation induced by UV light in human mammary carcinoma BT-20, murine fibroblast cell line 3T3, and human myeloid leukaemia HL-60 cells (Wright et al., 1994). In these cells, the TPCK target appears to be a 24 kD chymotrypsin-like serine protease with the synthetic
substrate Ala-Ala-Pro-Val p-nitroanilide but not the Ala-Ala-Pro-Phe-AMC as a preferred substrate. It is possible that in THP.1 cells, TPCK also inhibits a similar protease, leading to the inhibition of internucleosomal cleavage of DNA. TPCK but not TLCK induced apoptosis in HL-60 cells possibly through inhibiting the chymotrypsin-like protease activity in the proteasomes (Drexler, 1997). It is likely that the differential effects of TPCK and TLCK on apoptosis is due to their ability to inhibit distinct intracellular proteases.

Although caspase-1 is continuously expressed in THP.1 cells, it may not be involved in apoptosis. This conclusion is supported by various observations: lysates from apoptotic cells do not cleave IL-1β or the fluorogenic substrate YVAD-AMC (MacFarlane et al., 1997); YVAD.CMK, a caspase-1 selective inhibitor, has no effect on the induction of apoptosis (Fig. 5.10). In contrast, caspase-3 or its related proteases may be a key effector of apoptosis, as suggested by the following facts: caspase-3 and 7 were processed during the induction of apoptosis (Fig. 5.9); a potent inhibitor of caspase-3 effectively inhibited the induction of apoptosis (Fig. 5.8); an increase in the Z-DEVD-AFC cleavage activity is associated with the induction of apoptosis; the Z-DEVD.AFC cleavage activity was not detected when apoptosis was prevented by Z-VAD.FMK (Fig. 6.1).

6.5 Summary

1. TPCK but not TLCK showed significant inhibition of the Boc-Val-Leu-Lys-AMC cleavage activity in THP.1 cell lysates. Whether this inhibition is responsible for the induction of apoptosis by TPCK is unclear.

2. The induction of apoptosis by diverse stimuli is associated with the increased Z-DEVD-AFC cleavage activity, suggesting that caspase-3 or a related caspase is a common effector of apoptosis.
CHAPTER 7

GENERAL DISCUSSION
7.1 The role of proteases in the execution of apoptosis

7.1.1. Caspases control the execution phase of apoptosis

Apoptotic cell death has been proposed as a two phase process (Earnshaw, 1995). First, cells react to an external or internal stimulus and become committed to die. After this variable phase, cells cross into the second phase, the execution phase. Based on the morphological similarity observed among diverse cell types in the process of apoptotic cell death, it has been suggested that there is likely to be a shared apoptotic mechanism operating in most, if not all, cells of the body. The goal of the research over the years has been to determine the sequence of biochemical events that leads to cell death following a variety of model injuries with the overall aim of determining a final common pathway. The molecular basis of the final apoptotic pathway is beginning to be defined. It is now clear that the execution phase of apoptosis requires the participation of caspases.

Using THP.1 cells as a model, the present study demonstrated that diverse agents were capable of inducing apoptosis and that Z-VAD.FMK abrogated the induction of apoptosis regardless of the apoptotic stimuli (Table 5.1). These results imply that multiple signalling pathways lead from death-triggering extracellular agents to a central death pathway in which Z-VAD.FMK inhibitable caspase activity is involved. Consistent with the present study, Z-VAD.FMK effectively inhibits apoptosis in a wide range of cellular systems (Table 7.1), suggesting that Z-VAD.FMK sensitive caspases are required for the induction of apoptosis in a majority of mammalian cell types.

In THP.1 cells the inhibition of apoptosis by Z-VAD.FMK was complete, since no morphological or biochemical changes of apoptosis had been left unaffected (Fig. 5.6, Fig. 5.3). In addition, the protection could be observed for up to 24 h (Fig. 5.2). This result was in contrast with that obtained by McCarthy et al. In their system, Rat-1 fibroblasts were induced to undergo apoptosis by a number of stimuli. Z-VAD.FMK failed to inhibit membrane blebbing in these cells, and delayed cell death was observed (McCarthy et al., 1997). In another study using Jurkat T cells, Z-VAD.FMK (50 μM) inhibited Bax-induced caspase activity together with the fragmentation of DNA, however, partial chromatin condensation, membrane blebbing, dramatic cytoplasmic vacuolation,
Table 7.1. Effects of caspase inhibitors on apoptotic cell death

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treatment</th>
<th>Inhibitors (µM)</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP.1</td>
<td>CHX, THG, STS, ETOP</td>
<td>YVAD.CMK (100)</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Z-VAD.FMK (5-20)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Z-DEVD.FMK (5-20)</td>
<td>yes</td>
</tr>
<tr>
<td>Jurkat</td>
<td>Fas</td>
<td>YVAD.CMK (10-120)</td>
<td>yes (Juo et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Z-VAD.FMK (50-100)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Etoposide</td>
<td>YVAD.CMK (120)</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Z-VAD.FMK (100)</td>
<td>yes</td>
</tr>
<tr>
<td>Ramos-BL</td>
<td>Anti-IgM, inomycin</td>
<td>Z-VAD.FMK (50-100)</td>
<td>partially (An and Knox 1996)</td>
</tr>
<tr>
<td>PC12</td>
<td>NGF withdrawal</td>
<td>Z-VAD.FMK (100)</td>
<td>yes (Park et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Serum withdrawal</td>
<td>Z-VAD.FMK (10-50)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-YVAD.CMK (250)</td>
<td>partially (Stefanis et al., 1996)</td>
</tr>
<tr>
<td>Thymocytes</td>
<td>Fas</td>
<td>Z-VAD.FMK (10)</td>
<td>yes (Deshmukh et al., 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAF (100)</td>
<td>partially</td>
</tr>
<tr>
<td>Sympathetic</td>
<td>NGF withdrawal</td>
<td>Z-VAD.FMK (&gt;100)</td>
<td>yes</td>
</tr>
<tr>
<td>neurons</td>
<td></td>
<td>BAF (100)</td>
<td>yes</td>
</tr>
<tr>
<td>GM701</td>
<td>STS</td>
<td>Z-VAD.FMK (10-100)</td>
<td>yes (Jacobson et al., 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-DEVD.CHO (1-100)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ac-YVAD.CHO (100)</td>
<td>no</td>
</tr>
<tr>
<td>SKW6.4</td>
<td>Fas</td>
<td>Z-VAD.FMK (1-100)</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>STS</td>
<td>Z-VAD.FMK (10-100)</td>
<td>yes</td>
</tr>
<tr>
<td>Four-cell mouse</td>
<td>STS</td>
<td>Z-VAD.FMK (100)</td>
<td>no</td>
</tr>
<tr>
<td>embryos</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat-1</td>
<td>Etoposide</td>
<td>Z-VAD.FMK (100)</td>
<td>Delayed but not inhibited cell death (McCarthy et al., 1997)</td>
</tr>
</tbody>
</table>

GM701, an SV40-transformed human fibroblast cell line.

PC12, pheochromocytoma cell line.

Ramos-BL, Burkitt lymphoma B cell line.

SKW6.4 cell, a human B lymphoma cell line.
and the reduction in mitochondrial membrane potential still occurred (Xiang et al., 1996). The implication from these observations is that Z-VAD.FMK inhibits the apoptotic program downstream of an irreversible point. On the other hand, a caspase inhibitor Boc-aspartyl (OMe)-fluoromethyl ketone (BAF) offered a long term protection against neuronal death induced by nerve growth factor deprivation (Deshmukh et al., 1996). This compound, lacking the amino acids in P2-P4 position, would be expected to be less selective for caspases. Thus the full protection of cell death may be due to its non-specific inhibition of more caspases. It is likely that the effects of caspase inhibitors on apoptosis depend on the set of caspases involved in certain systems, although it has been suggested that there may be a caspase-independent apoptotic pathway (Xiang et al., 1996).

Caspase-3 appears to be an important component of the cell death pathway in several cell types (Nicholson et al., 1995; Schlegel et al., 1996; Chinnaian et al., 1996; Darmon et al., 1996). Also, importantly, more enzymes closely related to caspase-3 exist in a single cell type, and they are all able to cleave PARP in vitro (Orth et al., 1996; Takahashi et al., 1996), and therefore, may function to replace caspase-3. Caspase-3 can efficiently cleave PARP into fragments identical in size to those seen in apoptotic cells (Tewari et al., 1995; Nicholson et al., 1995). Ced-3 is also a functional PARP protease (Xue et al., 1995), although the cleavage site used in mammalian apoptosis does not appear to be conserved in *C. elegans* PARP. Besides being a substrate for caspase-3, PARP has also been shown to be degraded by other caspases, including caspase-7 and caspase-6. In vitro, caspase-1 also can cleave PARP although it requires 50-100-fold higher concentration than for the processing of pro-IL-1β (Gu et al., 1995). Thus it is possible that there are several caspases capable of cleaving PARP *in vivo*. Other targets of caspase-3 include sterol regulator element-binding protein-1 (SREBP-1) and SREBP-2 (Wang et al., 1996), the 70 kD subunit of the U1 small ribonucleoprotein (U1-70 kD) (Casciola-Rosen et al., 1996), the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) (Han et al., 1996), PKC δ (Emoto et al., 1995), D4-GDI (Na et al., 1996). Cleavage of these proteins was found to be associated with the induction of apoptosis. Other well characterized protein substrates for caspases during apoptosis are lamins, a family of intermediate filament proteins which form a meshwork that provides structural support for the inner nuclear membrane. Caspase-6 is capable of cleaving lamins (Takahashi et al., 1996).
In THP.1 cells, caspase-3 and its closely related family member, caspase-7, were both activated during the induction of apoptosis (Fig. 5.9, Fig. 4.3). Caspase-3 inhibitor Z-DEVD.FMK inhibited apoptosis together with the processing of both caspase-3 and caspase-7 (Fig. 5.9), supporting a role for caspase-3 related caspases as key effectors of apoptosis in this cell type, although it is difficult to ascertain which of these caspases play the major role in the execution of apoptosis. Induction of apoptosis by two different treatments (CHX/TLCK and TPCK) (Fig. 5.10, Fig. 5.11) in THP.1 cells involved the cleavage of lamins into fragments similar to those produced after cleavage by caspase-6 \textit{in vitro} (Takahashi et al., 1996), suggesting that caspase-6 was also involved in the execution phase of apoptosis in these cells. Clearly, this study provides further evidence that caspase-3 and its related family members have a key role in the execution of apoptotic cell death.

\subsection*{7.1.2. Contribution of proteolysis to nuclear events of apoptosis}

It has been suggested that in the chain of events leading to apoptosis, proteolysis and DNA fragmentation are linked. Arguments in favour of this hypothesis are that several protease inhibitors reverse the effect of cytotoxic drugs on DNA fragmentation in different systems and that a proteolytic step precedes, and is required for, apoptosis (Bruno et al., 1992; Higuchi et al., 1995). Internucleosomal DNA cleavage induced by different apoptotic stimuli in a range of cell types was inhibited by the serine protease inhibitor TPCK (Wright et al., 1994; Ghibelli et al., 1995), suggesting the existence of a common TPCK-inhibitable DNA degradation pathway. In this regard, a 24 kD chymotrypsin-like protease has been identified to be the target of TPCK for the inhibition of internucleosomal DNA cleavage in different cell types (Wright et al., 1994).

In THP.1 cells, CHX/TLCK caused fully apoptotic morphological features of apoptosis, including DNA condensation and nuclear fragmentation. These morphological changes were associated with the proteolysis of PARP and lamins and the degradation of nuclear DNA into internucleosomal cleavage fragments. In contrast, cells treated with TPCK showed some features of the intermediate phase of apoptosis (Fig. 3.6 C), which was described by Ghibelli et al (1995). Most cells lost their normal irregular shape and become rounded with the chromatin being condensed in patches at the nuclear edge. The cytoplasmic condensation was accompanied by dilation of the endoplasmic reticulum. However, the nuclei of these cells were not fully condensed or fragmented. The
biochemical changes induced by TPCK included the proteolysis of PARP and lamins and the degradation of DNA into large kbp fragments (Fig. 4.5 and Fig. 4.2). Thus the only difference between the biochemical changes induced by CHX/TLCK and by TPCK was the absence of internucleosomal cleavage of DNA in TPCK-induced apoptosis. It seems that the proteolysis of PARP and lamins together with the degradation of DNA into large kbp fragments was not responsible for the full condensation of nucleus. A study in a cell free system showed that when the lamin protease was inactivated in the lysates from apoptotic cells, the PARP cleavage and the internucleosomal DNA fragmentation proceeded in isolated nuclei, however the nuclear condensation did not occur (Takahashi et al., 1996). The data from studies in intact cells and in cell free systems strongly suggest that it is the co-operation of proteolysis of lamin and internucleosomal cleavage of DNA that mediates the full condensation of the nucleus.

Studies in nuclei isolated from thymocytes have revealed that a Ca\textsuperscript{2+} -regulated serine protease in the nuclear scaffold may also mediate lamin proteolysis (Clawson et al., 1993). This enzyme was resistant to calpain action and was characterized as a serine protease with a predominantly chymotrypsin-like substrate preference (Clawson et al., 1993). Since lamins are intrinsically connected and linked to chromatin loops by means of DNA-protein interactions and scaffold associated proteins, lamin dissociation may result in detachment of large chromatin regions, resulting in sufficient chromatin unfolding to allow endonuclease access. In thymocytes, lamin degradation precedes the formation of large kbp DNA fragments in apoptosis, and inhibition of the Ca\textsuperscript{2+} -regulated serine protease prevents lamin and DNA breakdown induced by methylprednisolone (Zhivotovsky et al., 1995), suggesting that lamin cleavage is responsible for the DNA degradation. However another study showed that the degradation of lamin B\textsubscript{1} in apoptotic thymocytes can precede DNA fragmentation (Neamati et al., 1995), but the inhibition of lamin proteases by TLCK does not prevent internucleosomal cleavage of DNA (Lazebnik et al., 1995). Moreover in THP.1 cells, YVAD.CMK (100 μM), which inhibited lamin cleavage (Fig. 5.10), had little effect on DNA cleavage induced by several stimuli, indicating that DNA degradation is not always linked to lamin cleavage.

A kinetic study of apoptosis induced by CHX/TLCK showed that large kbp DNA degradation preceded PARP cleavage (Fig. 3.13), suggesting that this form of DNA degradation is independent of PARP proteolysis. However, both events were inhibited by
a caspase-3 selective inhibitor, Z-DEVD.FMK. These results suggest that one or more
caspase-3-related family members are required for the degradation of DNA and PARP.

Taken together, these studies demonstrated that the PARP protease and the
fragmentation nuclease acting alone or in combination are insufficient to drive the final
disassembly of the nuclei, and the combination of the PARP and lamin cleavage protease
is also not sufficient to drive the apoptotic pathway. It seems that nuclear events begin
with the activation of multiple caspases and then ramify into a number of pathways that
operate in parallel to effect the rapid disassembly of the entire cell.

7.2. Multiple proteases are involved in the regulation of apoptosis at
early stages

Studies of the effects of protease inhibitors on apoptosis have revealed a complex
picture. In THP.1 cells, TPCK had three distinct effects dependent on the concentration
and apoptotic stimulus. First, at a low concentration TPCK (10 μM) inhibited
internucleosomal cleavage of DNA induced by other apoptotic stimuli (Fig. 4.6), while
TLCK had no such effect. Secondly, at the same concentration, TPCK potentiated CHX-
induced apoptosis but inhibited etoposide-induced apoptosis (Fig. 4.7), similar effects
which were observed with TLCK. Since these two compounds are structurally related, it
is expected that in some instances they produce overlapping effects, due to reacting with
the same intracellular components. The common targets of TLCK and TPCK may play
distinct roles in different apoptotic pathways. Thus even at the same concentration, TPCK
showed different effects on apoptosis possibly by reacting with two distinct targets. One is
involved in an early stage of apoptosis, which is TLCK sensitive, and another is involved
in the internucleosomal DNA cleavage, which is resistant to TLCK. Thirdly, higher
concentrations of TPCK (50-75 μM) were required for the induction of apoptosis (Fig.
4.1), indicating another distinct intracellular target was involved.

Proteases of different classes have been implicated in some forms of apoptosis. Calpain
inhibitors prevent dexamethasone-induced apoptosis in thymocytes, but they have no
effect on CHX-induced apoptosis in HL-60 cells (Squier et al., 1994; Lu and Mellgren,
1996) and ultraviolet light-induced apoptosis in Hela cells (Casciola-Rosen et al., 1994).
TLCK inhibited diverse stimuli induced apoptosis in thymocytes. On the other hand,
inhibitors of calpain, cysteine, serine proteases and proteasome all have been
demonstrated to induce apoptosis (Squier et al., 1994; Zhu et al., 1995; Lu and Mellgren et al., 1996; Drexler, 1997). The induction of apoptosis by all these protease inhibitors was inhibited by caspase inhibitors (Lu and Mellgren et al., 1996; Drexler, 1997).

Taken together, the death program in mammalian cells is operated by multiple pathways and the regulation of apoptosis requires more than one class of protease (a proposed cell death pathway in THP.1 cells and the putative acting site of different protease inhibitors is illustrated in Fig. 7.1). Serine proteases, calpain, and the proteasome have fundamentally different roles compared to the caspases. The former may participate in the regulation of "upstream" events in apoptosis that may differ depending on the apoptotic stimuli and cell type. However, since the inhibitors of these proteases can induce apoptosis, it is clear that these inhibitors do not inhibit a process which is fundamental to apoptosis in general. In contrast, the caspases appear to play a pivotal role in the execution of the apoptotic pathway.

7.3 Future work

Recent work has supported a central role for caspase family members as effectors of apoptosis. Despite the growing knowledge of the structure, expression, and substrate-recognition properties of caspases, there are still many issues that need to be resolved.

**What is the initial upstream caspase?** Activation of the Fas receptor leads to the recruitment of FADD, that in turn associates with caspase-8 (Muzio et al., 1996; Boldin et al., 1996). The resulting activation of caspase-8 probably causes further activation of other downstream caspases. The CTL-specific protease granzyme B activates caspase-3 and induces cell death (Darmon et al., 1995; Quan et al., 1996).

However, what is the upstream caspase which activates caspase-3 in THP.1 cells in response to different stimuli? Clearly, certain caspases can activate others, for example, caspase-1 can activate caspase-3 (Tewari et al., 1995a), caspase-4 (TX) can activate caspase-1 (Faucheu et al., 1995) and caspase-3 can activate caspase-6 (Takahashi et al., 1996a); however, whether these cleavage are relevant to an apoptotic cascade in vivo is not clear. In THP.1 cells, multiple caspases are proteolytically processed into their active forms. The caspase-3 inhibitor Z-DEVD-FMK failed to inhibit the processing of caspase-3 (Fig. 5.9), indicating that the activation of caspase-3 is not due to the autoprocessing but to the processing by a distinct Z-VAD.FMK-sensitive caspases.
Do caspases function in specific cell types or in response to specific apoptotic stimuli? Accumulating evidence suggests that apoptosis is mediated by combinations of caspases acting in concert that may be cell type-specific and stimulus-dependent. Since the expression of caspases differs between various cell types, it is possible that the repertoire of caspases involved in the execution of apoptosis differs between cell types, resulting in different sensitivity of cell death to a given caspase-inhibitor (Table 7.1).

High expression of caspase-3 is restricted mostly to lymphoid and myeloid cells and their precursors (Fernandes-Alnemri et al., 1994). Caspase-1 is expressed in many types of cells including thymocytes and THP.1 cells, but not in sympathetic neurons (Deshmukh et al., 1996). It is unlikely that caspase-1 is responsible for some neuron deaths. Caspase-2 but not caspase-3 was cleaved during neuronal apoptosis (Deshmukh et al., 1996). Caspase-2 is required for apoptosis after trophic factor withdrawal but not superoxide dismutase downregulation in sympathetic neurons and PC12 cells (Troy et al., 1997), indicating that neuronal death trigged by different initial causes may be mediated by distinct members of the caspase family. In THP.1 cells, although pro-caspase-2 was decreased during the induction of apoptosis by TPCK (Fig. 4.4, Fig. 5.5), only a small amount of active subunits was detected. Z-VAD.FMK effectively inhibited the induction of apoptosis but failed to effectively prevent the decrease of the proform enzyme (Table 5.1, Fig. 5.5). It seems that caspase-2 is not essential to the induction of apoptosis in this cell type. The significance of the processing of caspase-2 in the induction of apoptosis in this system needs further investigation. Thus instead of a “universal” cell death pathway operated by a single gene ced-3 in C.elegans cell death, alternative pathways, in which more than one caspase is involved, may function in mammalian cell death. Further studies of the effects of more specific caspase inhibitors on apoptosis may ultimately provide a means of controlling apoptotic cell death in a tissue specific manner or to induce apoptosis in selected cell populations.

Which caspase is pivotal in apoptosis? Inhibitors of caspase-3 inhibit apoptosis, suggesting that caspase-3 may be a key effector of apoptosis. However, there are number of caspase-3 related protease activities existing in apoptotic cells, proving that it is difficult to ascertain which one is pivotal. It is unclear whether the various caspases function in parallel with each other or in a cascade. (Fig. 5.4; Fig. 7.1). It seems that in THP.1 cells, a number of caspases was activated in a cascade fashion (Fig. 7.1). Although
caspases appear to play fundamental roles in apoptosis, their individual contribution to apoptosis and the mechanisms of their activation, enzymatic specificity, and physiologically relevant apoptotic substrates remain to be established.

The role of Bcl-2 proteins in apoptosis. The cell death suppressor, Bcl-2, may act upstream of caspases. This has been suggested by a number of recent studies demonstrating that the activation of caspases is inhibited by the Bcl-2 or its related proteins. Overexpression of Bcl-2 inhibits caspase-2 activation and apoptosis in GT1-7 cells treated with STS (Srinivasan et al., 1996). Bcl-2 and Bcl-xL inhibit STS-induced apoptosis and activation of caspase-3 (Chinnaiyan et al., 1996). The existence of Bcl-2 in THP.1 cells may provide protection against cell death. It has not been shown in the present study whether Bcl-2 family proteins function to inhibit the induction of apoptosis by any of the apoptotic stimuli tested. Elucidating the function of the apoptosis suppressors might be expected to provide new approaches for therapeutic intervention in certain tumors.

The role of other classes of proteases. Previous studies suggested that serine proteases were involved in the internucleosomal cleavage of DNA and lamin cleavage (Wright et al., 1994; Clawson et al., 1992). The present study with the serine and cysteine protease inhibitors TLCK and TPCK showed differential effects on apoptosis. Clearly the role of proteases in the control of apoptosis is more complex than was originally thought and needs to be further elucidated.
Apoptotic pathway in THP.1 cells

Fig. 7.1 A proposed apoptotic pathway in THP.1 cells. Diverse apoptotic signals emerge to activate a Z-VAD.FMK inhibitable caspase, which in turn activates downstream caspases. Caspase-3 may be upstream of caspase-6, indicated by the observation that PARP cleavage occurred earlier than lamin B1 cleavage (Fig. 5.5). These caspases participate in the execution of apoptosis in parallel. Z-DEVD.FMK inhibits the induction of apoptosis, possibly due to the inhibition of more than one caspase-3 like enzyme. TLCK modifies the induction of apoptosis at early stages, whereas TPCK can activate the apoptotic pathway and also can inhibit the activation of an endonuclease which is responsible for the degradation of DNA into nucleosomal fragments.


124


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