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**AN ANALYSIS OF NUCLEAR EVENTS DURING HEAT-INDUCED
APOPTOSIS IN RESTING MOUSE SPLENOCYTES.**

**by
CAROLINE TERESA SODJA, B. Sc. (Maj.)**

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of**

Master of Science

Department of Biology

**Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario.**

July, 1997.

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Chair, Department of Biology

Thesis Supervisor

Carleton University,
September, 1997.

ABSTRACT

The behaviour of nuclear proteins and DNA was compared during spontaneous and heat-induced apoptosis in resting mouse splenocytes. DNA content, distribution and cleavage, and nuclear antigen organization and degradation were monitored by flow cytometry, immunoblotting, and fluorescence microscopy. Apoptosis was hyper-induced by heat, but proceeded mostly like spontaneous apoptosis. DNA cleavage was detected first. Subsequently, progressive chromatin collapse and loss of Nuclear Mitotic Apparatus (NuMA) protein, lamin B and PI2 immunolabelling were temporally, but not spatially, independent of one another. On immunoblots, NuMA and lamin B, but not PI2, were degraded, and NuMA appeared ubiquitinated before and during apoptosis. However: heating resulted immediately in unique NuMA labelling in T splenocytes; heat induced apoptosis preferentially in T splenocytes; and DNA cleavage/collapse were protein synthesis-dependent during heat-induced, but not spontaneous, apoptosis. This suggests different regulatory pathways for spontaneous and heat-induced apoptosis, with NuMA as an early apoptotic marker in heated T cells.

ACKNOWLEDGEMENTS

I would like to thank Dr. Nathalie Chaly for her encouragement and excellent supervision during my thesis, and Dr. David L. Brown for co-supervising the project. I would also like to thank my entire committee: Dr. Chaly, Dr. Brown, Dr. P. Roy Walker and Dr. Ron Mitchel for their support and guidance throughout the project.

Special thanks to Dr. Walker and Dr. Sikorska for the use of their lab facilities for flow cytometry and immunoprecipitation experiments, and to Dr. Mitchel for providing me with a water bath for heat-treating cells. Thanks to Christine Carson, not only for her help with flow cytometry, but for always making me feel welcome at the NRC. I thank the donors of the antibodies D. Compton, T. K. Tang, and N. Chaudhary.

I would also like to thank Xia Chen for her training and companionship over the years, Bea Valentine for her help with the EM course, Andrew Vaillant for training on the confocal microscope and for sending my images to Carleton, Warren Hagar for stimulated splenocyte samples.

I would like to thank my parents, John and Marga Sodja, for their never-ending support, and Allison, Dorothy, Brian, and Ingrid for their understanding and friendship. Special thanks to Dr. Gabriela Obrocea and Dr. Mary Morris for convincing me to do graduate studies.

My husband, Pierre Ouellet, I can't thank enough for his encouragement, love and support.

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LIST OF ABBREVIATIONS

ABRP	attachment region binding protein
BHK-21	baby hamster kidney cell line
BrDU	bromodeoxyuridine
BrUTP	bromouridine triphosphate
cAMP	cyclic adenosine monophosphate
CaSki	human cervical epidermoid carcinoma cell line
cDNA	complementary DNA
CENP A	centromere protein A
CH	cycloheximide
CHO	Chinese hamster ovary cell line
Con A	concanavalin A
CPSR-2	controlled process serum replacement-2
CY3	Cyanine 3
DAPI	4',6-diamidino-2-phenylindole
DHCC	3,3'-dihexyloxacarbocyanin
DPA	diphenylamine
DNase	deoxyribonuclease
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
FC	fully collapsed
FITC	fluorescein isothiocyanate
GAP	GTPase-activator protein
GTP	guanidine triphosphate

HeLa	cervical carcinoma cell line derived from Henrietta Lacks
HL-60	human promyelocytic leukemia cell line
hnRNP	heterogenous nuclear ribonucleoprotein
HSP	heat shock protein
HT	heat-treated
ICE	interleukin-1 β converting enzyme
IgG	immunoglobulin G
MAR	matrix attachment region
mRNA	messenger RNA
kb	kilobase
kDa	kilodalton
NuMA	Nuclear Mitotic Apparatus Protein
PAGE	polyacrylamide gel electrophoresis
PARP	poly (ADP-ribose) polymerase
PCNA	proliferating cell nuclear antigen
PBS	phosphate buffered saline
PC	partially collapsed
PtK2	kangaroo rat kidney cell line
Ran	Ras-related nuclear protein
RCC1	Regulator of Chromatin Condensation
rDNA	ribosomal DNA
RPMI	Roswell Park Memorial Institute
RNase	ribonuclease
SAF-A	scaffold attachment factor A
SAR	scaffold attachment region
SAT-B1	special AT-rich sequence binding protein 1

SDS	sodium dodecyl sulfate
snRNP	small nuclear ribonucleoprotein
SPN	Spindle Pole Nucleus
SW13	human adrenal cortex carcinoma cell line
Topo I	DNA topoisomerase I
Topo II	DNA topoisomerase II
tsBN2	temperature-sensitive hamster kidney cell line
TUNEL	terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick end labelling
TUNEL-normal	TUNEL-positive DAPI-normal
U-251	human glioma cell line
VM-26	teniposide
WGA	wheat germ agglutinin

CHAPTER 1.

Literature Review

I am studying the spatial and temporal interrelationships of chromatin and nuclear protein organization during apoptosis. Using the nucleus of the resting mouse splenocyte as a model system, I have compared nuclear disassembly during control and heat-induced apoptosis. In this literature review, I will discuss: the organization of chromatin and proteins in the nucleus; the changes in nuclear organization that accompany degradation of these components during apoptosis; the lymphocyte as a model system for studying apoptosis; and the effects of hyperthermia on apoptosis induction and nuclear morphology.

A. Organization of the Nucleus

Evidence is accumulating that the nucleus is assembled in an ordered fashion through interactions between chromatin and nuclear proteins. In this section of the literature review, I will discuss the current model of nuclear organization, in which DNA loops are anchored at the nuclear envelope and in the nucleoplasm by a protein scaffold called the nuclear matrix.

A.1 Organization of DNA into Loops

In the first level of DNA organization, DNA is wrapped around an octamer of core histones forming a fibre with a diameter of 10 nm. The 10 nm fibre, or nucleosome chain, is then folded into a solenoid with a diameter of 30 nm. In current models of the nucleus, the 30 nm fibre is further folded into loops that are anchored at the nuclear periphery and within the nucleoplasm by interaction with the nuclear matrix [reviewed in Davie, 1996].

Recently, a model for folding of DNA was proposed based on the size distribution of DNA fragments from autodigestion of cells and isolated nuclei, or from treatment of cells with DNA topoisomerase II (Topo II) inhibitors [Filipski *et al.*, 1990]. In this model, 50 kb loops of 30 nm chromatin fibre are organized into rosettes containing an average of 300 kb of DNA [Filipski *et al.*, 1990].

Early evidence in support of a loop model of chromatin was derived from the treatment of "nucleoids" with ethidium bromide, a DNA intercalating dye. Nucleoids are nuclear structures that retain all DNA but are depleted of histones and other proteins. Addition of ethidium bromide to nucleoids results in the formation of DNA halos visible by fluorescence microscopy. The appearance of DNA halos, and their subsequent disappearance with increasing concentrations of ethidium bromide, was interpreted to be the result of progressive positive supercoiling introduced by ethidium bromide. It was proposed that negatively supercoiled loops of DNA are anchored to a nuclear skeleton [Vogelstein *et al.*, 1980; reviewed in Razin *et al.*, 1995]. In another early study, electron microscopy was used to visualize DNA loops in interphase nuclei extracted with high salt [Hancock and Hughes, 1982]. DNA loops with an average size of 50 kb were observed attached to a skeletal structure [Hancock and Hughes, 1982]. It is now accepted that the 30 nm fibre is organized into loops constrained by attachment to a non-chromatin skeletal structure [reviewed in Razin *et al.*, 1995]. A more recent study of the loop organization of chromatin focussed on the organization of the highly repetitive rDNA sequences in nucleoli, examining the DNA fragments excised by Topo II-mediated cleavage [Iarovaia *et al.*, 1995; reviewed in Razin *et al.*, 1995]. Permeabilized cells extracted with high salt

were treated with Topo II inhibitors, and DNA fragments were analyzed by pulse field gel electrophoresis and Southern blotting. The results suggested that nucleolar genes are organized into loops of the same size as the rDNA repeat, and are separated by attachment to the nuclear matrix [Iarovaia *et al.*, 1995; reviewed in Razin *et al.*, 1995].

The association of chromatin loops with the nuclear matrix is thought to be mediated by DNA sequences termed matrix (MARs) or scaffold attachment regions (SARs) [reviewed in Bode *et al.*, 1995; Boulikas, 1995]. MARs/SARs have an average size of 500 bp, occur in non-transcribed regions, and are thought to define borders between chromatin loop domains containing single genes, segments of genes, or groups of genes [reviewed in Boulikas, 1995]. Such sequences were first identified by their association with the nuclear matrix after removal of DNA and/or proteins [Mirkovitch *et al.*, 1984], and by *in vitro* binding of radiolabelled DNA fragments to isolated nuclear matrix proteins [Cockerill and Garrard, 1986]. Although there is no consensus sequence for MARs/SARs, they generally contain AT-rich sequences as well as sequences similar to Topo II cleavage sites, and are characterized by a strong tendency for extensive unpairing when exposed to superhelical strain [reviewed in Bode *et al.*, 1995].

A number of nuclear matrix proteins have been shown to bind MARs/SARs *in vitro*. Competition assays showed that radio-labelled *Drosophila* histone MAR binds with a high affinity to purified Topo II [Adachi *et al.*, 1989], and B-type lamins [Ludérus *et al.*, 1992], and with a lower affinity to NuMA protein and A-type lamins [Ludérus *et al.*, 1994]. SAT-B1 (special AT-rich sequence binding protein 1) is a thymus-specific nuclear matrix protein that binds naturally occurring MARs/SARs from a number of species [Dickinson

et al., 1992]. SAT-B1 was identified during screening of a human cDNA library with a synthetic MAR/SAR probe [Dickinson et al., 1992]. Using the same synthetic MAR/SAR probe in an affinity column, a 100 kDa MAR/SAR binding protein was isolated and identified as nucleolin [Dickinson and Kohwi-Shigematsu, 1995]. Other proteins that bind MARs/SARs have been identified in the nuclear matrix fractions of chicken oviduct [von Kries et al., 1991], HeLa cells [Romig et al., 1992], and rat brain [Tsutsui et al., 1993] using a DNA binding protein blot assay. These proteins bind with high affinity to MARs/SARs of different species, and were named attachment region binding protein (ABRP) [von Kries et al., 1991], scaffold attachment factor A (SAF-A) [Romig et al., 1992], and SP120 [Tsutsui et al., 1993]. It is currently unknown whether these proteins regulate loop attachment to the nuclear matrix *in vivo* [reviewed in Boulikas, 1995].

A.2 The Nuclear Matrix

In 1974, Berezney and Coffey described a residual nuclear structure, the nuclear matrix, isolated from rat liver nuclei treated with high concentrations of salt, a nonionic detergent, DNase and RNase. These treatments removed 75-90% of nuclear protein and nearly all of the chromatin from the nucleus. The remaining residual structure was composed of approximately 98% non-histone nuclear proteins, 1% RNA, and 0.1% DNA. At the electron microscope level, the isolated nuclear matrix was shown to consist of a residual nuclear pore complex-lamina, a residual nucleolus, and an internal fibrogranular network [Berezney and Coffey, 1974; reviewed in Berezney et al., 1995]. This structure was similar in morphology to the interchromatinic regions of the intact nucleus revealed by

regressive EDTA staining [Berezney and Coffey, 1974; reviewed in Berezney *et al.*, 1995].

In subsequent studies, it was found that the structure of the internal component of the nuclear matrix was dependent on the isolation protocol [reviewed in Stuurman *et al.*, 1992; Berezney *et al.*, 1995]. For example, treatment of rat liver nuclei with RNase during DNA digestion resulted in a sparse granular internal network in comparison to matrices isolated without RNase treatment. However, if the addition of RNase was preceded by treatment with sodium tetrathionate, an agent that promotes the formation of disulphide bonds, the internal network was more extensive and contained residual nucleoli [Kaufmann and Shaper, 1984].

A number of protocols were developed in an effort to avoid the possible formation of artefacts from conditions used to isolate nuclear matrices [Cook, 1988; Stuurman *et al.*, 1992]. Procedures based on the original protocol of Berezney and Coffey [1974] use high concentrations of salt to extract chromatin. Alternatively, the ionic detergent lithium 3,5-diiodosalicylate (LIS) has been used instead of salt for chromatin extraction from heat-stabilized or divalent cation-stabilized nuclei [Mirkovitch *et al.*, 1984]. These structures were called "nuclear scaffolds" and contained a pore-complex lamina and a granular internal matrix [Mirkovitch *et al.*, 1984]. However, if the heating step was omitted from the LIS procedure, "nuclear shells" were isolated which contain a pore-complex lamina, but no internal fibrogranular network [Mirkovitch *et al.*, 1984; Ludérus *et al.*, 1992].

To avoid precipitation or aggregation of nuclear components from the use of non-physiological strength buffers, Jackson and Cook [1988] embedded cells in agarose, and

removed digested DNA by electrophoresis in a physiological strength buffer. In such preparations, a filamentous network was observed in the interior of the residual nucleus by resinless thick section electron microscopy [Jackson and Cook, 1988]. A filamentous internal network has also been reported by He et al. [1990] using resinless thick section electron microscopy of HeLa cells. Nuclear matrices isolated from whole cells extracted with ammonium sulphate contained a complex fibrogranular network [He et al., 1990]. Further extraction of cells with salt solutions resulted in a highly branched network of 8-10 nm filaments named "core filaments". The core filaments have been interpreted to represent a framework upon which other nuclear matrix components are assembled [He et al., 1990; reviewed in Berezney et al., 1995].

Despite the differences in procedures, all of the above studies resulted in isolation of a structure composed of a residual nuclear lamina and internal granular or fibrogranular network [Stuurman et al., 1992]. HeLa cell nuclear matrices prepared by salt and by LIS procedures have been shown to be similar in ultrastructure by electron microscopy [Belgrader et al., 1991]. Recently, Eberharter et al. [1993] used gel electrophoresis to examine the protein composition of nuclear matrices isolated from renal epithelial cells using various protocols. They found no differences in protein composition for matrices isolated using NaCl, LIS or ammonium sulphate to extract chromatin [Eberharter et al., 1993]. Furthermore, addition of Cu^{2+} or Ca^{2+} ions, sulfhydryl reducing agents, or chelating agents did not change the protein composition of the isolated nuclear matrix [Eberharter et al., 1993]. Such results have led to the conclusion that the nuclear matrix is a stable and reproducible structure, and that matrices isolated using the different

procedures are likely functionally similar to each other [Stuurman *et al.*, 1992; Eberharter *et al.*, 1993].

Although there is still some debate, the existence of a nuclear matrix in eucaryotic cells is now widely accepted, and there is evidence for its involvement in the nuclear functions of replication, transcription, and RNA processing [Berezney *et al.*, 1995]. For instance, it has been proposed that nuclear functions such as replication are carried out in domains assembled on the nuclear matrix [Berezney *et al.*, 1995]. DNA replication and replication-associated proteins have been localized to discrete nuclear matrix-associated foci by immunodetection of biotin-dUTP or BrDU incorporation [Nakayasu and Berezney, 1989; Hassan, 1995; Jackson, 1995]. Similarly, BrUTP labelling has shown that transcription also occurs in discrete nucleoplasmic foci [Jackson *et al.*, 1993; Wansink *et al.*, 1993; Hassan, 1995]. Furthermore, newly transcribed RNA has been localized to visible "tracks" in the nucleus [Lawrence *et al.*, 1989]. Replication foci, transcription foci, and RNA track patterns are retained in nuclear matrices, suggesting that the nuclear matrix is involved in organizing these functional domains [Nakayasu and Berezney, 1989; Xing and Lawrence, 1991; Jackson *et al.*, 1993; Berezney *et al.*, 1995; Hassan, 1995]. It has been proposed that assembly of functional domains in the nucleus may involve nuclear matrix components such as lamins, Topo II and NuMA [Cleveland, 1995; Moir *et al.*, 1995; Poljak and Käs, 1995].

A.3 Proteins of the Nuclear Matrix

This study focuses on the distribution of three nuclear matrix proteins during apoptosis: the nucleoplasmic protein NuMA, and the nuclear envelope proteins lamin B and PI2. The following sections will review the structural and functional properties of these proteins.

A.3.1 NuMA

NuMA was first identified, by gel electrophoresis, as a high molecular weight non-histone nuclear protein present in human cells, and in human/hamster hybrid cells. Electrophoretic analysis of HeLa cells treated with DNase I and 2M NaCl showed that NuMA co-purified with the nuclear matrix. A mouse antiserum generated against partially purified HeLa cell NuMA immunoprecipitated a 300 kDa band from HeLa cell chromatin extracts, but not from Chinese hamster cell chromatin extracts. These results suggested that NuMA was a human-specific nuclear matrix protein [Lydersen and Pettijohn, 1980].

The same authors then investigated the subcellular localization of NuMA by immunofluorescence microscopy. In HeLa cells, NuMA showed a distinct cell cycle-dependent distribution. In the interphase nucleus, NuMA fluorescence was nucleoplasmic, non-nucleolar, and distributed in a diffuse pattern punctuated by brighter patches or spots. In mitotic cells, NuMA was detected at the polar regions of the spindle apparatus. Similar NuMA labelling patterns were observed in other human cell lines, and in a human/hamster hybrid cell line. However, NuMA fluorescence was not observed in cells from non-human origins, including *Drosophila*, mouse, rabbit, and Chinese hamster. These results

supported the conclusion that NuMA was a human-specific protein [Lyderson and Pettijohn, 1980]. However, subsequent immunolabelling and immunoblotting studies by the same group using different anti-NuMA antibodies detected NuMA as a 240 kDa protein in a variety of mammalian cell lines including mouse, rat, and Chinese hamster cell lines [Van Ness and Pettijohn, 1983; Price *et al.*, 1984; Price and Pettijohn, 1986].

A number of proteins with a subcellular distribution similar to NuMA were subsequently identified by immunofluorescence staining with autoimmune antibodies [Maekawa *et al.*, 1991] and with monoclonal antibodies generated against nuclear [Kallajoki *et al.*, 1991; Tang *et al.*, 1993] or chromosome extracts [Compton *et al.*, 1991; Tousson *et al.*, 1991]. Each antibody detected a protein with a molecular weight between 210-240 kDa on immunoblots [Compton *et al.*, 1991; Kallajoki *et al.*, 1991; Maekawa *et al.*, 1991; Tousson *et al.*, 1991; Tang *et al.*, 1993]. These proteins were named centrophilin [Tousson *et al.*, 1991], SPN [Kallajoki *et al.*, 1991], SP-H [Maekawa *et al.*, 1991], 1H1/1F1 [Compton *et al.*, 1991], and W1 [Tang *et al.*, 1993]. Recently, comparison of amino acid or nucleotide sequences has shown that all of the above proteins are NuMA [Compton *et al.*, 1992; Yang *et al.*, 1992; Kallajoki *et al.*, 1993; Maekawa and Kuriyama, 1993; Tang *et al.*, 1993].

The various studies provided similar descriptions of the distribution of NuMA during mitosis. During prophase, NuMA is localized between the condensing chromosomes. NuMA is first observed at the spindle poles during prometaphase, and remains there until telophase. During telophase, NuMA reassociates with the reforming nucleus [Price and Pettijohn, 1986; Compton *et al.*, 1992; Yang *et al.*, 1992; Tang *et al.*, 1993]. However,

the mechanism for this reassociation is unclear. In one study, rhodamine-conjugated wheat germ agglutinin (WGA) was microinjected into metaphase cells and the distribution of NuMA was monitored by immunofluorescence microscopy [Compton *et al.*, 1992]. In the subsequent interphase, NuMA was located in the cytoplasm of daughter cells [Compton *et al.*, 1992]. Since WGA impairs transport through nuclear pores, it was concluded that re-entry of NuMA into the nucleus requires nuclear pore function [Compton *et al.*, 1992]. However, immunofluorescence microscopy of mitotic cells double-labelled for lamin A/C and NuMA detected NuMA in association with telophase chromosomes before lamin A/C [Yang *et al.*, 1992]. These results suggested that NuMA relocates to the nucleus prior to nuclear envelope assembly [Yang *et al.*, 1992].

In contrast to the mitotic distribution of NuMA, the interphase distribution of NuMA varies somewhat with different anti-NuMA antibodies. NuMA labelling during interphase has most often been described as uniformly diffuse or punctate [Tousson *et al.*, 1991; Kallajoki *et al.*, 1991; Maekawa *et al.*, 1991; Compton *et al.*, 1992; Yang *et al.*, 1992; Tang *et al.*, 1993]. However, patches or spots of brighter NuMA labelling have been observed in addition to the diffuse labelling in some studies [Lydersen and Pettijohn, 1980; Zeng *et al.*, 1994b]. In one study, patches of NuMA labelling were reported to colocalize with snRNP-containing nuclear speckles [Zeng *et al.*, 1994b]. The different nuclear patterns may be due to recognition of different epitopes by various anti-NuMA antibodies, differences in fixation protocols, or recognition of different NuMA isoforms by the different antibodies [Compton *et al.*, 1992; He *et al.*, 1995].

NuMA is a phosphoprotein [Compton and Luo, 1995; Sparks *et al.*, 1995; Weaver *et*

al., 1996] that has been estimated to be present at approximately 2×10^5 copies per cell in K562 erythroleukemic cells [Compton et al., 1992]. According to the nucleotide sequence for the human NuMA gene, the predicted structure of NuMA consists of globular head and tail domains of 24 and 45 kDa separated by a discontinuous alpha-helical region of approximately 1500 amino acids containing hydrophobic heptad repeats [Compton et al., 1992; Yang et al., 1992; Maekawa and Kuriyama, 1993; Tang et al., 1993]. However, recent studies have suggested the existence of NuMA isoforms that differ in the length of the rod domain or the carboxyl domain [Tang et al., 1993; 1994]..

During sequence analysis of overlapping cDNA clones for NuMA, Tang et al. [1993] identified two sequence blocks of 75 and 42 bp that were absent from some of the clones. The 42 bp sequence codes for a 14 amino acid peptide, and was reported in the NuMA sequence from Yang et al. [1992], but not from Compton et al. [1992]. These results indicated the possible existence of multiple NuMA isoforms [Tang et al., 1993]. It has also been proposed that the multiple bands on immunoblots may represent different NuMA isoforms [Tang et al., 1993; Zeng et al., 1994a]. Doublet or triplet bands between 180-240 kDa on immunoblots probed with anti-NuMA antibodies have been observed for HeLa cells [Kalajoki et al., 1991; Tousson et al., 1991; Zeng et al., 1994a] and human erythroleukemic cells [Compton et al., 1992]. However, multiple bands for NuMA, observed on immunoblots, have alternatively been interpreted to represent degradation products of NuMA produced during sample preparation [Price and Pettijohn, 1986; Tousson et al., 1991].

In support of the existence of NuMA isoforms, three species of NuMA mRNA were

detected by PCR amplification of mRNA from a human glioma cell line (U-251) [Tang *et al.*, 1993]. Southern blot analysis of U-251 genomic DNA indicated that the three mRNAs are most likely generated from a single gene. The proteins encoded by the mRNAs differ mainly in the length of the carboxyl region and have been named NuMA-l (long), NuMA-m (medium) and NuMA-s (small). These proteins have 391, 52, and 39 amino acids in the carboxyl region, respectively [Tang *et al.*, 1993]. Human NuMA-l expressed in a hamster cell line showed the conventional NuMA distribution, located in the nucleoplasm during interphase and at the spindle poles during mitosis [Tang *et al.*, 1994]. NuMA-m and NuMA-s, both of which lack the 36 amino acid nuclear localization signal in the tail domain, were located in the cytosol throughout the cell cycle [Tang *et al.*, 1994]. It is currently unknown whether multiple NuMA isoforms are present in all cell types [He *et al.*, 1995].

Although the exact function of NuMA during mitosis is unknown, there is evidence that NuMA may participate in stabilizing microtubules of the mitotic spindle. Mitotic spindle organization was perturbed in HeLa cells microinjected with anti-NuMA antibodies during interphase and allowed to progress to mitosis [Kallajoki *et al.*, 1991; Yang and Snyder, 1992]. Instead of a bipolar distribution of spindle microtubules, a multipolar spindle was observed in these cells [Kallajoki *et al.*, 1991; Yang and Snyder, 1992]. Similarly, expression of a mutant form of NuMA that fails to target the mitotic spindle resulted in spindles with an aberrant morphology in hamster BHK cells [Compton and Luo, 1995]. Furthermore, immunodepletion of NuMA results in disorganization of taxol-induced microtubule asters in a mitotic extract of HeLa cells [Gaglio *et al.*, 1995] as

well as disorganization of mitotic spindles assembled in *Xenopus* egg extracts [Merdes et al., 1996].

NuMA may also be involved in post-mitotic nuclear reassembly. Microinjection of anti-NuMA antibodies into the cytoplasm of HeLa or PtK2 cells resulted in mitotic arrest or the formation of micronuclei in daughter cells [Kalajoki et al., 1991; 1993]. Similarly, micronuclei formed in BHK cells after transfection with human NuMA carrying deletions in the head or tail domains [Compton and Cleveland, 1993; Gueth-Hallonet et al., 1996] or after transfection with human NuMA carrying point mutations in predicted phosphorylation sites of the carboxyl terminus [Compton and Luo, 1995]. In cells transfected with the entire human NuMA gene, on the other hand, single nuclei formed after mitosis [Compton and Cleveland, 1993; Gueth-Hallonet et al., 1996].

The formation of micronuclei has also been observed, at the restrictive temperature, in a temperature-sensitive hamster cell line, tsBN2, which was derived from the BHK-21 cell line [Nishitani et al., 1991]. These cells enter prematurely into mitosis, fail to segregate chromosomes at the end of mitosis, and form micronuclei in the subsequent interphase [Nishitani et al., 1991]. In tsBN2 cells synchronized at the G1/S boundary, then shifted to the restrictive temperature, endogenous NuMA was not imported into the micronuclei but was apparently degraded in the cytoplasm [Compton and Cleveland, 1993]. Lamins and hnRNP, on the other hand, were observed in the micronuclei by immunofluorescence microscopy [Compton and Cleveland, 1993]. Furthermore, over expression of wild type human NuMA in these cells prevented micronucleation, and the human NuMA was immunolocalized to the nucleus [Compton and Cleveland, 1993]. Neither the mechanism

by which NuMA prevents micronucleation in tsBN2 cells nor its function in normal cells is known. Moreover, the phenotype of tsBN2 cells has not yet been satisfactorily explained.

The tsBN2 cell line carries a point mutation in a gene encoding a chromatin-associated protein called RCC1 (Regulator of Chromosome Condensation). The precise role of RCC1 in the cell cycle is unknown, but RCC1 has been implicated in a number of cellular processes including RNA transcription and processing, DNA replication, nuclear transport, and cell cycle control. RCC1 acts as a guanine nucleotide exchange factor for Ran (Ras-related nuclear protein), a nuclear GTPase required for nucleocytoplasmic transport. It has been proposed that RCC1 functions in a signal transduction pathway through Ran and a GAP (GTPase-activator protein), that inhibits the production of mitosis-inducing factors until DNA replication is complete. Alternatively, it has been proposed that RCC1 may function in maintaining chromatin structure in interphase [Dasso, 1993; Moore and Blobel, 1994].

In the initial studies of NuMA, electrophoretic analysis showed that NuMA was enriched in HeLa cell fractions prepared from isolated nuclei treated with DNase I and 2M NaCl [Lydersen and Pettijohn, 1980]. These results suggested an association of NuMA with the nuclear matrix of HeLa cells [Lydersen and Pettijohn, 1980]. NuMA has also been detected in nuclear matrix fractions of a human cervical carcinoma cell line (CaSki) [Zeng *et al.*, 1994a] and rat thymocytes [Weaver *et al.*, 1996] by immunoblotting with anti-NuMA antibodies. Immunofluorescence microscopy has been used to examine NuMA distribution during *in situ* nuclear matrix preparation from SW13 [Kallajoki *et al.*, 1991] and CaSki [Zeng *et al.*, 1994a; 1994b] human carcinoma cell lines. In nuclei

extracted with detergents, DNase, and 2 M salt [Kallajoki *et al.*, 1991] or DNase, ammonium sulphate, and 2 M salt [Zeng *et al.*, 1994a; 1994b], DNA was not detected but NuMA fluorescence was similar in intensity to unextracted nuclei. Furthermore, immunogold electron microscopy has shown that NuMA is a component of a subset of nuclear matrix core filaments [Zeng *et al.*, 1994a].

The function of NuMA in the interphase nucleus is still unclear, but the association of NuMA with the nuclear matrix is suggestive of a structural role [Lydersen and Pettijohn, 1980; Kallajoki *et al.*, 1991; Zeng *et al.*, 1994a, 1994b]. NuMA binds MARs/SARs *in vitro* and may, therefore, participate directly in the formation of chromatin loops [Ludérus *et al.*, 1994]. As in intermediate filament proteins, the predicted structure of the rod domain of NuMA contains segments of heptad periodicity with hydrophobic amino acids in positions a and d of the heptad [Compton *et al.*, 1992; Yang *et al.*, 1992]. The hydrophobic amino acids may form a spine capable of interacting with the spine of another NuMA helix to form a coiled-coil dimer [Compton *et al.*, 1992; Yang *et al.*, 1992]. The structure of the rod domain has led to the proposal that NuMA may oligomerize to form nuclear filaments [Compton *et al.*, 1992; Yang *et al.*, 1992; Zeng *et al.*, 1994a; Cleveland, 1995]. In agreement with this proposal, recombinant NuMA *in vitro* formed coiled-coiled dimers [Harborth *et al.*, 1995]. The dimers did not further oligomerize *in vitro*, however, and the authors concluded that a lack of filament formation *in vitro* may be the result of experimental conditions [Harborth *et al.*, 1995]. Recently, the formation of a network of cytoplasmic NuMA filaments *in vivo* has been demonstrated by transient expression of human NuMA lacking a nuclear localization signal in BHK cells [Tang *et al.*, 1994; Saredi

et al., 1996]. These results, in combination with the immunolocalization of NuMA to nuclear matrix core filaments [Zeng et al., 1994a], support the hypothesis that NuMA is a component of nuclear matrix filaments.

A.3.2 Lamins

The lamins are a family of polypeptides that are the main components of the nuclear lamina, a filamentous layer underlying the nuclear envelope of interphase cells. The nuclear lamina of isolated *Xenopus* oocyte nuclear envelopes has been observed as a meshwork of 10 nm filaments when examined by electron microscopy using freeze-dried/metal shadowing and negative staining [Aebi et al., 1986]. During mitosis, when the nuclear envelope breaks down, lamins are depolymerized in a phosphorylation-dependent manner. Lamins A and C become solubilized, while lamin B associates with nuclear membrane vesicles. Lamins are then dephosphorylated as the nuclear envelope reassembles after mitosis [Gerace and Blobel, 1980; reviewed in Moir et al., 1995].

Vertebrate lamins range in molecular weight from 60-80 kDa, and have been grouped into two families, the neutral A-type lamins and the acidic B-type lamins. The A-type lamins, lamins A and C, are transcribed from the same gene but differ in the C-terminal region due to differential mRNA splicing. In contrast, lamin B is transcribed from a distinct gene. In vertebrate cells, lamin B isotypes have been identified that are closely related in sequence, but are encoded by separate genes. For example, two somatic lamin B isotypes, lamins B₁ and B₂, have been found in mouse and human cells [reviewed in Moir et al., 1995].

cDNA analysis has shown that lamins are members of the intermediate filament family of proteins. Lamins have an α -helical central rod domain of approximately 350 amino acids flanked by N- and C- terminal globular domains. The N-terminal domain consists of about 30-40 amino acids and contains a phosphorylation site for p34^{cdc2} kinase. The C-terminal domain is about 210-300 amino acids long and contains sequences not found in intermediate filament proteins, including a nuclear localization signal and a number of phosphorylation sites. In addition, A- and B-type lamins contain a CaaX box for isoprenylation and carboxymethylation, the posttranslational modifications believed to be required for targeting of lamins to the nuclear envelope [reviewed in Moir *et al.*, 1995].

All vertebrate cells contain B-type lamins, but expression of A-type lamins is developmentally regulated [reviewed in Moir *et al.*, 1995]. For example, in resting mouse lymphocytes from peripheral blood, bone marrow, thymus or spleen, lamins A/C were not detected by immunoblotting or immunofluorescence microscopy [Röber *et al.*, 1990]. Lamin B, on the other hand was detected in cells from all of the lymphocyte sources [Röber *et al.*, 1990]. Similarly, two-dimensional gel electrophoresis detected lamin B in nuclear matrices from resting and stimulated mouse splenocytes and bovine lymphocytes [Setterfield *et al.*, 1985; Bladon *et al.*, 1988]. Lamins A/C, on the other hand, were only detected in matrices prepared from stimulated cells [Setterfield *et al.*, 1985; Bladon *et al.*, 1988].

The lamins have been identified as components of nuclear matrices isolated from a wide range of cells from lower eucaryotes to humans [reviewed in Berezney *et al.*, 1995]. Two-dimensional gel electrophoresis and immunoblotting of mouse nuclear matrix

fractions has identified lamins B as a "minimal matrix" protein, common to mouse cells in different stages of differentiation [Stuurman *et al.*, 1990]. *In situ* nuclear matrix preparation of mouse 3T3 fibroblasts has also shown that lamins are resistant to extraction during nuclear matrix preparation [Chaly *et al.*, 1985].

The function of the nuclear lamina is not fully understood. The lamina is believed, however, to provide structural support for the nuclear membrane and to participate in post-mitotic nuclear reassembly [reviewed in Moir *et al.*, 1995]. *In vivo* nuclear assembly studies using *Xenopus* oocyte extracts immunodepleted of lamins are in agreement with this proposal [Newport *et al.*, 1990]. Upon immunodepletion of the major lamin present in these extracts, lamin B₃, nuclei formed around exogenous DNA, but the nuclei were fragile [Newport *et al.*, 1990]. Lamins may also function in the reformation of the nuclear envelope at the end of mitosis, although this function for lamins is still controversial [Lourim and Khron, 1994]. Newport *et al.* [1990] reported that nuclear envelopes formed in *Xenopus* egg extracts upon immunodepletion of lamins, suggesting that lamins are not involved in nuclear envelope assembly. However, using the same system Daubauvalle *et al.* [1991] reported that addition of antibodies against lamins B₃ and B₂ prevented the formation of nuclear envelopes. Furthermore, electron microscopic analysis showed that nuclear envelope formation around exogenous chromatin was inhibited upon addition of anti-lamin antibodies to a mammalian cell free system [Burke and Gerace, 1986], or to *Drosophila* embryonic extracts [Ulitzer *et al.*, 1992]. These results suggest that lamins are necessary for assembly of the nuclear envelope at the end of mitosis. The contradiction in these results is most likely due to incomplete depletion of lamins with

anti-lamin B₃ by Newport *et al.*, [1990] since it is now known that *Xenopus* oocytes also contain lamin B₂ [Lourim and Khroné, 1994].

In the interphase nucleus, lamins are believed to anchor chromatin at the nuclear periphery. In a mammalian cell free system, coating of chromosomes with lamins A/C preceded nuclear envelope formation, and addition of anti-lamin antibodies prevented both nuclear envelope formation and chromosome decondensation [Burke and Gerace, 1986]. Similarly, chromosome decondensation was inhibited upon injection of mitotic PtK2 cells with anti-lamin antibodies [Benavente and Khroné, 1986]. Recently, the relative distribution of lamin B and chromatin in CHO cells has been examined by electron microscopy of immunogold-labelled thick sections using three dimensional reconstruction [Belmont *et al.*, 1993]. This study has demonstrated a close association of chromatin with thick regions of the lamin B immunogold labelling at the nuclear periphery, consistent with a possible interaction between lamins and chromatin [Belmont *et al.*, 1993].

A number of studies have also demonstrated an association of lamins with chromatin *in vitro*. Bacterially expressed *Drosophila* lamins were observed by immunofluorescence microscopy to coat sperm chromatin in *Drosophila* embryonic extracts [Ulitzer *et al.*, 1992]. *In vitro* studies have shown that synthetic and bacterially expressed lamins bind chromatin [Höger *et al.*, 1991; Glass *et al.*, 1993], and that lamin B₁ binds specifically to MARs/SARs [Ludérus *et al.*, 1992].

In addition, recent immunofluorescence and electron microscopy studies have identified lamin foci in the nuclear interior [Bridger *et al.*, 1993; Moir *et al.*, 1994; Hozák *et al.*, 1995; Sasseville and Raymond, 1995]. Immunogold electron microscopy of

resinless thin sections identified lamin A at nodes in the internal nucleoskeleton of HeLa cells [Hozák *et al.*, 1995]. Lamin foci in the interior of G1 nuclei from human dermal fibroblasts appeared to be associated with propidium iodide-labelled chromatin by confocal microscopy [Bridger *et al.*, 1993]. In addition, confocal microscopy of mouse 3T3 cells labelled with anti-lamin B and anti-BrDU has shown that lamin B foci colocalize with sites of BrDU incorporation in mid-late S phase cells [Moir *et al.*, 1994]. Although the function of lamin foci has not been clearly determined, the association of lamin B foci with newly replicated DNA in S phase suggests that lamins may participate in chromatin organization during replication, possibly providing a scaffold upon which nuclear functions such as replication are organized [Moir *et al.*, 1994; Moir *et al.*, 1995].

A.3.3 PI2

The PI2 antigen was first identified by immunoblotting with a monoclonal antibody against mouse splenic lymphocyte nuclear matrix [Chaly *et al.*, 1984]. The antibody labelled three bands at 35 kDa, 70 kDa and 140 kDa [Chaly *et al.*, 1984]. During interphase, antibody PI2 labelled the nuclear periphery of mouse 3T3 fibroblasts in a pattern similar to that of lamins by immunofluorescence microscopy [Chaly *et al.*, 1984]. However, it localizes to the cytoplasmic face of the nuclear envelope by immunoelectron microscopy [Chaly *et al.*, 1989]. Like lamins, peripheral PI2 labelling dispersed and reappeared in parallel with the nuclear envelope during mitosis [Chaly *et al.*, 1984]. Peripheral nuclear labelling for PI2 has also been observed in a wide variety of animal cells in interphase including human, mouse and hamster cell lines [Chaly *et al.*, 1986], and

human, bovine, murine, and rat lymphocytes [Chaly *et al.*, 1986; Weaver *et al.*, 1996], indicating that this antigen is conserved among the animal kingdom. In human and mouse cell lines, but not in mouse splenocytes, antibody PI2 also labels the nucleoplasm in a speckled pattern [Chaly *et al.*, 1984; Chaly *et al.*, 1985]. The peripheral component of PI2 was resistant to extraction during nuclear matrix preparation of mouse 3T3 fibroblasts [Chaly *et al.*, 1985]. The nucleoplasmic PI2 labelling, on the other hand, was extracted during nuclear matrix preparation [Chaly *et al.*, 1985]. PI2 does not redistribute during stimulation of lymphocytes, or during treatment of stimulated lymphocytes with a reversible transcriptional inhibitor, suggesting that PI2 is not involved in metabolic processes [Chaly *et al.*, 1988]. Taken together, these results suggest that PI2 may be a structural component of the nuclear envelope.

B. Organization of the Nucleus During Apoptosis

Apoptosis is defined by characteristic changes in cellular morphology and biochemistry, many of which involve the nucleus. I will begin this section of the literature review with a brief introduction to cell death by apoptosis. I will then focus on the nuclear events in apoptosis, including changes in nuclear morphology, and degradation of DNA and nuclear proteins.

B.1 Apoptosis

Apoptosis was first described, in rat liver cells, as a death process with morphological features distinguishable from those associated with accidental death, or necrosis [Kerr *et*

al., 1972]. Necrosis involves swelling of the cell and subsequent membrane rupture.

During apoptosis, on the other hand, cells shrink while membranes and organelles appear intact. Surface protuberances appear on apoptotic cells which are eventually released as membrane bound bodies [Kerr et al., 1972; Wyllie et al., 1980]. The nucleus also undergoes striking changes in morphology that will be described in section B.3.

In vitro, apoptotic cells eventually disintegrate, a process called "secondary necrosis". However, apoptotic cells are phagocytosed *in vivo*, without release of cytoplasmic contents into the intracellular space. Apoptosis, therefore, does not elicit an inflammatory response *in vivo*. Necrosis, on the other hand, involves the rupture of membranes, typically affects groups of cells, and is normally accompanied by an inflammatory response *in vivo* [Wyllie et al., 1980; Earnshaw, 1995].

Apoptosis occurs in two physiological stages. During the condemned phase, cells are committed to apoptosis, but exhibit no morphological changes. The condemned phase varies in length depending on the apoptotic stimulus. Furthermore, the length of the condemned phase varies within the same population of cells so that apoptosis occurs asynchronously. The execution stage is rapid and includes all of the morphological changes associated with apoptosis [Earnshaw, 1995].

Unlike necrosis, which is a passive process, apoptosis can be accompanied by *de novo* protein synthesis [Patterson et al., 1995] and, in some cases, may require synthesis of macromolecules [Wyllie et al., 1984; Walker et al., 1991; Weaver et al., 1993]. Gene products such as p53 and Bcl-2 have been shown to modulate the process of apoptosis [reviewed in Williams and Smith, 1993]. However, the effect of these proteins appears to

be cell type- and stimulus-specific, implying that apoptosis can follow a number of pathways to the condemned phase. For example, expression of p53 is required for cell death of lymphocytes induced by DNA damage, but causes growth arrest and DNA repair in other cell types. Glucocorticoid induced apoptosis in thymocytes, on the other hand, occurs independently of p53 expression. The proto-oncogene product Bcl-2 suppresses apoptosis under some conditions, such as radiation-induced death of lymphoid cells, but not others, including negative selection of T cells. Although it appears that different pathways can lead to the condemned phase of apoptosis, the execution phase apparently involves a highly conserved series of changes in cell structure and biochemistry. These changes include destruction of nuclear organization, DNA degradation and proteolysis of nuclear proteins [Veis *et al.*, 1993; Williams and Smith, 1993; Earnshaw, 1995].

B.3 Nuclear Morphology during Apoptosis

The nucleus undergoes striking changes in appearance during apoptosis. One of the most dramatic changes is the loss of chromatin organization. During apoptosis, chromatin marginates into smooth masses that abut the nuclear periphery [Wyllie *et al.*, 1980]. The chromatin in apoptotic nuclei appears compact and uniformly electron dense by electron microscopy [Wyllie *et al.*, 1980]. The chromatin then takes on one of a number of characteristic morphologies. Chromatin may fragment into a number of membrane bound bodies. Alternatively, all of the chromatin collapses into a single ball or crescent shape at the periphery of the nucleus [Wyllie *et al.*, 1980; Earnshaw, 1995].

Changes in chromatin structure during apoptosis are accompanied by morphological

changes in the nuclear envelope and nucleoplasm. At the electron microscope level, the nuclear membrane often appears intact in apoptotic cells, but nuclear pores change distribution and accumulate between masses of collapsed chromatin [Wyllie *et al.*, 1980; Falcieri *et al.*, 1994; Oberhammer *et al.*, 1994; Weaver *et al.*, 1996]. Nucleoli become enlarged and segregate [Wyllie *et al.*, 1980; Arends *et al.*, 1990].

The nuclear matrix of apoptotic thymocytes has been reported to retain a recognizable pore complex-lamina and fine internal fibres, suggesting that the nuclear matrix remains largely intact during apoptosis [Arends *et al.*, 1990]. However, other studies at the electron microscope level have indicated changes in the organization of the nucleoplasm during apoptosis. Weaver *et al.* [1996] have reported that the nucleoplasm of apoptotic thymocytes became amorphous and disorganized, with few visible granules and filaments. Recently, Zweyer *et al.* [1997] have observed fibrogranular nuclear bodies late in apoptosis of HL60 cells. The bodies contain the nuclear matrix proteins NuMA, SATB1, proliferating cell nuclear antigen (PCNA), a 125 kDa protein, and a 160 kDa protein. The authors have suggested that nuclear matrix proteins aggregate in these bodies during apoptosis.

Immunofluorescence microscopy studies on whole cells, or on isolated nuclei in apoptotic extracts, have shown a redistribution and/or loss of specific nuclear matrix antigens during apoptosis. Disassembly of the nuclear lamina during apoptosis has been suggested by a reduction or loss of immunolabelling for lamins [Lazebnik *et al.*, 1993; Oberhammer *et al.*, 1994; Tinnemans *et al.*, 1995; Rao *et al.*, 1996; Weaver *et al.*, 1996]. In agreement with electron microscopy studies, immunofluorescence labelling of the

nuclear membrane with antibody PI2 [Weaver *et al.*, 1996] and fluorescence labelling with the lipophilic dye 3,3'-dihexyloxacarbocyanin (DHCC) [Lazebnik *et al.*, 1993] showed that the nuclear membrane appears intact in apoptotic cells. Nucleoplasmic antigens such as snRNPs and NuMA have been reported to concentrate in the nucleoplasm next to masses of condensed chromatin and/or decrease in fluorescence labelling intensity [Roy *et al.*, 1992; Miller *et al.*, 1993; Casciola-Rosen *et al.*, 1994a; Tinnemans *et al.*, 1995; Weaver *et al.*, 1996].

B.4 DNA Degradation during Apoptosis

Biochemically, apoptosis was originally defined by the production of DNA fragments, multiples of 200 bp in size, that appeared on agarose gels as a "DNA ladder" [Wyllie, 1980]. These fragments were interpreted to be the result of activation of an endonuclease which digests chromatin in the linker regions between nucleosomes [Wyllie, 1980]. More recently, pulse field gel electrophoresis has demonstrated the formation of 200-300 kbp and 30-50 kbp DNA fragments during apoptosis [Walker *et al.*, 1991; Roy *et al.*, 1992; Brown *et al.*, 1993; Oberhammer *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994]. The production of large DNA fragments has been shown to precede internucleosomal DNA fragmentation during apoptosis. In these experiments, large DNA fragments were formed in the absence of oligonucleosomal DNA fragments upon treatment of thymocytes with Zn^{2+} , protease inhibitors, or Mg^{2+} in the absence of Ca^{2+} [Brown *et al.*, 1993; Oberhammer *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994]. The sequential

degradation of DNA during apoptosis is thought to reflect the higher order folding of chromatin in the nucleus, and to result from release of loops (50 kb) and rosettes (300 kb) of DNA [Filipski *et al.*, 1990; Walker *et al.*, 1991; Cohen *et al.*, 1992; Roy *et al.*, 1992; Brown *et al.*, 1993; Oberhammer *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994].

There is evidence that different nuclease activities are involved in the production of high and low molecular weight fragments of DNA. Studies of thymocyte apoptosis have shown that oligonucleosomal DNA fragmentation requires Ca^{2+} and Mg^{2+} ions, and is inhibited by Zn^{2+} or serine proteases inhibitors [Brown *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994]. Cleavage of DNA into large fragments, on the other hand, requires Mg^{2+} ions and is not inhibited by Zn^{2+} or serine protease inhibitors [Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994]. The identity of the nuclease responsible for production of large DNA fragments is unknown, but it is possible that some of these fragments may be due to Topo II cleavage [Walker *et al.*, 1991; Roy *et al.*, 1992; Oberhammer *et al.*, 1993; Sun and Cohen, 1994]. The identity of the nuclease involved in oligonucleosomal DNA fragmentation is also unknown, although a number of nucleases have been suggested including DNase I, DNase II, NUC18, and endo-exonuclease [reviewed in Peitsch *et al.*, 1994; Fraser *et al.*, 1996].

Agarose gel electrophoresis, and more recently pulse field gel electrophoresis, have provided biochemical approaches for the study of apoptotic DNA fragmentation in populations of cells. Flow cytometry provides another approach to study apoptotic

populations, and is based on the extraction of low molecular weight DNA from ethanol-permeabilized cells during sample preparation [Darzynkiewicz *et al.*, 1994; Sgonc and Wick, 1994]. Apoptotic cells exhibit a reduced DNA content and produce a "subdiploid" peak upon staining with fluorescent DNA dyes [Darzynkiewicz *et al.*, 1994; Sgonc and Wick, 1994]. Recently, another method to detect DNA fragmentation was developed that allows for the examination of DNA fragmentation *in situ* [Gavrieli *et al.*, 1992]. Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick end labelling (TUNEL) exploits the ability of the enzyme terminal transferase to add biotin-labelled nucleotides to broken ends of DNA [Gavrieli *et al.*, 1992]. DNA strand breaks can then be visualized using streptavidin tagged with fluorescent compounds or with enzymes [Gavrieli *et al.*, 1992]. Other similar methods use different enzymes such as DNA polymerase I to detect DNA fragmentation [Falcieri *et al.*, 1994]. Unlike the biochemical approaches, these methods allow for direct comparison of cellular morphology and DNA fragmentation in individual cells.

Recently, confocal microscopy of thymocytes labelled by *in situ* nick translation [Falcieri *et al.*, 1994] or TUNEL [Weaver *et al.*, 1996] has shown that DNA cleavage is initiated before collapse of chromatin during apoptosis. However, the relationship between DNA fragmentation and chromatin collapse is still unclear. In some studies, the appearance of an apoptotic chromatin morphology has been related to the appearance of oligonucleosomal DNA fragments [Arends *et al.*, 1990; Lazebnik *et al.*, 1993]. However, in other studies, chromatin collapse has been reported in the absence of internucleosomal DNA fragmentation [Oberhammer *et al.*, 1993]. Furthermore, analysis of DNA

fragmentation by pulse field gel electrophoresis, which allows both large and small DNA fragments to be resolved on the same gel, has suggested that oligonucleosomal DNA fragments comprise only a small fraction of the fragmented DNA [Walker *et al.*, 1994; Weaver *et al.*, 1996].

There is evidence that chromatin collapse towards the nuclear periphery involves DNA digestion to large fragments [Weaver *et al.*, 1993; Cohen *et al.*, 1994; Walker *et al.*, 1994]. In thymocytes treated with glucocorticoids or Topo II inhibitors in the presence of protease inhibitors or Zn^{2+} , the production of large DNA fragments correlates with partial condensation of chromatin towards the margin of the nucleus [Weaver *et al.*, 1993; Sun *et al.*, 1994; Walker *et al.*, 1994]. Thymocytes with a similar partial collapse of chromatin have been isolated from glucocorticoid- or etoposide-treated populations, exhibit a size and density intermediate between normal and apoptotic cells, and contain large DNA fragments only [Cohen *et al.*, 1994]. During apoptosis in HL60 cells, protease inhibitors did not prevent the onset of chromatin collapse, but prevented oligonucleosomal DNA cleavage and nuclear fragmentation [Hara *et al.*, 1996]. These results indicate that initial collapse of chromatin is a result of the formation of large DNA fragments [Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun *et al.*, 1994; Walker *et al.*, 1994]. It has been proposed further that the conformational changes in chromatin upon formation of large DNA fragments allow access to chromatin for subsequent internucleosomal DNA cleavage [Cohen *et al.*, 1994; Sun *et al.*, 1994].

B.5 Protein Degradation during Apoptosis

Early studies on *Caenorhabditis elegans* indicated that proteolysis plays an important role in apoptosis. It was found that mutations in *ced-3*, a gene coding for a protein that resembles the cysteine protease, interleukin-1 β converting enzyme (ICE), resulted in survival of cells that would normally die during development. ICE homologues such as CPP32 or YAMA, ICE_{ind}II, and ICE_{ind}III have been implicated in proteolysis during apoptosis. However, to date, every class of protease has been shown to be involved in apoptosis including serine proteases, caspases, granzymes, and calpains. Furthermore, the involvement of ubiquitin-mediated proteolysis has recently been suggested for radiation- and adenovirus E1A-induced apoptosis [Delic *et al.*, 1993; Nakajima *et al.*, 1996; reviewed in Patel *et al.*, 1996].

Degradation of specific nuclear proteins into discrete reproducible fragments has been reported in different cell types, and with a variety of apoptotic stimuli, suggesting that site-specific proteolysis of nuclear proteins is a characteristic feature of apoptosis [Casciola-Rosen *et al.*, 1994b; Oberhammer *et al.*, 1994; Casciola-Rosen *et al.*, 1995; Lazebnik *et al.*, 1995a; 1995b; Tewari *et al.*, 1995; Casiano *et al.*, 1996; Hsu and Yeh, 1996; Rao *et al.*, 1996; Weaver *et al.*, 1996; Zweyer *et al.*, 1997]. The proteolytic fragments produced during apoptosis include: a 45 kDa fragment for lamins [Oberhammer *et al.*, 1994; Lazebnik *et al.*, 1995b; Casiano *et al.*, 1996; Rao *et al.*, 1996; Weaver *et al.*, 1996]; a 70 kDa fragment for Topo I [Casciola-Rosen *et al.*, 1995; Casiano *et al.*, 1996]; several low molecular weight fragments for Topo II [Casiano *et al.*, 1996]; a 40 kDa fragment for U1-70 kDa snRNP [Casciola-Rosen *et al.*, 1994b; Casiano *et al.*, 1996]; an

85 kDa fragment for poly (ADP-ribose) polymerase (PARP) [Casciola-Rosen *et al.*, 1995; Lazebnik *et al.*, 1995a; Tewari *et al.*, 1995]; and a 200 kDa fragment [Weaver *et al.*, 1996; Zweyer *et al.*, 1997] or 180 kDa fragment for NuMA [Casiano *et al.*, 1996; Hsu and Yeh, 1996]. In contrast, some other nuclear proteins have been shown to remain intact during apoptosis, including the nuclear envelope antigen PI2 [Weaver *et al.*, 1996], and DNA-associated proteins RCC1 [Lazebnik *et al.*, 1995a], PCNA [Casiano *et al.*, 1996; Zweyer *et al.*, 1997], CENP A, CENP B, CENP C, histone H2B, and Ku [Casiano *et al.*, 1996]. These results support the hypothesis that apoptosis is accompanied by site-specific proteolysis of key proteins. The signals involved in targeting nuclear proteins for proteolysis are unknown, but the phosphorylation of NuMA during thymocyte apoptosis suggests that post-translational protein modifications, like phosphorylation, may be involved [Weaver *et al.*, 1996]. It has been proposed that site-specific proteolysis of major structural proteins may expose DNA to attack by endonucleases, leading to DNA degradation and nuclear collapse [Weaver *et al.*, 1996]. In agreement with this hypothesis, DNA degradation and chromatin collapse were inhibited in rodent cells expressing mutant lamins A or B that are not cleaved during apoptosis [Rao *et al.*, 1996].

C. The Lymphocyte Cell System

C.1 Morphology

Unstimulated lymphocytes are small cells (5-8 μ m in diameter) that contain roughly spherical nuclei surrounded by a thin layer of cytoplasm with few organelles [Setterfield *et*

al., 1983; Daev et al., 1994]. Electron microscopic autoradiography has shown that resting lymphocytes are metabolically inactive, exhibiting very little protein synthesis and no DNA synthesis [Setterfield et al., 1983; Brown et al., 1989]. The nuclei of unstimulated cells contain large aggregates of condensed chromatin adjacent to the nuclear envelope and extending into the nuclear interior. Morphometric analysis of resting lymphocytes examined by electron microscopy indicated that condensed chromatin occupies approximately half of the volume of resting lymphocytes. The small interchromatinic regions in these cells have been visualized by regressive EDTA staining and contain fibrils and some granules [Setterfield et al., 1983].

Activation of resting lymphocytes with mitogens such as concanavalin A (Con A) results in a greater than 5 fold increase in nuclear volume and a nearly 10-fold increase in the volume of interchromatinic material [Setterfield et al., 1983]. Upon stimulation, the interchromatinic material visualized by regressive EDTA staining showed granular material and a more extensive network of fine fibrillar material. The volume of condensed chromatin changes little during stimulation but chromatin redistributes into smaller aggregates dispersed throughout the nucleus [Setterfield et al., 1983]. Nuclear restructuring during lymphocyte activation coincides with increased polypeptide composition [Setterfield et al., 1985; Bladon et al., 1988]. Individual nuclear matrix antigens, such as snRNP antigens, fibrillarin, Topo II, and P11 have been shown to redistribute and/or increase in quantity in response to lymphocyte stimulation [Setterfield et al., 1985; Chaly et al., 1988; Davis et al., 1993; Chaly et al., 1996]. It has been suggested that the morphological changes occurring in stimulated lymphocyte nuclei are

related to the assembly of transcription and RNA processing domains [Setterfield *et al.*, 1983; Chaly *et al.*, 1988; Davis *et al.*, 1993]. This hypothesis is based on the dependence of these changes on protein and RNA synthesis, but not on DNA replication [Setterfield *et al.*, 1983; Chaly *et al.*, 1988; Davis *et al.*, 1993].

C.2 Apoptosis in Lymphocytes

Apoptosis is thought to play a central role in the immune system, both in physiological control of T and B cell levels and in the prevention of autoimmune responses [reviewed in Cohen, 1995; Abbas, 1996]. Furthermore, increased apoptosis of CD4⁺ lymphocytes in HIV and other viral infections suggests that apoptosis may be involved in pathological immune responses [reviewed in Cohen, 1995].

A variety of stimuli have been reported to induce apoptosis of lymphocytes *in vitro* including treatment with glucocorticoids and chemotherapeutic drugs [e.g. Walker *et al.*, 1991; Roy *et al.*, 1992; Weaver *et al.*, 1993; Daev *et al.*, 1994], and radiation [Sellins and Cohen, 1991; Delic *et al.*, 1993; Cregan *et al.*, 1994]. However, lymphocytes also die in culture in the absence of an exogenous apoptotic stimulus. Spontaneous apoptosis of 20-50% of the cell population by 16 hours of culture has been reported for cells isolated from mouse spleen and lymph nodes [Illera *et al.*, 1993; Perandones *et al.*, 1993; Reap *et al.*, 1995; Zhang *et al.*, 1995], mouse thymus [Migliorati *et al.*, 1992; Clarke *et al.*, 1993], rat spleen and thymus [Rinner *et al.*, 1996] and human thymus [Mentz *et al.*, 1995]. Human T cells [Giordano *et al.*, 1995; Mentz *et al.*, 1995] and B cells [Lomo *et al.*, 1995] isolated from peripheral blood, on the other hand, show much lower levels of apoptosis.

Death of less than 10% of the cells has been reported to occur by 48 hours in culture [Giordano *et al.*, 1995; Mentz *et al.*, 1995; Lomo *et al.*, 1995].

There is evidence that apoptosis of lymphocytes can follow multiple pathways that differ in dependence on the expression of certain genes, and on synthesis of macromolecules in general. In mouse thymocytes, p53 expression is required for apoptosis induced by agents that damage DNA, such as radiation, but is not required for apoptosis induced by other agents such as glucocorticoids [Clarke *et al.*, 1993; Lowe *et al.*, 1993]. Bcl-2 expression in immature thymocytes prevents apoptosis induced by glucocorticoids and radiation, but does not prevent negative selection of T cells [Sentman *et al.*, 1991]. In rat thymocytes, protein synthesis is required for apoptosis induced by glucocorticoids and inhibitors of Topo II [Wyllie *et al.*, 1984; Walker *et al.*, 1991; Weaver *et al.*, 1993; Sun *et al.*, 1994; Rinner *et al.*, 1996]. However, inhibition of protein synthesis had no effect on spontaneous apoptosis of rat thymocytes as detected by gel electrophoresis or flow cytometry [Sellins and Cohen, 1991; Weaver *et al.*, 1993; Rinner *et al.*, 1996]. There are also reports that heat-induced apoptosis of mouse thymocytes is independent of protein synthesis [Sellins and Cohen, 1991; Migliorati *et al.*, 1992].

Furthermore, lymphocyte subsets may respond differently to a given apoptotic stimulus. By labelling for cell surface markers and DNA, and analyzing samples by flow cytometry, Reap *et al.* [1995] determined that spontaneous apoptosis in total mouse splenocyte populations affected 22% of the B cell population but only 8.2% of the T cell population [Reap *et al.*, 1995]. Flow cytometric analysis of lymphocytes double-labelled for cell surface markers and DNA strand breaks has also shown that CD4⁺ cells are more

resistant to irradiation *in vivo* than are CD8+ or CD4-CD8- cells [Zhang *et al.*, 1995].

D. Hyperthermia

D.1 Hyperthermia and apoptosis

Treatment with hyperthermia induces apoptosis in a variety of cell types, including murine [Sellins and Cohen, 1991; Migliorati *et al.*, 1992] and rat thymocytes [Sikora *et al.*, 1993], and a number of human, murine and rat cell lines [Harmon *et al.*, 1990; Takano *et al.*, 1991; Mosser and Martin, 1992; Li *et al.*, 1994; Li *et al.*, 1996; Yonezawa *et al.*, 1996]. Induction of apoptosis has been confirmed in these populations by detection of DNA fragmentation using gel electrophoresis [Sellins and Cohen, 1991; Takano *et al.*, 1991; Migliorati *et al.*, 1992; Mosser and Martin, 1992; Sikora *et al.*, 1993; Li *et al.*, 1996; Yonezawa *et al.*, 1996], and by variations on the TUNEL method [Gavrieli *et al.*, 1992; Yonezawa *et al.*, 1996]. DNA fragmentation has also been suggested by flow cytometry data showing a fractional DNA content of the cells [Migliorati *et al.*, 1992; Li *et al.*, 1994; Yonezawa *et al.*, 1996]. As well, morphological changes associated with apoptosis have been observed for heat-treated cells by light and electron microscopy [Takano *et al.*, 1991; Mosser and Martin, 1992; Li *et al.*, 1996; Yonezawa *et al.*, 1996].

The response of cells to hyperthermia is cell type-dependent. The differential response of cells to elevated temperatures has implications in the treatment of diseases such as cancer [Fuller *et al.*, 1994; Yonezawa *et al.*, 1996]. Takano *et al.* [1991] showed that the same heat treatment, 43°C for 30 min, resulted in a variable apoptotic response for two human Burkitt's lymphoma cell lines, and a murine mastocytoma cell line. Furthermore,

heating at 43°C did not affect the viability of rat fibroblasts [Sellins and Cohen, 1991; Yonezawa *et al.*, 1996] but resulted in apoptosis of rat thymocytes [Sellins and Cohen, 1991] and a malignant rat histiocytoma cell line [Yonezawa *et al.*, 1996].

The cell death response due to heat treatment is also dependent on the duration and severity of treatment. The rate of apoptotic cell death in human cells increases as a function of length of exposure to a given temperature [Mosser and Martin, 1992; Fuller *et al.*, 1994]. In general, heating cells at temperatures below 42°C does not affect cell viability [Ishiyama *et al.*, 1996; Yonezawa *et al.*, 1996]. On the other hand, hyperthermic treatment of 42–43°C induces apoptosis in many cell types [Sellins and Cohen, 1991; Takano *et al.*, 1991; Migliorati *et al.*, 1992; Mosser and Martin, 1992; Sikora *et al.*, 1993; Li *et al.*, 1994; Yonezawa *et al.*, 1996]. However, slight increases in treatment temperature above these levels (e.g. from 43°C to 44°C for rat histiocytoma cells [Yonezawa *et al.*, 1996], or from 44°C to 46°C for murine mastocytoma cells [Harmon *et al.*, 1990]) can result in a shift from apoptotic to necrotic cell death.

D.2 Hyperthermia and Nuclear Organization

There have been a number of reports that heat induces changes in nuclear organization. Rod-like inclusion bodies of actin have been observed in rat fibroblast nuclei upon heating at 42–43°C for 3 hours [Welch and Suhan, 1985]. As well, heat-induced alterations in immunofluorescence labelling patterns have suggested that redistribution of many nuclear proteins occurs upon heat treatment. In HeLa cells, a 125 kDa nuclear matrix antigen lost its spotted organization upon heat treatment, and instead showed a diffuse distribution in

the nucleoplasm [Neri *et al.*, 1995]. Immunofluorescence and immunoelectron microscopy showed that diffuse labelling of a 160 kDa nuclear matrix antigen became clustered upon heat treatment of HeLa cells [DeGraaf *et al.*, 1992]. In heat-treated *Drosophila* embryo nuclei, anti-Topo II labelling changed from a diffuse nucleoplasmic pattern to a ring around nucleoli [McConnell *et al.*, 1987]. The speckled distribution of anti-Sm labelled snRNPs in HeLa cells appeared unchanged with a 42°C treatment [DeGraaf *et al.*, 1992], but became diffusely distributed with a 45°C treatment [Welch and Mizzen, 1988]. The significance of these heat-induced alterations in nuclear structure is unclear. However, as discussed below, the morphological changes observed after heat treatment may reflect a dynamic association of nuclear proteins with the nuclear matrix.

In contrast to internal nuclear matrix proteins, nuclear envelope antigens such as lamins and a *Drosophila* nuclear pore complex glycoprotein, gp188, showed no detectable change in distribution upon heat treatment when analyzed by immunofluorescence microscopy [McConnell *et al.*, 1987; DeGraaf *et al.*, 1992]. However, after heat treatment of *Drosophila* embryos, lamins were converted from a 76 KDa isoform to a 74 kDa isoform, apparently due to a dephosphorylation event [McConnell *et al.*, 1987; Smith *et al.*, 1987]. A similar dephosphorylation of lamins A and C was detected after heat treatment of Erlich ascites tumor cells [Krachmarov and Traub, 1993]. These results show that the absence of gross morphological changes in intact cells after heating does not necessarily indicate a lack of alterations at the biochemical level [McConnell *et al.*, 1987; Krachmarov and Traub, 1993].

D.3 Hyperthermia and Nuclear Matrix Composition

Heat treatment of isolated nuclei or of intact cells results in an increase in the amount of insoluble protein associated with the nucleus [Evan and Hancock, 1985; Littlewood *et al.*, 1987; Chu *et al.*, 1993; Wachsberger and Coss, 1993; Warters *et al.*, 1993].

Furthermore, electron microscopy of resinless thin sections has shown that changes in the architecture of nuclear matrices isolated after heat treatment correlate with this increased nuclear protein content [Wachsberger and Coss, 1993]. The nuclear matrices of heat-treated cells showed a more extensive network of fibres than in unstressed cells, and also showed an overall increase in electron density [Wachsberger and Coss, 1993].

Furthermore, heat-induced Topo II redistribution by immunofluorescence microscopy correlated with an increased association of the protein with the nuclear matrix fraction on immunoblots [McConnell *et al.*, 1987].

Autoradiography of fixed intact cells, and of nuclei isolated before fixation, have shown that the increase in protein content after heat treatment is not due to excessive import of protein from the cytoplasm [Chu *et al.*, 1993]. Immunoblotting of soluble, DNA-associated, and nuclear matrix fractions from CHO cells suggested that increased nuclear protein content after heat treatment is largely due to tighter association of nuclear proteins with the nuclear matrix and with DNA [Warters *et al.*, 1993]. Some of the nuclear proteins enriched in heated nuclei have been identified by immunoblot analysis, and include the enzymes Topo I, Topo II [McConnell *et al.*, 1987; VanderWaal *et al.*, 1996], and the oncoproteins p53 and p62c-myc [Littlewood *et al.*, 1987]. Histones, on the other hand were equally extractable from control and heated nuclei [Warters *et al.*,

1993].

The biological significance of increased protein content in heated nuclei is unknown. However, it has been proposed that adsorption of proteins onto nuclear DNA may affect cellular functions associated with the nuclear matrix, particularly DNA replication and transcription [Littlewood *et al.*, 1987; Wachsberger and Coss, 1993], and may contribute to heat-induced cytotoxicity [Littlewood *et al.*, 1987; Kampinga *et al.*, 1989]. Heat shock proteins (HSP) may be involved in prevention of or recovery from heat-induced nuclear protein aggregation and in cellular survival after heat treatment [Littlewood *et al.*, 1987; Kampinga *et al.*, 1989; Stege *et al.*, 1994].

This literature review has described the current model of nuclear organization, in which chromatin loops are anchored at the nuclear periphery and in the nucleoplasm by interaction with the nuclear matrix. During apoptosis, there are major changes in nuclear organization, including degradation of DNA, chromatin aggregation and collapse, and redistribution and/or loss of nuclear proteins. This study was originally intended to test the hypothesis that disassembly of the nuclear matrix and chromatin during apoptosis would be spatially and temporally ordered, reflecting the original organization of the nucleus. I have tested this hypothesis by studying nuclear disassembly during spontaneous and heat-induced apoptosis in resting mouse splenocytes (Chapter 2). In addition, however, a unique labelling pattern for NuMA observed after heat treatment led to another series of studies that came to form a major part of the thesis, and that effectively constituted a comparison of spontaneous and heat-induced apoptosis in mouse splenocytes

(Chapters 3-5).

The thesis has been separated into Chapters, written in the form of manuscripts. Chapters 2 and 3 are being submitted for publication. Chapters 4 and 5 require some further experimentation prior to submission for publication.

CHAPTER 2.

Unique behaviour of NuMA during heat-induced apoptosis of lymphocytes

INTRODUCTION

Apoptotic cell death is characterized by major changes in nuclear organization, including degradation of DNA, chromatin aggregation and collapse, and redistribution and/or loss of nuclear proteins [reviewed in Earnshaw, 1995]. Pulsed field gel electrophoresis has shown that DNA is first cleaved into 300 kbp fragments and then into 50 kbp fragments, with the nucleosomal ladder appearing rather later in apoptosis [Brown *et al.*, 1993; Oberhammer *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994]. These results are thought to reflect the higher order folding of the 10 nm chromatin fibre into loops of 50 kbp and rosettes of 300 kbp [Filipski *et al.*, 1990; Walker *et al.*, 1991; Roy *et al.*, 1992; Brown *et al.*, 1993; Oberhammer *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994]. Moreover, DNA fragmentation appears to be linked to chromatin collapse. If proteases or nucleases are inhibited during thymocyte apoptosis induced by glucocorticoids or inhibitors of DNA topoisomerase II, DNA fragments smaller than 50 kbp are not detected and chromatin morphology exhibits only partial collapse [Weaver *et al.*, 1993; Sun *et al.*, 1994; Walker *et al.*, 1994].

These data also imply that proteolysis is a necessary factor for the apoptotic destruction of chromatin morphology. Consistent with this hypothesis, several immunofluorescence analyses have demonstrated loss of nuclear antigens such as NuMA and lamin B during apoptosis [Lazebnik *et al.*, 1993; Miller *et al.*, 1993; Oberhammer *et al.*, 1993; Tinnemans *et al.*, 1995; Rao *et al.*, 1996; Weaver *et al.*, 1996]. Moreover, it has

been shown that a number of nuclear proteins undergo degradation by ICE-like and other proteases during apoptosis. Notably, site-specific proteolysis appears to be limited to a subset of nuclear proteins, including Nuclear Mitotic Apparatus protein (NuMA), lamin B, DNA topoisomerase I and II, poly(ADP) ribose polymerase (PARP), the 70 kDa U1 snRNP protein, and hnRNP proteins C1 and C2 [Casciola-Rosen *et al.*, 1994b; Oberhammer *et al.*, 1994; Casciola-Rosen *et al.*, 1995; Lazebnik *et al.*, 1995a, 1995b; Tewari *et al.*, 1995; Casiano *et al.*, 1996; Hsu and Yeh, 1996; Rao *et al.*, 1996; Waterhouse *et al.*, 1996; Weaver *et al.*, 1996; Zweyer *et al.*, 1997]. Other nuclear proteins, including the nuclear envelope antigen PI2 [Chaly *et al.*, 1984, 1985, 1988, 1989; Weaver *et al.*, 1996], RCC1 [Lazebnik *et al.*, 1995a], PCNA [Casiano *et al.*, 1996; Zweyer *et al.*, 1997], CENP A, CENP B, CENP C, histone H2B, Ku, and fibrillarin [Casiano *et al.*, 1996] remain intact. Since many of the affected proteins identified to date are components of the nuclear matrix [Berrios *et al.*, 1985; Chaly *et al.*, 1985; Kallajoki *et al.*, 1991; Zeng *et al.*, 1994a, 1994b], it has been proposed that proteolysis of key structural proteins destabilizes the nucleus and contributes to apoptotic nuclear destruction [Weaver *et al.*, 1996].

However, the causal relationships among DNA fragmentation, protein degradation and chromatin collapse remain unclear. Recent studies have attempted to establish the relative timing of proteolysis and DNA fragmentation, and to determine whether the proteins are degraded sequentially. Studies in which DNA fragmentation and protein degradation were monitored in cell populations or in an *in vitro* apoptotic model system concluded that the lamins are early targets for proteolysis [Lazebnik *et al.*, 1993; Oberhammer *et al.*, 1994;

Lazebnik *et al.*, 1995b; Neamati *et al.*, 1995], and led to the conclusion that chromatin collapse resulted from disruption of the nuclear lamina.

We have approached this problem by attempting to establish the temporal sequence of events microscopically, at the level of individual cells [Weaver *et al.*, 1996]. Combined fluorescence labelling of nuclear antigens and of DNA strand breaks in dexamethasone-treated rat thymocytes has indicated that DNA fragmentation is a very early feature of apoptotic nuclei and that solubilization of the lamina is not required for DNA fragmentation [Weaver *et al.*, 1996].

In the present study, we have used similar approaches to analyze apoptosis in mouse splenic lymphocytes. Unlike thymocytes, freshly isolated mouse splenocytes are a non-cycling population of mature B and T cells, fully arrested in G₀. They exhibit very low levels of transcription and protein synthesis, and do not synthesize DNA [Setterfield *et al.*, 1983; Brown *et al.*, 1989]. The nuclear morphology reflects this low metabolic rate. The nuclei are small (ca. 5 μ m diameter), with large masses of condensed chromatin, small nucleoli, and very little nucleoplasm and nuclear matrix [Chaly *et al.*, 1988; Setterfield *et al.*, 1985; Bladon *et al.*, 1988]. Mouse and rat spleen and thymus lymphocytes have been reported to undergo apoptosis spontaneously, with 20-50% of the population succumbing within 16 h in culture [Migliorati *et al.*, 1992; Clarke *et al.*, 1993; Illera *et al.*, 1993; Perandones *et al.*, 1993; Zhang *et al.*, 1995; Rinner *et al.*, 1996]. Apoptosis in mouse splenocytes is also readily induced by agents such as VM-26 [Roy *et al.*, 1992; Daev *et al.*, 1994].

In the present work, we have compared events during spontaneous and heat-induced

apoptosis at the level of individual cells by double- or triple-label conventional and confocal fluorescence microscopy, and have correlated the data with analyses at the population level by flow cytometry and immunoblotting. Chromatin fragmentation has been evaluated by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) [Gavrieli *et al.*, 1992; Weaver *et al.*, 1996], overall chromatin organization has been monitored by staining with 4,6'-diamidino-2-phenylindole (DAPI), and the behaviour of nuclear protein antigens NuMA, PI2 and lamin B was followed by immunofluorescence labelling.

We have shown that heat treatment increases the level of apoptotic splenocytes to double that of spontaneous apoptosis within 6 hours. The organization and disassembly of nuclear envelope antigens is identical during spontaneous and heat-induced apoptosis, and proceeds in a temporally and spatially ordered manner relative to DNA fragmentation and chromatin collapse. On the other hand, NuMA organization is affected by heat treatment. Further, NuMA reorganization and the hyperinduction of apoptosis by heat are correlated, suggesting that splenocytes with reorganized NuMA are destined to undergo apoptosis.

MATERIALS AND METHODS

Splenocyte isolation and culture

Splenocytes were isolated from male Balb/c mice (Charles River) as previously described [Davis *et al.*, 1993] (see Appendix III). Cells were resuspended at 2.5×10^6 cells/ml in complete RPMI 1640 containing 10% CPSR-2 (Sigma), 2 mM glutamine (Gibco), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml Fungizone, and divided into 1 ml aliquots in culture tubes.

Control unstimulated samples were cultured at 37°C and 5% CO₂. For stimulation, Con A was added to the tubes at a final concentration of 4 μ g/ml, as in Daev *et al.* [1994], and the samples cultured at 37°C and 5% CO₂ for up to 48 hours.

Heat Treatment

Aliquoted cells were incubated for 30 minutes at $42.0 \pm 0.1^\circ\text{C}$ in a Grant circulating water bath equipped with a Julabo PCH heater. At the end of the heat treatment, designated as 0 hours (t_0), some samples were fixed for flow cytometry and immunofluorescence labelling. The remaining samples were cultured at 37°C for up to 6 hours before processing.

Treatment with VM-26

Cells were incubated for 2 hours at 37°C in complete medium containing 5 μ M VM-26 (Bristol Laboratories), as described in Daev *et al.* [1994]. At the end of the treatment,

some samples were fixed for flow cytometry or immunofluorescence labelling. The remaining samples were resuspended in complete medium without VM-26, and cultured for a further 2, 4, or 6h prior to fixation.

Flow Cytometry

Cells were pelleted in a benchtop centrifuge, resuspended in 1 ml phosphate-buffered saline (PBS), fixed by addition of 4 ml absolute ethanol (-20°C), and stored in PBS-ethanol at -20°C. Before analysis, samples were processed for propidium iodide fluorescence as in Walker *et al.* [1991]. Red fluorescence was measured at 610 nm on a Coulter Elite ESP flow cytometer, with 20,000 cells counted for each sample. Data were then analyzed using Multicycle Cell Cycle software (Phoenix Flow Systems).

Immunofluorescence Labelling

Fixation, permeabilization, and immunofluorescence staining of cells layered onto poly-L-lysine-coated coverslips were carried out as previously described [Chaly *et al.*, 1984, 1988] (see Appendix IV). Samples were then incubated sequentially in PBS-diluted primary and secondary antibodies.

Primary antibodies: Antibody PI2, a mouse monoclonal IgM (lyophilized supernatant, 1:100) [Chaly *et al.*, 1984]; anti-NuMA, a mouse monoclonal IgG1 (1:25, A-204) (Matritech); anti-lamin B, a rabbit anti-peptide serum (1:500) (gift of N. Chaudhary) [Chaudhary and Courvalin, 1993].

Secondary antibodies: CY3- or fluorescein isothiocyanate (FITC)-conjugated goat

anti-mouse IgM (μ chain-specific)(1:100) (Cappel); FITC- or CY3-conjugated donkey anti-rabbit IgG (heavy-and light-chain specific) (1:200) (Jackson Immunochemicals); CY3-conjugated goat anti-mouse IgG (Fc fragment specific)(1:200) (Jackson Immunochemicals).

All samples were counterstained with 0.2 $\mu\text{g}/\text{ml}$ of 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 min and mounted in Vectashield mounting medium (Vector Laboratories). Samples were viewed on a Zeiss photomicroscope III with a Plan-Neofluar X 63, N.A. 1.25 oil immersion objective, and photographed using Ilford XP2-400 film. Optical sections 0.5 μm apart were collected by confocal microscopy with a Leica Confocal Scanning Laser Microscope equipped with a Krypton/Argon laser and a Zeiss X 63, N.A. 1.4 Planapo objective.

In 2h control and heat-treated samples double-labelled for lamin B and PI2, 500 FC nuclei were scored as having intact or partial labelling of lamin B or PI2. Cell counts were repeated on samples from three separate experiments.

Labelling of DNA strand breaks

Samples fixed and permeabilized as above were assayed for DNA strand breaks using the method of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) [Gavrieli *et al.*, 1992], as described in Weaver *et al.* [1996]. After permeabilization, samples were incubated for 1 h with 300 U/ml of terminal deoxynucleotidyl transferase in cacodylate buffer (Gibco BRL) with 10 μM biotin-16-dUTP (Boehringer Mannheim) and the reaction was terminated by transferring samples to

buffer containing 300 mM NaCl and 30 mM sodium citrate. The cells were then incubated sequentially in 2% bovine serum albumin in PBS for 10 min to block non-specific labelling, and in streptavidin-CY3 (1:100, Jackson) or avidin-FITC (1:100, Vector) in PBS. All steps were performed at room temperature. For samples double labelled for immunofluorescence and TUNEL, antibody labelling was carried out first, as described above. To block non-specific binding, samples were incubated in 0.15% gelatin in PBS for 20 min. Samples were then washed in PBS and processed for TUNEL. Samples were then counterstained with DAPI, mounted and viewed as described above.

In 2h heat-treated samples double-labelled for TUNEL and nuclear antigens, labelling patterns were quantified in 10-50 TUNEL-normal nuclei, 45-150 PC nuclei, and 500 FC nuclei. In 2h control samples, labelling patterns were quantified in 300-500 FC nuclei. All cell counts were repeated on samples from three separate experiments.

Gel electrophoresis and Immunoblotting

Control and heated samples were pelleted, washed in PBS, flash frozen in liquid nitrogen, and solubilized on ice in sample buffer (0.5 M Tris-HCl, pH 6.8, with 10% sodium dodecyl sulphate and 10% glycerol). After determination of protein concentration by UV absorbance [Kalb and Bernlohr, 1977], β -mercaptoethanol was added to a final concentration of 0.25%. Protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer onto nitrocellulose membranes (0.45 μ m, Bio-Rad Laboratories) were carried out by standard protocols (Bio-Rad Manual), with 20 μ g of protein loaded into each lane.

For immunodetection, membranes were incubated in 2% skim milk in PBS, or in PVP (polyvinyl pyrrolidone) -buffer (1% PVP, 0.05% Tween-20, 150 mM NaCl, 20 mM Tris-HCl pH 7.4), to block nonspecific binding, and then in anti-NuMA (1:100 in PBS-milk), anti-lamin B (1:1000 in PBS-milk), or antibody PI2 (1:200 in PVP-buffer) for 1 hour at room temperature. Membranes were washed in PBS with 0.1% Tween-20, and then incubated with peroxidase-conjugated anti-rabbit IgG (1:3000) (Promega), goat anti-mouse IgG (τ -chain specific)(1:500) (Sigma Chemical Co.) or goat anti-mouse IgM (μ -chain specific)(1:1000) (Sigma Chemical Co.) as appropriate for 1 hour, and washed in PBS. Detection was carried out by chemiluminescence (BM Chemiluminescence substrate, Boehringer Mannheim) using hyperfilm-ECL (Amersham).

RESULTS

Kinetics of apoptosis

Preliminary experiments showed that a heat treatment of 42°C for 30 min caused no immediate effect on cell viability as evaluated by cellular exclusion of Trypan Blue (data not shown), but resulted in significant induction of apoptosis over a 6 hour period with respect to controls. These observations were confirmed using flow cytometry to monitor cellular DNA content (Fig. 1A-B), and DAPI staining to examine nuclear morphology (Fig. 1C). Control and heat-treated samples fixed at t_0 produced a single DNA peak corresponding to the DNA content of resting cells (Fig. 1A-B). There was still little difference between control (6%) and treated (9%) populations at t_2 . In controls, apoptotic cells then increased gradually to 18% sample at t_6 . On the other hand, the percentage of apoptotic cells in heated samples tripled to 27% between t_2 and t_4 , and had increased to 39% by t_6 .

As well, chromatin morphology in control and heat-treated samples was evaluated microscopically over the 6 hour period. As shown in Figure 3A', and as previously described [Chaly *et al.*, 1988; Davis *et al.*, 1993; Daev *et al.*, 1994], the size and appearance of freshly isolated, t_0 control splenocyte nuclei are quite uniform. The nuclear outline is roughly circular and somewhat irregular, and DAPI staining shows a broad irregular band of condensed chromatin at the edge of the nuclei and, usually, a large mass of chromatin in the centre. There is also some faint diffuse DAPI staining throughout the nucleoplasmic regions. DAPI-stained nuclei with this morphology were classified as

Figure 1: Kinetics of spontaneous and heat-induced apoptosis. Splenocytes were cultured at 37°C (control) or heat-treated at 42°C for 30 min, then cultured at 37°C for 0, 2, 4, or 6 hours.

(A) Flow cytometric profiles of propidium iodide fluorescence from a single experiment for control and heat-treated splenocytes. Twenty thousand cells were counted for each sample.

(B) Histogram showing the percent apoptotic cells detected by flow cytometry in control (open bars) and heat-treated (solid bars) samples. Results are the means from 15 experiments; error bars show the standard deviation from the mean. At t_4 and t_6 , the percentage of apoptotic cells in control samples was significantly different from that in heat-treated samples with $p \leq 0.001$.

(C) Histogram showing the proportion of cells with FC nuclei in control (open bars) and heat-treated (solid bars) samples as determined by microscopy.

Chromatin morphology by DAPI staining was evaluated as normal or fully collapsed for 500 nuclei in each sample. The data are the means from three experiments; error bars show the standard deviation from the mean. At t_4 and t_6 , the percentage of apoptotic cells in control samples was significantly different from that in heat-treated samples with $p \leq 0.006$.

"DAPI-normal".

Cells with a DAPI-normal morphology still represented over half of the control population 6 hours after heat treatment (not shown), but many of the nuclei exhibited a morphology characteristic of apoptotic cells [Weaver *et al.*, 1996]. The chromatin showed a variety of profiles: collapsed into a single near spherical body, with (Fig. 2D') or without (Fig. 8D') associated small spherical bodies; arranged as a smooth ring at the nuclear margin (Fig. 8C'); or collapsed to the margin of the nucleus in a cup-shaped mass (Fig. 9D'). A notable feature of the chromatin in these nuclei was the very uniform DAPI staining, which resulted in a "textureless" appearance. There was little or no diffuse DAPI labelling elsewhere in the nuclei. In quantifying the accumulation of apoptotic cells in the samples, nuclei with these patterns of DAPI staining were classified as fully collapsed (FC).

The data obtained by scoring the samples for DAPI-normal and FC nuclei were consistent with the flow cytometric results (Fig. 1C). In both control and heat-treated samples, FC nuclei represented about 2% of the population at t_0 , and increased to about 13% by t_2 . There was little change in the percentage of FC nuclei in control samples thereafter. In heat-treated populations, however, FC nuclei nearly doubled to 23% by t_4 , and represented 33% of the population at t_6 .

These data show first that mouse splenocytes undergo considerable spontaneous apoptosis in the first six hours in culture. This is in contrast to human peripheral blood lymphocytes, which exhibit only about 5% spontaneous apoptosis over the first 24 hours of culture [Giordano *et al.*, 1995; Lomo *et al.*, 1995; Mentz *et al.*, 1995]. The data also

show, however, that heat treatment significantly increased the rate of splenocyte death after 2 hours in culture, with heat-treated populations exhibiting about twice the control level of apoptotic cells by t_6 .

DNA fragmentation and chromatin morphology

To examine the relationship between DNA fragmentation and chromatin collapse during apoptosis, samples were labelled with TUNEL and DAPI (Fig. 2).

Qualitatively, the results were identical for control (not shown) and heat-treated populations. All cells categorized as FC by DAPI staining were labelled with TUNEL, confirming that these cells were indeed apoptotic (Fig. 2D-D'). The TUNEL in these cells was either uniformly distributed throughout the chromatin body(ies), or formed a bright rim around each chromatin body, with the interior of the bodies unlabelled (Fig. 2D-D').

In addition to FC cells, a small number of nuclei with a DAPI-normal morphology were labelled with TUNEL (Fig. 2A-A'), with the signal distributed uniformly throughout the entire nucleus. Such TUNEL-labelled DAPI-normal nuclei will be referred to as TUNEL-normal. TUNEL-normal nuclei were observed in both control and heat-treated populations but were extremely rare in all samples. In heat-treated samples, they represented 1% of all DAPI-normal cells at t_2 , an even lower proportion at later time points, and were too rare to count in controls (data not shown). According to our earlier interpretation, these cells are in very early stages of DNA fragmentation [Weaver *et al.*, 1996].

Figure 2: Chromatin morphology of nuclei with DNA strand breaks in heat-treated samples fixed at t_2 , labelled with TUNEL (A-D) and counterstained with DAPI (A'-D'). The right-hand cells in A'-C' are TUNEL-negative, DAPI-normal, and non-apoptotic. The other cells are TUNEL-positive and show varying extents of chromatin collapse. A-A' - DAPI-normal nucleus with diffuse TUNEL (TUNEL-normal); B-B' - PC nucleus with chromatin rimmed by TUNEL; C-C' - PC nucleus with diffuse TUNEL; D-D' - FC nuclei with diffuse TUNEL (bottom) and with chromatin outlined by TUNEL (top). Magnification 2600X.

In both heat-treated and control samples, TUNEL also labelled nuclei with a morphology intermediate between DAPI-normal and FC (Fig. 2B-B', 2C-C'). In such nuclei, condensed chromatin still formed a broad irregular band at the nuclear margin and a large mass at the nuclear centre (Fig. 2B', 2C'). However, the chromatin was stained quite uniformly by DAPI and exhibited the textureless appearance characteristic of FC nuclei (Fig. 2B', 2C'). Such nuclei were categorized as partially collapsed (PC) and were invariably labelled with TUNEL. As for FC nuclei, TUNEL-labelling of PC nuclei was either uniformly diffuse (Fig. 2C-C') or formed a rim around the periphery of chromatin masses (Fig. 2B-B'). PC nuclei were observed more frequently than TUNEL-normal nuclei but were still infrequent. They represented less than 3% of the total cell population in heat-treated samples at t_2 , and an even lower proportion in other samples.

The TUNEL data demonstrate that apoptotic changes in chromatin fragmentation and chromatin morphology occur indistinguishably in control and heat-treated samples. They also confirm that cells with PC and FC chromatin morphology by DAPI staining are, in fact, apoptotic. Moreover, the existence of TUNEL-normal nuclei indicates that DNA fragmentation is initiated before detectable changes in chromatin morphology during apoptosis.

Behaviour of NuMA during control and heat-induced apoptosis

Anti-NuMA and DAPI - The behaviour of NuMA during splenocyte apoptosis was examined by immunofluorescence labelling.

In control DAPI-normal nuclei at all time points, NuMA labelling was diffuse or

slightly granular, was distributed throughout the nucleoplasm, and was excluded from masses of condensed chromatin (Fig. 3A-A', 3C-C', 3E-E'). A similar pattern has been previously described for rat thymocytes and a variety of other cell types in interphase [reviewed in He *et al.*, 1995; Weaver *et al.*, 1996]. In the majority of control PC nuclei (>90%), NuMA labelling was identical in intensity and distribution to that in DAPI-normal cells (Fig. 3C-C'), and was faint in the remaining PC cells (not shown). The labelling intensity in most control FC nuclei was similar to, or greater than, that in DAPI-normal cells, and NuMA was still concentrated in the nucleoplasm between the chromatin masses (Fig. 3E-E'). These results are similar to the changes in NuMA organization we observed during dexamethasone-induced apoptosis of rat thymocytes [Weaver *et al.*, 1996].

However, in splenocyte FC nuclei with a single spherical chromatin mass, we further observed that NuMA labelling was consistently undetectable (not shown; see Fig. 3G-G').

NuMA staining was somewhat different in heat-treated populations. At t_0 , about half of the cells exhibited the control diffuse NuMA pattern (Fig. 3B-B'). The other half of the splenocyte population, however, contained many bright spots in addition to diffuse nucleoplasmic labelling (Fig. 3B-B'). The spots appeared to be nucleoplasmic, often located in clusters adjacent to condensed chromatin. NuMA spots were observed in DAPI-normal nuclei of heat-treated samples throughout the 6 hour period (e.g. Fig. 3G-G').

Apoptotic cells in heat-treated samples also showed this dichotomy in staining pattern.

Figure 3: Distribution of NuMA during control and heat-induced apoptosis. Control (A-A', C-C', E-E') and heated (B-B', D-D', F-F', G-G') samples were fixed at t_0 (A-A', B-B') or at t_2 (C-G'), and were labelled with anti-NuMA (A-G) and DAPI (A'-G'). The right-hand nucleus in (A-F') is DAPI-normal, and non-apoptotic. Diffuse NuMA labelling is shown for control DAPI-normal (A-A'), PC (C-C') and FC (E-E') nuclei. In heat-treated samples (B-B', D-D', F-F'), nuclei on the left are spotty (arrowheads). NuMA labelling is diffuse (B-B', D-D', F-F'), or absent (G-G') in the other nuclei. Magnification 2600X.

In some PC and FC nuclei, the NuMA distribution was indistinguishable from that in controls (Fig. 3C-C', 3E-E'). However, bright spots against diffuse fluorescence were characteristic of most heat-treated PC nuclei (Fig. 3D-D'). Moreover, NuMA spots and diffuse NuMA labelling were observed in most FC nuclei, including nuclei with chromatin forming a hollow shell at the nuclear periphery (Fig. 3F-F') or collapsed into various irregular shapes (not shown). As in control samples, however, nuclei with a compact spherical chromatin mass showed no NuMA label (Fig. 3G-G').

NuMA and TUNEL - To examine the relationship between NuMA organization and DNA fragmentation at the level of individual cells during spontaneous and heat-induced apoptosis, samples were double-labelled with anti-NuMA and TUNEL. This approach also allowed us to begin investigating the significance of spotty NuMA labelling to apoptotic induction in heated populations.

In controls, all TUNEL-negative nuclei were DAPI-normal and showed the diffuse nucleoplasmic NuMA labelling described above (Fig. 4B-B", 4D-D"). TUNEL-positive nuclei were PC (Fig. 4B-B") or FC (Fig. 4D-D") by DAPI, and the NuMA labelling patterns (Fig. 4B, 4D) were as expected for these cells (Fig. 3).

In heated samples, TUNEL-negative nuclei included nuclei with diffuse nucleoplasmic labelling (not shown) and nuclei with both spotty and diffuse labelling (Fig. 4A-A"). NuMA staining in TUNEL-normal nuclei was indistinguishable from that in TUNEL-negative cells (Fig. 4A-A"). As well, all PC and FC nuclei were labelled by TUNEL, and NuMA staining changed progressively with chromatin disorganization, as described

Figure 4: NuMA distribution and DNA fragmentation. Control (B-B", D-D") and heat-treated (A-A", C-C", E-E") samples were fixed at t_2 , labelled with anti-NuMA (A-E) and TUNEL (A'-E'), and counterstained with DAPI (A"-E"). Spotted NuMA labelling is shown for heat-treated TUNEL-negative, non-apoptotic nuclei (lower nucleus, A-A", E-E"), as well as TUNEL-normal (A-A"), PC (C-C") and FC (E-E") nuclei. Diffuse NuMA labelling is shown for control TUNEL-negative, non-apoptotic nuclei (lower nucleus, B-B", D-D"), as well as control PC (B-B") and FC (D-D") nuclei. Magnification 2600X.

above (Fig. 3). These results indicate that the presence of NuMA spots does not affect the progress of apoptotic DNA fragmentation.

To determine the spatial relationship of NuMA to fragmented chromatin, these samples were examined by confocal microscopy (Fig. 5). At all stages of DNA fragmentation and collapse, NuMA and TUNEL labelling were spatially separate in both control and heat-treated samples. A single midsection of NuMA from a control nucleus and the corresponding section of TUNEL showing fully collapsed chromatin are presented in Figures 5A and 5A' respectively. The merged image shows NuMA distributed diffusely in the nucleoplasm, with chromatin unlabelled (Fig. 5A"). Both the diffuse NuMA component and the NuMA spots were also located in the nucleoplasm in heat-treated TUNEL-normal (Fig. 5B-B"), PC (Fig. 5C-C") and FC (Fig. 5D-D") nuclei. However, the NuMA spots were not distributed at random, but were generally associated with the surface of condensed chromatin masses at all three stages of chromatin collapse (Fig. 5B-B", 5C-C", 5D-D").

Our observations had suggested that most TUNEL-positive cells in heat-treated samples contained NuMA spots. The proportion of TUNEL-normal, PC and FC nuclei with NuMA spots was therefore determined in heat-treated samples from three separate samples at t_2 . In each experiment, >90% of TUNEL-normal nuclei, >80% of PC nuclei, and >45% of FC nuclei had NuMA spots. The relatively low proportion of spotty FC nuclei is likely due to several factors. On the one hand, FC nuclei with a single spherical chromatin mass are not labelled at all, as noted above. On the other hand, NuMA labelling in some FC nuclei is so concentrated as to make spots undetectable. Moreover,

Figure 5: Confocal fluorescence microscopy of NuMA distribution in relation to fragmented DNA and lamin B. Control (A-A") and heat-treated (B-E") samples were fixed at t_2 and labelled with anti-NuMA (A'-E'), and with TUNEL (A-D) or anti-lamin B (E); the corresponding merged images are shown in A"-E". Labelling is shown for TUNEL-normal (B-B"), PC (C-C"), and FC (A-A", D-D", E-E") nuclei. Magnification 3000X.

A	A'	A''
B	B'	B''
C	C'	C''
D	D'	D''
E	E'	E''

some part of the FC population will probably have been derived from splenocytes that were not spotty after heat treatment.

These results indicate that the presence of NuMA spots is related to the induction of apoptosis. To determine whether NuMA spots were a general feature of splenocyte apoptosis, we performed experiments on splenocytes treated with VM-26, an inhibitor of DNA topoisomerase II. We have previously shown that treating freshly isolated splenocytes with VM-26 resulted in a rapid increase in the proportion of apoptotic cells [Roy *et al.*, 1992]. In two separate experiments, splenocytes were treated with 5 μ M VM-26 for 2 hours and cultured without the drug for up to 6 hours. Samples were taken at intervals after treatment and analyzed by flow cytometry of propidium iodide fluorescence and by microscopy with anti-NuMA. VM-26 had little effect on the proportion of apoptotic cells within the first 2h. By 6 hours, as previously reported [Roy *et al.*, 1992], >50% of the treated population but <15% of the control population was apoptotic (see Appendix II, Fig. 28A). Despite the massive induction of apoptosis by VM-26, however, the patterns of NuMA labelling in DAPI-normal and apoptotic nuclei were identical to those in control samples (see Appendix II, Fig. 28B-B'). Specifically, NuMA spots were observed in <3% of DAPI-normal and FC cells in both types of samples (not shown). These results indicate that the presence of NuMA spots is related specifically to the induction of apoptosis by heat.

NuMA spots and the cell cycle

Freshly isolated mouse splenocytes are uniformly arrested in G₀. Since NuMA spots

have not been previously reported in interphase nuclei, we wished to determine whether they were observed naturally at other stages of the splenocyte cell cycle.

Upon addition of a mitogen such as concanavalin A (Con A), splenocytes become stimulated and enter G1 asynchronously. There is massive upregulation of cell metabolism and remodelling of nuclear structure in the first 24 hours, at which time DNA replication begins. By 48 hours, approximately 5% of the population is in mitosis [Setterfield *et al.* 1983, 1985; Chaly *et al.*, 1988; Chaly and Brown, 1988; Davis *et al.*, 1993].

To examine NuMA organization during the splenic lymphocyte mitotic cycle, freshly isolated populations were stimulated with Con A and processed at 12 hour intervals up to 48 hours for immunofluorescence labelling with anti-NuMA. As we have previously described, the nuclei more than doubled in diameter during stimulation, the chromatin masses disaggregated, and large nucleoli became elaborated (Fig. 6A') [Setterfield *et al.*, 1985; Chaly *et al.*, 1988]. NuMA labelling was exclusively diffuse in all interphase nuclei at all time points (Fig. 6B-B'). Moreover, despite the well-documented increases in metabolic rate and in the elaboration of a complex nuclear matrix, the labelling intensities of resting and stimulated nuclei were similar. In mitotic cells, the staining was concentrated at the spindles poles (Fig. 6A-A'), as previously described for many cell types [reviewed in He *et al.*, 1995].

These results show that formation of NuMA spots is not a natural feature of any part of the lymphocyte proliferative cell cycle. They further indicate that the appearance of NuMA spots is specific to heat-induced apoptosis.

Figure 6: The organization of NuMA (A, B) and DAPI staining (A', B') during the proliferative cell cycle. Samples fixed 48 hours after addition of Con A show a mixed population of resting (bottom left), partially (middle), and fully (top) stimulated interphase cells (A-A'), as well as some cells in mitosis (B-B').
Magnification 1700X.

Behaviour of peripheral nuclear antigens during apoptosis

To determine whether heat treatment affected the organization of other nuclear antigens during apoptosis, samples at t_2 were labelled with antibodies to the nuclear envelope antigens PI2 or lamin B, processed for TUNEL, and counterstained with DAPI.

In DAPI-normal TUNEL-negative control cells at t_0 and throughout culture, PI2 labelled the nuclear periphery as has been previously reported for lymphocytes and other cell types [Chaly *et al.*, 1984, 1986, 1988; Weaver *et al.*, 1996]. Anti-lamin B labelling was also as previously described in these cells [Chaly *et al.*, 1988], and was indistinguishable from that produced by PI2. The labelling of PI2 and lamin B in DAPI-normal TUNEL-negative cells in heat-treated samples was identical to that in controls at t_0 and throughout culture (Fig. 7A-A", 8A-A"). Furthermore, though the distribution of PI2 and lamin B showed characteristic changes during apoptosis, the pattern of changes was identical in control (not shown) and heat-treated samples (Fig. 7, 8), and was overall similar to that reported in apoptotic rat thymocytes [Weaver *et al.*, 1996]. For brevity, the behaviour of these antigens in heat-treated samples only will be described.

In TUNEL-normal nuclei, the entire periphery was labelled with antibody PI2 (Fig. 7A-A") and anti-lamin B (Fig. 8A-A"). The continuity in the staining of both antigens in TUNEL-normal nuclei was confirmed by confocal microscopy. As shown for lamin B in serial optical sections in Figure 9A-A", there were no interruptions in the peripheral nuclear labelling in such cells. Quantitation of the proportion of TUNEL-normal nuclei with intact labelling in three separate experiments confirmed that they were invariably identical to DAPI-normal TUNEL-negative nuclei in both the brightness and distribution

Figure 7: Organization of PI2 during apoptosis. Heat-treated samples were fixed at t_2 , labelled with PI2 (A-E) and TUNEL (A'-E'), and counterstained with DAPI (A''-E''). The lower nucleus in each figure is DAPI-normal, TUNEL-negative and non-apoptotic, with continuous peripheral labelling of PI2. In apoptotic cells, the PI2 labelling shown is continuous (A-C), interrupted (D), or absent (E) in TUNEL-normal (A'-A''), PC (B'-B'') and FC (C'-C'', D'-D'', E'-E'') nuclei. Magnification 2600X.

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Figure 8: Organization of lamin B during apoptosis. Heat-treated samples were fixed at t_2 , labelled with anti-lamin B (A-E) and TUNEL (A'-E') and counterstained with DAPI (A''-E''). The lower nucleus in each figure is DAPI-normal, TUNEL-negative and non-apoptotic, with continuous peripheral labelling of lamin B. In apoptotic cells, the lamin B labelling shown is continuous (A-C), interrupted (D), or absent (E) in TUNEL-normal (A'-A''), PC (B'-B'') and FC (C'-C'', D'-D'', E-E'') nuclei. Magnification 2600X.

Figure 9: Confocal microscopy of PI2, lamin B and TUNEL. Heat-treated samples were fixed at t_2 and labelled with TUNEL (A, B) or PI2 (C), and anti-lamin B (A'-C'); the corresponding merged images are shown in A"-C". A-A" - FC nucleus with ring-shaped collapsed chromatin and continuous lamin B staining; images labelled 1 to 12 are the complete through-focus series of lamin B staining in this nucleus. B-B" -FC nucleus with a lamin B discontinuity in a region of the envelope lacking apposed chromatin. C-C" - FC nucleus with coincident discontinuities in PI2 and lamin B staining. Magnification 3160X.

A	A'	A''
1	2	3
4	5	6
7	8	9
10	11	12
B	B'	B''
C	C'	C''

of both antigens. These experiments were also used to quantify the labelling patterns in PC and FC nuclei described below.

Similarly, most of the PC nuclei showed intact labelling for these antigens that was similar in intensity to labelling of DAPI-normal cells (Fig. 8B-B") or somewhat fainter (Fig. 7B-B"). Quantitative evaluation of PC nuclei found that 78-80% had intact PI2 labelling and 77-79% had intact lamin B labelling. In the remainder, staining of the nuclear rim was interrupted to varying extents (not shown). In FC nuclei, however, the majority showed some discontinuities in the nuclear envelope antigens, and the extent of the interruptions was related to the morphology of the collapsed chromatin. Nuclei with chromatin forming a continuous shell at the margin mostly retained continuous labelling (Fig. 7C-C", 8C-C"). In FC nuclei with chromatin collapsed into cup-shaped or irregularly shaped masses at the rim, large extents of the peripheral labelling were absent (Fig. 7D-D", 8D-D"). Confocal microscopy showed that the discontinuities in lamin B occurred in regions of the nucleus periphery lacking apposed condensed chromatin (Fig. 9B-B"). Finally, as for NuMA, FC nuclei with chromatin compacted into a spherical mass were unlabelled (Fig. 7E-E", Fig. 8E-E").

Quantitation confirmed that these antigens were no longer continuous in most FC nuclei. Although the absolute values varied between experiments, only 13-24% of FC nuclei showed intact lamin B staining, with PI2 labelling intact in 17-32% of the nuclei. However, in each experiment, the proportion of FC cells with intact PI2 labelling was slightly (4-8%) larger than the proportion exhibiting uninterrupted lamin B.

These results implied that some FC nuclei might exhibit partial lamin B-staining while

retaining intact labelling for PI2. To examine this possibility, as well as to determine the relative location of interruptions in PI2 and lamin B labelling, samples were double-labelled for the two antigens and examined by conventional (Fig. 10) and confocal (Fig. 9C-C") microscopy. In DAPI-normal nuclei, and in FC nuclei with continuous labelling, PI2 and lamin B were coincident (Fig. 10A-A"). As well, partial labelling with the two antibodies appeared to be colocalized in most FC nuclei, and was normally in contact with condensed chromatin (Fig. 10D-D"). Confocal microscopy confirmed that these two antigens were co-localized in both normal (not shown) and most FC nuclei (Fig 9C-C"). Occasionally, however, FC cells were observed with intact (Fig. 10B-B") or partial (Fig. 10C-C") PI2 labelling and no detectable lamin B labelling.

To quantify these observations, the proportion of FC nuclei with continuous lamin B and/or PI2 labelling was determined for three experiments by cell counting. Within each experiment the trends were the same, and similar to those obtained above, with 10-25% of the FC nuclei labelled continuously with both antibodies. Again, there was variability between experiments as to the absolute values, but 2-4% more FC nuclei exhibited intact PI2 than intact lamin B. These results confirm the existence of FC nuclei that show no lamin B staining, even though PI2 is still continuous.

These results show that, unlike NuMA, the behaviour of the nuclear envelope antigens lamin B and PI2 is identical during spontaneous and heat treatment-induced apoptosis. They also indicate that loss of these proteins is spatially coincident, but may be temporally independent, with loss of PI2 occurring slightly later than loss of lamin B. With respect to DNA fragmentation, the data demonstrate that cleavage is initiated before detectable

Figure 10: Relative organization of lamin B and PI2 during apoptosis. Heat-treated samples were fixed at t_2 , labelled for PI2 (A-D) and lamin B (A'-D'), and counterstained with DAPI (A''-D''). The lower nuclei in A-A'', B-B'', C-C'' are DAPI-normal with both antigens continuous at the nuclear rim. The other nuclei are FC, with both antigens continuous (A-A''), PI2 continuous and lamin B absent (B-B''), PI2 discontinuous and lamin B absent (C-C''), or both PI2 and lamin B discontinuous and co-localized (D-D''). Magnification 2600X.

changes in peripheral nuclear antigens. Notably, however, these proteins are initially lost in regions where the envelope is no longer in contact with condensed chromatin.

Biochemical changes in NuMA, PI2 and lamin B during apoptosis

Earlier studies have shown specific proteolysis of nuclear proteins such as NuMA and lamin B during apoptosis [Oberhammer *et al.*, 1994; Lazebnik *et al.*, 1995b; Casiano *et al.*, 1996; Rao *et al.*, 1996; Hsu and Yeh, 1996; Weaver *et al.*, 1996; Zweyer *et al.*, 1997]. To examine the degradation of nuclear antigens in this study, whole cell lysates were prepared from control splenocytes immediately after isolation, and from control and heat-treated samples at intervals between t_0 and t_6 , and were processed for immunoblotting with anti-NuMA, anti-lamin B, and antibody PI2 (Fig. 11).

As previously reported [Weaver *et al.*, 1996], anti-NuMA detected a strong band at 230 kDa in all samples (Fig. 11A). A faint band at 200 kDa was also observed in most of the samples. A third band at 170 kDa was observed within 2 to 4 hours of t_0 in both control and heated samples. This band increased in intensity over the 6 hour period in both samples, but became much more pronounced in heated samples (Fig. 11A, see also Fig. 21A).

Anti-lamin B labelled a 67 kDa band in all samples (Fig. 11B). A second band at 46 kDa was detected in heated samples by t_2 , and this band increased in intensity over time (Fig. 11B, lanes 8-13). The 46 kDa band was also detected in control samples beginning at t_2 , but the band was consistently less intense than in the corresponding heat-treated samples (Fig. 11B, lanes 1-7). These results are consistent with other reports of lamin B

Figure 11: Immunoblotting of nuclear matrix proteins during spontaneous and heat-induced apoptosis. Immunoblotting with anti-NuMA (A), anti-lamin B (B) , and antibody PI2 (C) was carried out on 7.5 (A) or 10 (B-C) % SDS-PAGE gels loaded with 20 µg of protein per lane. Samples were whole cell lysates prepared from control splenocytes immediately after isolation (lane 1), and from control (lanes 2-7) and heat-treated samples (lanes 8-13) processed immediately after heat treatment (2, 8), or after culture at 37°C for 30 minutes (3, 9), 1 hour (4, 10), 2 hours (5, 11), 4 hours (6, 12) or 6 hours (7, 13). Molecular weight standards are shown in kDa.

degradation during apoptosis [Oberhammer *et al.*, 1994; Lazebnik *et al.*, 1995b; Casiano *et al.*, 1996; Rao *et al.*, 1996; Weaver *et al.*, 1996].

Antibody PI2 consistently detected bands at 70 kDa and 35 kDa in all samples (Fig. 11C). As reported for dexamethasone-treated rat thymocytes [Weaver *et al.*, 1996], there was no evidence of apoptosis-related degradation of the PI2 antigens. The labelling intensity of the bands did not change and no additional bands appeared over the 6 hours of culture.

Both the cleavage patterns of NuMA and lamin B and the timing of the first detectable cleaved fragments were similar to those reported during dexamethasone-induced thymocyte apoptosis [Weaver *et al.*, 1996]. Moreover, these results show that NuMA and lamin B are cleaved in the same manner during spontaneous and heat-induced apoptosis. On the other hand, again as for thymocytes [Weaver *et al.*, 1996], the PI2 antigen showed no susceptibility to proteolysis. Overall, therefore, these data support the proposal that site-specific proteolysis of nuclear antigens is a general feature of apoptosis [Weaver *et al.*, 1996].

DISCUSSION

Kinetics of Apoptosis

Apoptosis of lymphocytes *in vivo* is important for physiological control of T and B cell levels, as well as for the prevention of autoimmune responses [reviewed in Cohen, 1995; Abbas, 1996]. As demonstrated in our study, this is manifested *in vitro* as spontaneous death of lymphocytes in culture in the absence of an exogenous apoptotic stimulus. We found that about 18% of splenocytes had died by apoptosis within 6 hours of culture. These data are similar to rates of spontaneous apoptosis measured for lymphocytes in other studies, which have reported that 20-50% of the cells isolated from mouse spleen and lymph nodes [Illera *et al.*, 1993; Reap *et al.*, 1995; Zhang *et al.*, 1995], mouse thymus [Migliorati *et al.*, 1992; Clarke *et al.*, 1993], rat spleen and thymus [Rinner *et al.*, 1996] and human thymus [Mentz *et al.*, 1995] had died by 16 hours of culture. On the other hand, human peripheral blood lymphocytes are anomalous in this regard, showing much lower levels (<10%) of apoptosis even over a 48 hour culture period [Giordano *et al.*, 1995; Lomo *et al.*, 1995; Mentz *et al.*, 1995].

Still higher rates of lymphocyte apoptosis can also be readily induced by a variety of stimuli, and the apoptotic effect on lymphocytes of glucocorticoids and chemotherapeutic drugs [Walker *et al.*, 1991; Roy *et al.*, 1992; Weaver *et al.*, 1993], and radiation [Sellins and Cohen, 1991; Delic *et al.*, 1993; Cregan *et al.*, 1994] has been widely investigated. However, although mild hyperthermia has been shown to induce apoptosis in a variety of human and rodent cell lines [Harmon *et al.*, 1990; Takano *et al.*, 1991; Mosser and Martin,

1992; Li *et al.*, 1994; Li *et al.*, 1996; Yonezawa *et al.*, 1996], few studies have examined its effect on lymphocytes. It has been reported, however, that murine and rat thymocytes treated for 20-60 minutes at 43°C develop the oligonucleosomal ladder of DNA fragments associated with apoptosis within a further 4 to 6 hours at 37°C [Sellins and Cohen, 1991; Migliorati *et al.*, 1992; Sikora *et al.*, 1993].

In our investigation of the response of resting mouse splenocytes to heat, we have similarly found that treatment at 42°C for 30 minutes hyper-induced apoptosis. After a 2-hour lag phase, during which there was little difference between control and treated samples, the proportion of cells with an apoptotic morphology and fragmented DNA in treated samples increased to double that in controls between t_2 and t_6 . Moreover, we conclude that the cells were dying by apoptosis, not necrosis, as there was no immediate effect of heating on plasma membrane integrity, as evaluated by Trypan Blue exclusion, or on the cellular DNA content measured by flow cytometry. Notably, other than for the appearance of NuMA spots in heated samples, the morphological and biochemical features of spontaneous and heat-induced apoptosis were qualitatively undistinguishable.

DNA Fragmentation and Chromatin Morphology

Perhaps the most dramatic change in the appearance of the nucleus during apoptosis is the loss of chromatin organization. In early electron microscopic studies, chromatin was reported to marginate into smooth, compact, uniformly electron dense masses at the nuclear periphery. The chromatin was then described as separating into a number of membrane bound bodies, or collapsing into a cup-shaped body or a single ball [Wyllie *et*

al., 1980; Earnshaw, 1995]. We have similarly observed by DAPI-staining that FC nuclei can be separated into three broad categories on the basis of the disposition of the collapsed chromatin in a ring shape, a cup shape, or a single ball.

The exact relationship between these morphological changes and the extent of DNA cleavage is still equivocal. On the one hand, it is widely accepted that the appearance of an apoptotic chromatin morphology is related to the production of fragmented DNA, and, on the basis of early reports, it was believed that the DNA was invariably cleaved into oligonucleosomal fragments [Wyllie et al., 1984; Arends et al., 1990; Lazebnik et al., 1993]. However, more recent evidence suggests that the extent of DNA fragmentation depends at least in part on the cell system. In some cell types, for instance, chromatin collapse has been observed in the absence of oligonucleosomal DNA fragmentation [Oberhammer et al., 1993]. On the other hand, studies of rat thymocyte apoptosis in which cleavage of the DNA is arrested at 50 kbp fragments upon application of protease or nuclease inhibitors have reported that chromatin collapses only partially in these samples [Weaver et al., 1993; Sun et al., 1994; Walker et al., 1994].

The temporal sequence of chromatin cleavage and chromatin collapse has also been examined. Confocal microscopy of thymocytes labelled by *in situ* nick translation [Falcieri et al., 1994] or TUNEL [Weaver et al., 1996] have suggested that DNA cleavage occurs before condensation of chromatin. By labelling with TUNEL and DAPI, we have identified splenocyte nuclei that contain DNA strand breaks with no apparent restructuring of chromatin. These results are further evidence that the onset of DNA fragmentation precedes the appearance of an apoptotic chromatin morphology. Moreover, such

TUNEL-normal nuclei were labelled only faintly with TUNEL, suggesting that DNA degradation in these cells is not yet extensive and may represent initial high MW cleavage. Since labelling for NuMA, PI2 and lamin B in TUNEL-normal nuclei was identical to that in non-apoptotic cells, we conclude further that chromatin cleavage begins before there is visible destruction of nuclear structure.

In contrast, PC nuclei were brightly labelled with TUNEL, indicating that DNA fragmentation was more pronounced in these nuclei. The evidence indicates that the PC nuclear morphology is transient and represents an apoptotic state intermediate between TUNEL-normal and FC nuclei. This interpretation is supported by observations of the frequency of PC nuclei. PC nuclei were not common in any sample, and were observed in appreciable numbers only in heat-treated samples at t_2 . It has been clearly established that apoptosis is completed very rapidly once initiated, within minutes [Wyllie *et al.*, 1980; Cohen *et al.*, 1994]. Since relatively few cells are dying in control samples, this speed would account for the rarity of intermediate apoptotic stages such as PC nuclei. On the other hand, since heating increased the proportion of dying cells dramatically, the probability of observing cells at intermediate stages of apoptosis would also be greater and the proportion of PC nuclei would increase. The higher concentration of PC nuclei in t_2 heated samples may reflect a partial synchrony in the development of the apoptotic pathway in response to heat during the lag phase referred to above.

In an earlier study [Cohen *et al.*, 1994], rat thymocytes with a similar partial collapse of chromatin were separated from dexamethasone- and etoposide-induced apoptotic populations. They exhibited a size and density intermediate between the normal and

apoptotic, and contained large DNA fragments only. Moreover, as noted above, rat thymocytes undergoing apoptosis in the presence of protease inhibitors exhibited only partially collapsed chromatin and also contained only large DNA fragments [Weaver *et al.*, 1993; Walker *et al.*, 1994]. Ours is the first report of such cells in populations undergoing spontaneous apoptosis, and strongly suggests that PC nuclei represent a generally-occurring transient stage of apoptosis, in which DNA fragmentation is extensive but not yet sufficient for complete chromatin collapse.

The size of the DNA pieces in PC nuclei remains to be determined. However, the existence of PC nuclei implies that apoptotic chromatin collapse occurs in at least two stages: first, collapse of chromatin fibres within a chromatin mass, resulting in a textureless DAPI staining, and then separation of the masses into several apoptotic bodies or their aggregation into a single body.

Chromatin Collapse and Nuclear Proteins

The morphological behaviour of non-chromatin nuclear components during apoptosis has been less well defined. Electron microscopy has shown that the nuclear membrane often appears intact, with nuclear pores accumulating between the margined chromatin masses [Wyllie *et al.*, 1980; Falcieri *et al.*, 1994; Oberhammer *et al.*, 1994; Weaver *et al.*, 1996]. As well, nucleoli enlarge and segregate [Wyllie *et al.*, 1980; Arends *et al.*, 1990], and, in apoptotic rat thymocytes, the nucleoplasm becomes amorphous and disorganized, with few visible granules and filaments [Weaver *et al.*, 1996]. Recently, Zweyer *et al.* [1997] have also described fibrogranular nuclear bodies that appear late in apoptosis of

HL60 cells [Zweyer *et al.*, 1997].

As noted in the Introduction, however, it is well established that destruction of proteinaceous nuclear components is effected by the action of site-specific proteases, and that it occurs in a non-random manner. Whereas some nuclear proteins, including lamins and NuMA, are targeted for cleavage [Oberhammer *et al.*, 1994; Lazebnik *et al.*, 1995b; Neamati *et al.*, 1995; Casiano *et al.*, 1996; Hsu and Yeh, 1996; Rao *et al.*, 1996; Weaver *et al.*, 1996; Zweyer *et al.*, 1997], others, including PI2, remain uncleaved [Weaver *et al.*, 1996]. It has been suggested that proteolysis of key structural nuclear proteins may potentiate subsequent chromatin degradation by exposing it to attack by endonucleases, followed by nuclear destruction [Weaver *et al.*, 1996]. Studies of cell populations and of *in vitro* apoptotic systems concluded more specifically that disruption of the nuclear lamina, presumably due to solubilization after cleavage, is the key to chromatin collapse [Lazebnik *et al.*, 1993; Neamati *et al.*, 1995].

However, the relationship between disassembly of the nuclear matrix and collapse of chromatin has not been studied extensively at the level of individual cells. We hypothesized that disassembly of the nuclear matrix and of chromatin would occur in a spatially and temporally ordered sequence, reflecting the original organization of the nucleus. Our data indicate that chromatin fragmentation and collapse precede massive reorganization of non-chromatin components.

PI2 and lamin B

These antigens occupy distinct locations on the nuclear envelope. Whereas PI2

immunolocalizes to the outer nuclear membrane, lamin B faces the nucleoplasm [see Chaly *et al.*, 1989]. Heating did not affect the localization of PI2 or lamin B. The organization of lamins and a *Drosophila* nuclear pore complex glycoprotein, gp188, in earlier immunofluorescence studies of nuclear envelope antigens in heat-treated samples was similarly unaffected [McConnell *et al.* 1987; DeGraaf *et al.*, 1992]. However, the antigens showed distinct behaviours by immunoblotting, with cleavage of lamin B detected at t_2 as in earlier studies [Oberhammer *et al.*, 1994; Lazebnik *et al.*, 1995b; Neamati *et al.*, 1995; Weaver *et al.*, 1996], and PI2 remaining uncleaved [Weaver *et al.*, 1996]. By immunofluorescence, on the other hand, their behaviour during apoptosis was very similar. Both showed intact labelling in all TUNEL-normal nuclei and in the great majority of PC nuclei (more than 80%), but only partial or no labelling in most FC nuclei. Exceptionally, PI2 and lamin B staining was largely continuous in FC nuclei with chromatin in a ring at the nuclear envelope. As well, double-labelling showed that the two antigens were both lost from the same region(s) of the nuclear periphery, with PI2 retained slightly longer than lamin B. These results are consistent with immunofluorescence and electron microscopic observations showing that the nuclear envelope remains intact until late in apoptosis [Wyllie *et al.*, 1980; Lazebnik *et al.*, 1993]. The data in our study also demonstrate that proteolytic cleavage is not essential for solubilization from the nucleus, since PI2 is released but not cleaved. Loss of PI2 labelling may result from the loss of nuclear envelope integrity due to extensive degradation of the lamina. However, though lamin B proteolysis was detected within 2 hours by immunoblotting, substantial loss of lamin B immunolabelling was not visualized until t_6 , when FC nuclei predominate. This

suggests, therefore, that proteolytic cleavage of lamin B does not result in the immediate disruption of the lamina. These observations also indicate that, contrary to some hypotheses [Lazebnik *et al.*, 1993; Neamati *et al.*, 1995], chromatin collapse, which is first evident in PC nuclei, does not depend entirely on destruction of the lamina.

The results do suggest, however, that chromatin cannot be released from the nuclear periphery if the nuclear envelope is intact. First, we observed that, in FC nuclei with only partial lamin B/PI2 staining, the portion of the envelope lacking lamin B/PI2 also lacked apposed chromatin. Second, we found that in FC nuclei with ring-shaped chromatin at the envelope, staining for lamin B and PI2 was largely indistinguishable from controls. Staining for these antigens was disturbed only in FC nuclei in which chromatin had pulled away from the envelope into a single spheroidal mass. Lazebnik *et al.* [1995a] similarly reported that, *in vitro*, chromatin collapse away from the nuclear margin requires solubilization of lamins, and suggested that release of chromatin from the nuclear lamina is required to free chromatin at the nuclear periphery. In agreement with this hypothesis, DNA degradation and chromatin collapse were inhibited in rodent cells expressing mutant lamins A or B that are not cleaved during apoptosis [Rao *et al.*, 1996].

NuMA

Recently, NuMA has been identified in rat thymocytes as an early target for proteolysis during apoptosis [Weaver *et al.*, 1996]. Further, it was proposed that NuMA may be one of the key structural proteins that stabilize nuclear morphology, and that proteolysis of NuMA contributes to apoptotic nuclear destruction [Weaver *et al.*, 1996]. In this study

also, we detected proteolysis of NuMA within 2 hours of culture in both control and heat-treated samples, a timeframe similar to that reported earlier [Weaver *et al.*, 1996].

Other than in cells with heat-induced NuMA spots, which are discussed separately below, NuMA organization in control and heat-treated non-apoptotic splenocytes was similar to that described in other cell types. Moreover, its behaviour during apoptosis, both morphologically and biochemically, was as we have reported for apoptotic rat thymocytes [Weaver *et al.*, 1996]. The NuMA labelling pattern in TUNEL-normal cells was identical to that in freshly isolated splenocytes, indicating that chromatin fragmentation is initiated without marked changes in the organization of the nucleoplasm. Indeed, both the pattern and the intensity of NuMA labelling were largely unaffected until chromatin collapse was well advanced. However, significant degradation of NuMA was already detectable in t_2 samples, which contain only about 3% PC cells and 13% FC cells. These data suggest that, as for lamin B, NuMA cleavage and solubilization proceed independently.

In PC nuclei, the organization of NuMA could not be differentiated from that in TUNEL-negative cells. As noted earlier, labelling for both PI2 and lamin B also is intact in over 80% of the cells. As well as supporting the interpretation that PC nuclei are still in the early stages of apoptotic disintegration, these observations further suggest that both DNA fragmentation and initiation of chromatin collapse precede large-scale nuclear disassembly.

In contrast, NuMA staining was absent in FC nuclei with a single spherical chromatin mass, indicating that NuMA is fully solubilized in such nuclei. Since, as noted above, such

FC nuclei also usually exhibited little or no lamin B and PI2, these results also further justify the conclusion that this category of FC nuclei represent the most advanced stage of apoptotic nuclear destruction in these cultures.

On the basis of these data, we propose the following sequence of events in apoptotic nuclei: DNA fragmentation necessarily precedes partial chromatin collapse, which is rapidly followed by full collapse of the chromatin. Proteolytic cleavage of NuMA and of lamin B are initiated and proceed independently of DNA cleavage, and are also independent of NuMA and lamin B solubilization. The evidence suggests that solubilization of nuclear envelope components, including lamin B and, perhaps, PI2 and other yet unidentified proteins, is necessary for release of collapsed chromatin from the envelope. The role of NuMA solubilization is still unclear. If, however, as discussed below, NuMA is a fundamental protein of the nuclear matrix filamentous network, NuMA proteolysis may underly the disassembly of the network and overall nuclear collapse.

NuMA Spots

The association of NuMA with the nucleus during interphase and with spindle poles during mitosis served as the origin for its name, Nuclear-Mitotic Apparatus protein [reviewed in He *et al.*, 1995]. As we have confirmed, NuMA is a large nuclear protein with a molecular mass of 210-240 kDa. In most reports, NuMA is described as non-nucleolar and nucleoplasmic during interphase, distributed in a diffuse pattern punctuated to a greater or lesser extent by brighter patches [Price and Pettijohn, 1986; Kallajoki *et al.*, 1991; Maekawa *et al.*, 1991; Tousson *et al.*, 1991; Compton *et al.*, 1992; Yang *et al.*,

1992; Tang *et al.*, 1993; Zeng *et al.*, 1994b]. Differences between labelling patterns have been ascribed to recognition of different epitopes by various anti-NuMA antibodies, differences in fixation protocols, or recognition of different NuMA isoforms by the different antibodies [Compton *et al.*, 1992; He *et al.*, 1995].

However, NuMA spots such as reported here have never been described previously. Their significance is still unclear, but the data suggest that they may be an early marker for splenocytes committed to the apoptotic pathway. NuMA spots are observed immediately after heat treatment, in samples devoid of other signs of apoptosis. Moreover, they not only persist throughout apoptosis, but PC and FC nuclei exhibit NuMA spots disproportionately. However, since TUNEL-normal, PC and FC nuclei without spots are also observed in heated samples, formation of the spots is not essential for cell death. The proportion of cells with spots was very consistent from one experiment to the next, which argues against spot formation occurring at random in the population. Since splenocytes are a mixed cell population, including both T and B cells, it is possible that the spots are restricted to a lymphocyte subset.

Considerable further work is necessary to determine the morphological and functional significance of the NuMA spots. In general, the function of NuMA in the interphase nucleus is unclear, but its co-fractionation with the nuclear matrix of a variety of cells, including rat thymocytes [Weaver *et al.*, 1996], is suggestive of a structural role [Lydersen and Pettijohn, 1980; Kalajoki *et al.*, 1991; Zeng *et al.*, 1994a, 1994b]. Furthermore, immunogold electron microscopy has shown that NuMA is a component of a subset of nuclear matrix core filaments [Zeng *et al.*, 1994a]. The similarity of the predicted

structure of the rod domain of NuMA to that in intermediate filament proteins suggested that NuMA may be able to form coiled-coil dimers and to oligomerize into filaments [Compton *et al.*, 1992; Yang *et al.*, 1992; Zeng *et al.*, 1994a; Cleveland, 1995]. Evidence in favour of this hypothesis has been provided by *in vitro* NuMA assembly studies [Harborth *et al.*, 1995], and a more recent *in vivo* study of cells overexpressing NuMA in the cytoplasm [Saredi *et al.*, 1996] showing that NuMA can form dimers [Harborth *et al.*, 1995] as well as filamentous networks. NuMA may be, therefore, a fundamental component of the fibrogranular network characteristic of nuclear matrices [Berezney *et al.*, 1995], which would account for its pervasive nucleoplasmic localization. Since NuMA binds MARs/SARs *in vitro* [Ludérus *et al.*, 1994], it may play a role in forming or maintaining chromatin loops.

The prominence of NuMA by immunofluorescence and immunoblotting in resting splenic lymphocytes is particularly interesting in view of our earlier studies showing microscopically and biochemically that the matrix is very sparse and of low protein complexity in these cells [Setterfield *et al.*, 1983, 1985; Bladon *et al.*, 1988]. This may be further indirect evidence of the importance of NuMA in maintaining nuclear structure.

The significance of the spots, however, remains highly speculative. Zweyer *et al.* [1997] have described the appearance of fibrogranular bodies of appropriate size in apoptotic HL-60 cells that contain NuMA, as well as other matrix proteins. However, these bodies were not observed until late in the apoptotic process and are therefore unlikely to account for the NuMA spots.

The nuclear matrix is generally described as fibrillar, but Cook and co-workers [Hozák

et al., 1995] recently reported the existence of larger non-random structures they have termed "nodes" within the meshwork of core filaments. Moreover, they have detected lamin A, and to a lesser extent lamin B at the nodes. It may be that the NuMA spots represent concentrations of NuMA in such nodes. If so, it remains unclear why NuMA spots become evident only after heating. One possibility is suggested by reports that heating cells increases the amount of protein in nuclei isolated after heat treatment [Evan and Hancock, 1985; Littlewood et al., 1987; Chu et al., 1993; Wachsberger and Coss, 1993; Warters et al., 1993]. The increase is not due to import of excessive protein from the cytoplasm [Chu et al., 1993], but, rather, arises through tighter association of proteins with DNA and the nuclear matrix [Warters et al., 1993] and presumably involves a change in protein conformation.

It is possible, therefore, that NuMA becomes stabilized at nuclear matrix nodes by heat treatment. The implied conformational change in NuMA may render it more accessible within the nodes to the monoclonal antibody used in this study, revealing the NuMA spots. Altered immunofluorescence labelling patterns have been reported for other internal nuclear matrix proteins upon heat treatment including Topo II [McConnell et al., 1987], and a 160 kDa nuclear matrix antigen [DeGraaf et al., 1992]. It may be that the heat-altered labelling patterns of these other nucleoplasmic nuclear matrix proteins also reflect a modified association of the proteins with the nuclear matrix in response to heat.

Notably, increased protein content in the nucleus, and the rate of its removal, have been linked to heat-induced cell death [Kampinga et al., 1989]. Thermotolerant cells recover more quickly from increased nuclear protein mass, leading to speculation that heat

shock proteins (HSP) aid in the recovery of these cells [Kampinga *et al.*, 1989]. We were unable to detect the heat-inducible HSP 70 protein in heat-treated splenocyte samples, although the constitutive form of the protein was clearly visible both before and after heat treatment (see Appendix I, Fig. 27). Protein synthesis in unstimulated splenocytes occurs at very low levels [Setterfield *et al.*, 1983], and splenocytes may not be able to respond to heat by a rapid production of HSP 70. This may inhibit their recovery from disturbances in the organization of key structural proteins, including NuMA, and may explain the persistence of NuMA spots in affected splenocytes throughout apoptosis.

CHAPTER 3.

Differential effect of heat treatment on NuMA distribution and apoptosis in

T and B splenocytes

INTRODUCTION

The nuclear mitotic apparatus protein (NuMA) was named for its localization in the nucleus during interphase and at spindle poles during mitosis [Lydersen and Pettijohn, 1980]. The evidence indicates a function for NuMA in mitotic spindle organization and in post-mitotic nuclear reassembly [reviewed in He *et al.*, 1995], but its function in the interphase nucleus is less clear. However, NuMA is an abundant nuclear protein that co-fractionates with the nuclear matrix [Kalajoki *et al.*, 1991; Zeng *et al.*, 1994a]. Moreover, analysis of the cDNA predicted a coiled coil structure for NuMA [Compton *et al.*, 1992; Yang *et al.*, 1992] and NuMA has been localized to a subset of nuclear matrix core filaments by immunoelectron microscopy [Zeng *et al.*, 1994b]. Collectively, these data have led to the suggestion that NuMA may play a key role in nuclear matrix organization as a major component of nuclear matrix filaments [reviewed in Cleveland, 1995].

In keeping with this view, we [Sodja *et al.*, 1996; Weaver *et al.*, 1996] and others [Casiano *et al.*, 1996; Hsu and Yeh, 1996; Zweyer *et al.*, 1997] have shown that NuMA is an early target of apoptotic proteolytic cleavage. In microscopic studies of dexamethasone-treated rat thymocytes [Weaver *et al.*, 1996] and of spontaneously apoptotic resting mouse splenocytes in culture [Sodja *et al.*, 1996], we also found that the normally diffuse NuMA labelling reorganized during apoptosis into a residual mass excluded from the condensed chromatin in fully collapsed (FC) apoptotic nuclei. In apoptotic mouse splenocytes, moreover, DNA fragmentation, as detected by TUNEL, and chromatin collapse visualized by DAPI staining were generally manifest before any

detectable change in the NuMA immunolabelling pattern [Sodja *et al.*, 1996]. The significance of the time lag between proteolytic cleavage of NuMA early in apoptosis and the disintegration of NuMA organization late in the process is not clear. One possibility, however, is that NuMA fragmentation may play a signalling or regulatory role as the death program is initiated in the nucleus.

We have also investigated the behaviour of NuMA in mouse resting splenic lymphocytes during apoptosis hyper-induced by heat treatment [Sodja *et al.*, 1996]. Immediately after heating, we found that about half of the lymphocytes exhibited a unique pattern of NuMA labelling, consisting of many bright spots within the customary diffuse nucleoplasmic labelling. The remaining cells in these samples showed the control NuMA labelling pattern. Importantly, staining was spotty in essentially all cells in early stages of DNA fragmentation, as detected by TUNEL, and the spots were retained throughout subsequent stages of DNA fragmentation and apoptotic nuclear collapse [Sodja *et al.*, 1996]. These data suggested that the appearance of NuMA spots during heat treatment might be an indication that the cells had initiated the death program.

We were also interested by the fact that the spotty pattern was observed in only about half of the splenocytes after heating. Since splenocytes are a mixed lymphocyte population, consisting of approximately equal proportions of T and B lymphocytes, it seemed possible that the novel NuMA pattern might be restricted to one of these lymphocyte subsets.

In the present study, we have examined more closely the significance of the spotty NuMA labelling pattern during heat-induced lymphocyte apoptosis. The results indicate

that heat-treated cells with this pattern are targeted for, or have initiated, the death program. Moreover, the data show that, whereas it is B cells which die preferentially during spontaneous apoptosis, heat treatment induces apoptosis preferentially in the T splenocyte subset.

MATERIALS AND METHODS

Preparation of cells

Splenocytes were isolated from male Balb/c mice and processed as control or heat-treated samples as described in Chapter 2 and Appendix III.

Flow Cytometry

Control and heat-treated cells were fixed for flow cytometry at t_0 , t_6 , and t_{24} . Flow cytometry of propidium iodide fluorescence was carried out by the methods of Walker *et al.* [1991], as described in Chapter 2 and Appendix IV.

Immunofluorescence Labelling

Total and fractionated splenocyte populations on poly-L-lysine coated coverslips were fixed and permeabilized as previously described [Chaly *et al.*, 1984; Chaly *et al.*, 1988 and Appendix IV]. For single labelling experiments, cells were labelled sequentially with anti-NuMA, a mouse monoclonal IgG1 (1:25, A-204, Matritech) followed by CY3-conjugated goat anti-mouse IgG (Fc fragment specific)(1:200, Jackson Immunochemicals). All samples were counterstained with DAPI, mounted and viewed by conventional and confocal fluorescence microscopy as described in Chapter 2.

Enrichment of cell suspension for T or B Cells

Enrichment of total splenocyte populations for T or B cells was carried out at t_0 .

Control and heated samples were enriched for T cells as previously described [Wysocki and Sato, 1978]. For each sample, 2.5×10^7 cells were pelleted by centrifugation in a benchtop centrifuge, resuspended in 3 ml of PBS with 5% fetal calf serum (Gibco), and incubated for 70 min at 4°C in bacteriological grade petri dishes (Fisher Scientific) pre-coated with affinity purified goat anti-mouse IgG (H+L) (Jackson Immunochemicals) at 5 µg/ml in 0.05 M Tris-HCl pH 9.5. Non-adherent cells were collected by aspiration.

B cell enrichment was carried out by complement-mediated lysis as described in Roy [1984]. For each sample, 5.0×10^7 cells were pooled by centrifugation, resuspended in 1ml RPMI 1640 containing monoclonal mouse anti-Thy1.2 (1:800, Sigma), and incubated on ice for 1h. Cells were washed with 1ml RPMI medium, collected by centrifugation, resuspended in 1ml RPMI with rabbit complement (1:20, Cedarlane), and incubated at 37°C for 1h. Unlysed cells were washed twice with 3ml of RPMI and collected by centrifugation.

To determine the proportion of B cells in total and fractionated splenocyte populations, samples were processed for immunofluorescence staining of cell surface IgG. Cells were incubated on ice for 20 min in RPMI containing CY3-conjugated goat anti-mouse IgG (Fc fragment specific) (1:100, Jackson Immunochemicals), layered onto poly-L-lysine coated coverslips, fixed for 1 minute in 3% paraformaldehyde in PBS, and permeabilized for 2 minutes in 0.2% Triton X-100 in PBS. Samples were then counterstained with DAPI, mounted and viewed by conventional fluorescence microscopy as described in Chapter 2.

RESULTS

Kinetics of apoptosis

Previous data from this study have shown that heat treatment increases the rate of apoptosis with respect to controls within 6 hours (Fig. 1). To determine the effect of heat treatment on the rate of apoptosis over longer periods in culture, flow cytometry was carried out on samples cultured for up to 24 hours (Fig. 12). Figure 12A shows flow cytometry data for a single representative experiment. In Figure 12B, the percentages of cells exhibiting reduced propidium iodide fluorescence are presented as the means from three experiments. As described in Chapter 2, in both control and heat-treated samples fixed at t_0 , propidium iodide fluorescence produced a single peak corresponding to the DNA content of resting cells (Fig. 12A-B). The proportion of cells with a sub-diploid DNA content increased over time in culture for both samples but, by t_6 , was approximately double in heat-treated samples (36%) with respect to controls (15%). By 24h, greater than 50% of the population was apoptotic in both control and heated samples. However, in each experiment the proportion of apoptotic cells at 24h was at least 7% higher in heated samples than in control samples.

These data show that isolation and culture of splenocytes resulted in apoptosis in approximately half of the population within 24 hours, and confirm that heating cells at 42°C for 30 minutes approximately doubled the proportion of apoptotic cells in heated samples with respect to controls. Moreover, they show that, though heated samples continue to exhibit more apoptotic cells than controls by t_{24} , the magnitude of the

Figure 12: Kinetics of spontaneous and heat-induced apoptosis. Splenocytes were cultured at 37°C (control) or heat-treated at 42°C for 30 min, then cultured at 37°C for 0, 6, or 24 hours.

(A) Flow cytometric profiles of propidium iodide fluorescence from a single experiment for control and heat-treated splenocytes. Twenty thousand cells were counted for each sample.

(B) Histogram showing the percent apoptotic cells detected by flow cytometry in control (open bars) and heat-treated (solid bars) samples. Results are the means from 3 experiments; error bars show the standard deviation from the mean. The difference between control and heat-treated samples was significant ($p \leq 0.001$) at t_6 and t_{24} .

difference is reduced.

Behaviour of NuMA during spontaneous and heat-induced apoptosis

1) Kinetics of the appearance of NuMA spots

We have shown that a 30 minute heat treatment results immediately in a spotted NuMA pattern in approximately half of the cell population, and that this spotted NuMA pattern is retained in many heat-treated apoptotic nuclei (Fig. 3). To quantify these observations, the proportion of DAPI-normal, PC and FC nuclei with NuMA spots was determined by cell counting in samples labelled with anti-NuMA and DAPI (Fig. 13). In control samples, DAPI-normal (Fig. 13A) and FC (Fig. 13C) nuclei with NuMA spots were rare (<5%) at all time points. PC nuclei with NuMA spots were more frequent, rising to 20% of the PC population by 6h (Fig. 13B).

On the other hand, NuMA spots were observed in greater than 45% of the DAPI-normal population fixed after heat treatment, at t_0 . Moreover, the proportion of nuclei with NuMA spots in heated samples from t_2 to t_6 was much higher than that observed in controls: 30-60% of DAPI-normal nuclei (Fig. 13A); about 80% of PC nuclei (Fig. 13B); and 40-50% of FC nuclei (Fig. 13C).

These data confirm that heat treatment results in an immediate induction of NuMA spots in approximately half of the DAPI-normal cell population. This labelling pattern persists in DAPI-normal nuclei cultured after heat treatment, and is retained in about half of the heat-treated population with FC nuclei. The appearance of NuMA spots in DAPI-normal nuclei may be an early indication that these cells have initiated the death program.

Figure 13: Kinetics of the appearance of NuMA spots after heat treatment. Control and heat-treated samples were fixed at intervals between t_0 and t_6 and labelled with anti-NuMA and DAPI. The chromatin morphology and NuMA distribution pattern were evaluated for 500 DAPI-normal (D-N) (A) 100 PC (B) and 250 FC nuclei (C) in control (open bars) and heat-treated (solid bars) samples. Histograms show the mean proportion of nuclei with NuMA spots from three separate experiments; error bars show the standard deviation from the mean. In each histogram at all time points, the data from control samples were significantly different from the data for heat-treated samples, with $p \leq 0.03$.

(A)

(B)

(C)

2) Relationship between NuMA spots and duration of heat treatment

To determine the length of heat treatment necessary for the appearance of NuMA spots, splenocytes were heated for various times, fixed immediately after heat treatment, and labelled with anti-NuMA and DAPI. DAPI-normal cells with NuMA spots were counted in control and heated samples as a proportion of the population (Fig. 14A). In all control samples, less than 5% of DAPI-normal cells contained NuMA spots. However, in heated samples the proportion of cells with NuMA spots varied with the different heat treatments (Fig. 14A). A 5 min treatment was sufficient to increase the proportion of cells with NuMA spots to 30%. However, heating for 10 min resulted in NuMA spots in 66% of the population. With longer heat treatments of 20 and 30 min, the proportion of cells with spots dropped to 53% and 41% respectively.

Induction of apoptosis in these samples was monitored by flow cytometry of t_6 samples (Fig. 14B). In all control samples, <20% of the population was apoptotic by t_6 . The 5 and 10 minute heat treatments did not significantly increase apoptosis above control levels, but with longer treatments of 20 and 30 min the proportion of apoptotic cells increased significantly to 40% and 35% respectively (Fig. 14B).

The detection of NuMA spots within 5 min of heat treatment suggests that protein synthesis during heat treatment may not be required to produce NuMA spots. Furthermore, these results suggest that, during the longer 20 and 30 minute heat treatments, some cells may recover from induction of NuMA spots, i.e. return to the diffuse pattern. The cells which do not recover may then be destined for apoptosis.

Figure 14: Relationship between the appearance of NuMA spots, induction of

apoptosis and duration of heat treatment. Samples were heat-treated for 5, 10, 20, or 30 minutes at 42°C. Heat-treated and parallel control samples were then fixed and labelled with anti-NuMA and DAPI, or were cultured for a further 6 hours and processed for flow cytometry.

(A) Histogram showing the proportion of DAPI-normal (D-N) nuclei with NuMA spots in control (open bars) and heat-treated samples (solid bars) as determined by microscopy. Chromatin morphology and NuMA distribution were evaluated for 500 DAPI-normal nuclei in each sample. The data are the means from three experiments; error bars show the standard deviation from the mean. The data for control samples was significantly different ($p \leq 0.05$) from that in heat-treated samples at all time points. For the heat-treated samples, the data was significantly different ($p \leq 0.08$) at all time points.

(B) Histogram showing the percent apoptotic cells detected by flow cytometry in control (open bars) and heat-treated (solid bars) samples. Results are the means from 3 experiments; error bars show the standard deviation from the mean. The difference between control and heat-treated samples was significant ($p \leq 0.04$) only with the 20 and 30 minute heat treatments.

(A)

(B)

NuMA distribution in T and B cells

Splenocytes are a mixed population consisting of approximately equal proportions of T and B lymphocytes. Since NuMA spots were observed in approximately half of the heat-treated population, it seemed possible that NuMA spots were restricted to one of these lymphocyte subsets. To test this hypothesis, total, B-enriched and T-enriched populations were labelled with anti-NuMA and DAPI. To test for extent of enrichment, the proportions of B cells in total and enriched populations were determined by labelling of the cell surface with CY3-conjugated goat anti-mouse IgG and DAPI.

As expected, cell surface IgG labelling showed 45-50% IgG⁺ cells in the total splenocyte population (Fig. 15A, Fig. 17A-B). In samples enriched for B cells, about 80% of the population was labelled for cell surface IgG (Fig. 15B, Fig. 17A). In samples enriched for T cells, on the other hand, less than 4% of the population showed labelling of the cell surface (Fig. 15C, Fig. 17B).

NuMA labelling of total and fractionated populations then showed that NuMA spots were rare (<5%) in all control samples (Fig. 16A, Fig. 17C-D). However, after heat treatment, 45-50% of the total population (Fig. 16B, Fig. 17C-D), but only 25% of the B-enriched population (Fig. 16C, Fig. 17C) had NuMA spots. Strikingly, moreover, greater than 90% of the cells were spotty in the T-enriched population (Fig. 16D, Fig. 17D).

These results indicate that NuMA spots were heat-induced preferentially in T lymphocytes. We have previously shown (see Chapter 2) that all heat-treated nuclei in early stages of apoptotic DNA fragmentation contain NuMA spots. These data suggest further that heat treatment hyper-induced apoptosis in T lymphocytes.

Figure 15: Cell surface IgG labelling of total and fractionated splenocyte populations.

Control and heat-treated samples were incubated with CY3-conjugated IgG (A-C), fixed, and counterstained with DAPI (A'-C'). Surface IgG labelling is shown for control total (A-A'), B-enriched (B-B') and T-enriched (C-C') populations.

Magnification 1400X.

Figure 16: Distribution of NuMA in total and fractionated splenocyte populations.

Control and heat-treated samples were fixed and labelled with anti-NuMA (A-D) and DAPI (A'-D'). NuMA labelling is shown for control (A-A') and heat-treated (B-B') total cell populations, and heat-treated B-enriched (C-C') and T-enriched (D-D') populations. Magnification 1400X.

Figure 17: Relationship between NuMA spots and lymphocyte subsets. Total cells and fractionated populations were labelled with CY3-conjugated IgG and DAPI (A-B) or NuMA and DAPI (C-D).

(A-B) Histograms showing the proportion of IgG⁺ cells in total (A-B), B-enriched (A) and T-enriched (B) samples as determined by microscopy. Control total (open bars), heat-treated total (solid bars), control enriched (cross-hatched bars) and heat-treated enriched (hatched bars) samples were evaluated for the presence or absence of surface IgG labelling. For each sample, 500 DAPI-normal nuclei were counted. Histograms show the mean values from three separate experiments; error bars show the standard deviation from the mean. The proportion of IgG⁺ cells in unenriched samples was significantly different ($p \leq 0.03$) from that in B- or T-enriched samples.

(C-D) Histograms showing the proportion of DAPI-normal nuclei with NuMA spots in total (C-D), B-enriched (C) and T-enriched (D) samples as determined by microscopy. Control total (open bars), heat-treated total (solid bars), control enriched (cross-hatched bars) and heat-treated enriched (hatched bars) samples were evaluated for the presence or absence of NuMA spots. For each sample, 500 DAPI-normal nuclei were counted. Histograms show the mean values from three separate experiments; error bars show the standard deviation from the mean. The proportion of cells with NuMA spots in unenriched samples was significantly different from that in B- ($p=0.08$) or T-enriched samples ($p=0.03$).

Ratio of B to T cells at 24h

The surface IgG labelling data showed that the total splenocyte population consists of approximately equal proportions of T and B cells (Fig. 15A, Fig. 17A-B). We predicted that, if heat treatment hyper-induces apoptosis in T cells, the ratio of T to B cells would decrease over time to reflect the more rapid loss of T cells in these samples. To test this hypothesis, control and heated samples were cultured for 24h, labelled with CY3-conjugated IgG and DAPI, and the proportion of DAPI-normal cells that were IgG⁺ was determined by cell counting (Table 1). Although the percentage of IgG⁺ cells in control samples varied between experiments, it was consistently lower than in the heat-treated samples from the same experiment. Moreover, the ratio of IgG⁻:IgG⁺ cells ranged from 5.2:1 to 22:1 for control samples, but did not rise above 3.7:1 for heated samples.

These data, therefore, support the conclusion that heat treatment hyper-induces apoptosis in T cells. Moreover, they also suggest that spontaneous apoptosis occurred preferentially in B splenocytes.

Table 1. Effect of culture on the ratio of IgG⁻: IgG⁺ lymphocytes.

Experiment Number	% IgG-labelled DAPI-normal cells ^a		Ratio of IgG ⁻ :IgG ⁺ cells	
	control ^b	heat-treated ^b	control	heat-treated
Expt 1	4.5	27	22:1	3.7:1
Expt 2	19	30	5.2:1	3.3:1
Expt 3	12	28	8:1	3.6:1

^a Nuclei were evaluated as normal or apoptotic based on the morphology of chromatin by DAPI staining.

^b Splenocytes were cultured for 24.5h at 37°C (control), or heated at 42°C for 30 min, then cultured at 37°C for 24h (heated). Samples were incubated with CY3-conjugated IgG for 20 minutes on ice, then fixed and counterstained with DAPI. In three separate experiments, 200 DAPI-normal cells were evaluated for the presence or absence of cell surface IgG labelling.

DISCUSSION

It is well known that lymphocytes die spontaneously in culture. Previous studies have indicated that spontaneous apoptosis affects 20 to 50% of the cell population within 16 hours for cultures of thymocytes [Migliorati *et al.*, 1992; Clarke *et al.*, 1993; Perandones *et al.*, 1993; Rinner *et al.*, 1996], isolated splenic B cells [Illera *et al.*, 1993] and isolated splenic T cells [Perandones *et al.*, 1993]. In this study, surface IgG labelling was used to determine whether spontaneous or heat-induced apoptosis preferentially affected one of the lymphocyte subsets in a mixed population of T and B lymphocytes from the spleen. The results showed that, by 24 hours in culture, most of the remaining DAPI-normal cells were IgG⁻, suggesting that spontaneous apoptosis affected mainly the B cell subset. These results are in agreement with a recent flow cytometric study by Reap *et al.* [1995]. Using a combination of cell surface antigen labelling and DNA staining of total mouse splenic lymphocyte populations, they showed by flow cytometry that the B cells were more susceptible to spontaneous apoptosis than the T cells [Reap *et al.*, 1995].

There have also been reports that lymphocyte subsets may respond differently to a given apoptotic stimulus. For example, flow cytometric analysis of lymphocytes double-labelled for cell surface markers and DNA strand breaks has also shown that CD4⁺ cells are more resistant to irradiation *in vivo* than are CD8⁺ or CD4⁻CD8⁻ cells [Zhang *et al.*, 1995]. As well, flow cytometric analysis of DNA content has shown that mouse splenic T cells are more resistant to glucocorticoid-induced apoptosis than are thymocytes [Perandones *et al.*, 1993].

My results demonstrate a differential response of splenic B and T cells to heat treatment. Microscopic evaluation of surface IgG labelling showed that, by 24 hours, the ratio of DAPI-normal T cells to B cells was consistently lower in heat-treated samples than in controls. This decrease in the relative frequency of T cells indicates that heat treatment increased the proportion of apoptotic cells in total splenocyte cultures by increasing apoptosis in T cells. An increase in the rate of thymocyte cell death in response to heat has been previously reported [Sellins and Cohen, 1991; Migliorati *et al.*, 1992]. However, to my knowledge, this study is the first to show that mature T cells from the spleen are more susceptible to heat treatment than mature B cells.

The basis for the differential response of B and T cells to heat is not clear. The simplest explanation is a difference in the ability of B and T cells to withstand heating, with higher temperatures or prolonged heating required to hyper-induce apoptosis in B cells. However, B and T splenocytes may also differ in their ability to become thermotolerant during heating. That is, some B cells may acquire thermotolerance during the 30 minute treatment whereas T cells may require different heating conditions to acquire thermotolerance. Indeed, experiments using shorter heat treatments and NuMA labelling (see below) suggest that some B cells may recover during the heat treatment. It is also possible, however, that rather than increasing the number of T cells undergoing apoptosis, the heat treatment increases the rate at which they progress through apoptosis. Further experiments, testing for the development of thermotolerance, for example, are necessary to differentiate between these possibilities.

Splenic T and B cells also showed a differential response to heat treatment with respect

to the NuMA labelling pattern. After a 30 minute heat treatment, essentially all cells in T-enriched samples exhibited NuMA spots. B-enriched cell populations, on the other hand, exhibited mainly the control diffuse NuMA pattern. However, the difference between total and enriched populations was less dramatic in B-enriched samples. This is most likely because the B-enrichment protocol was less effective, with B-enriched populations still containing approximately 20% IgG⁻ cells. Nonetheless, the proportion of cells with NuMA spots in these samples did correlate with the extent of removal of T cells, suggesting that the cells with spots in B-enriched populations represented the remaining T cells.

The development of NuMA spots preferentially in T cells, which form about half of the total splenic lymphocyte population, explains our initial observation that about half of the population exhibited NuMA spots after heat treatment. Moreover, the lack of NuMA spots in B cells after heat treatment suggests that these cells may undergo apoptosis in heated samples without developing NuMA spots. This would account for the absence of NuMA spots in some of the PC and FC nuclei in heat-treated cultures.

On the other hand, after shorter heat treatments of 10 and 20 minutes, the proportion of cells with NuMA spots was higher than the 50% expected if only the T cell subset was affected. After shorter heat treatments, therefore, some of the lymphocytes with NuMA spots must be B cells. However, as the duration of the heat treatment increased up to 30 minutes, the proportion of cells with spots decreased. This suggests that, as heat treatment is prolonged, some cells with spots, "recover" the original diffuse labelling pattern. The data imply that it is the B cell subset which recovers a diffuse labelling

pattern after longer heat treatments, and that the T cells do not recover and proceed to apoptosis.

The significance of NuMA spots in heat-treated samples is unclear. However, the very rapid appearance of NuMA spots in some cells after a 5 minute heat treatment suggests that this response does not require biosynthesis. Moreover, we have shown by conventional and confocal microscopy that all of the heat-treated cells in early stages of apoptotic DNA fragmentation contain NuMA spots. Therefore, NuMA spots may be a very early indicator of heat-induced splenic T cell apoptosis. However, they are clearly not a universal feature of NuMA organization in dying cells, since they were rare in both T and B cells in control samples, and were undetectable in some apoptotic cells even in heat-treated cultures.

Apoptosis is thought to play a central role in the immune system, functioning in the physiological control of T and B cell numbers, in the prevention of autoimmune responses, and in pathological immune responses [reviewed in Cohen, 1995; Abbas, 1996]. Moreover, T cell apoptosis has been implicated in immune deficiency associated with thermal injury [Teodorczyk-Injeyan *et al.*, 1995]. The results of this study, showing a differential response of mature T and B lymphocytes to heat, may aid in understanding of the response of the immune system to fever or thermal injury.

CHAPTER 4.

NuMA may be a ubiquitinated protein in resting mouse splenocytes

INTRODUCTION

Ubiquitin is a highly conserved 76 amino acid protein with a tightly packed globular structure. Ubiquitination is a widespread post-translational modification in eucaryotic cells that has been implicated in a number of cellular functions, including intracellular protein turnover, cell cycle control, DNA repair, the stress response, and apoptosis. Ubiquitin-protein conjugation involves the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and amino groups on lysine residues in the acceptor protein. This reaction involves adenylation of ubiquitin by a conjugating enzyme (E1), followed by direct transfer of ubiquitin to the target protein by the conjugating enzyme (E2), or conjugation of ubiquitin to the target protein by a ligase (E3). Many of the proposed functions for ubiquitin are believed to involve ubiquitin-mediated proteolysis. Following conjugation of multiple ubiquitin molecules, the protein is degraded by an ATP-dependent protease complex called the 26S protease. Finally, an isopeptidase releases ubiquitin from the proteolysed protein [reviewed in Ciechanover, 1994; Jennissen, 1995].

The ubiquitin system has been linked to proteolysis of the tumour suppressor protein p53 and mitotic cyclins during the cell cycle, and of damaged or misfolded proteins [reviewed in Ciechanover, 1994; Jennissen, 1995]. Recently, increased activity of the ubiquitin system has been linked to apoptosis [Delic *et al.*, 1993; Haas *et al.*, 1995; Nakajima *et al.*, 1996]. Immunoblotting has indicated an increased synthesis of ubiquitin during radiation-induced lymphocyte apoptosis [Delic *et al.*, 1993], and during developmentally programmed cell death of insect skeletal muscle [Haas *et al.*, 1995].

Moreover, the increase in ubiquitin synthesis during cell death of insect skeletal muscle was accompanied by increased conjugation of radiolabelled ubiquitin to the E1 and E2 conjugating enzymes [Haas *et al.*, 1995]. In a recent study of adenovirus E1A-induced apoptosis using apoptotic cell extracts, the nuclear matrix protein, Topo II, was poly-ubiquitinated and degraded in a ubiquitin- and ATP-dependent manner [Nakajima *et al.*, 1996]. These results imply that the ubiquitin system may be involved in protein degradation during apoptosis [Delic *et al.*, 1993; Haas *et al.*, 1995].

In this study, immunofluorescence microscopy and immunoblotting data suggest that NuMA is a high molecular weight ubiquitinated protein in resting mouse splenocytes.

MATERIALS AND METHODS

Immunofluorescence Labelling

Isolation of splenocytes, preparation of t_2 control and heat-treated samples, and processing for immunofluorescence was carried out as described in the Methods section of Chapter 2 (see Appendix III and Appendix V). For single labelling of NuMA, samples were incubated sequentially with anti-NuMA, a mouse monoclonal IgG1 (1:25 in PBS, A-204, Matritech) followed by CY3-conjugated goat anti-mouse IgG (Fc fragment specific)(1:200, Jackson). Samples were counterstained for 5 min with Sytox Green (1:100, 000, Molecular Probes Inc.) in 2X SSC buffer (0.3 M NaCl, 0.03 M sodium citrate). For double-labelling experiments, cells were incubated with anti-ubiquitin, a rabbit antiserum (1:10, Sigma, U-5379), followed by FITC-conjugated donkey anti-rabbit IgG (heavy- and light-chain specific) (1:200, Jackson Immunochemicals). Samples were then washed in PBS, transferred to 0.15% gelatin in PBS for 20 min to block nonspecific binding, then labelled with anti-NuMA as described above. Double-labelled samples were counterstained with DAPI, mounted and viewed as described in Chapter 2.

SDS-PAGE and Immunoblotting

Whole cell lysates of control and heat-treated splenocytes were prepared at t_0 , t_2 , t_4 , and t_6 as described in Chapter 2. For immunoblotting, 20 μ g of protein from each sample were separated on 10% SDS-PAGE gels. Membranes were incubated in 2% Carnation skim milk in PBS (PBS-milk) to block nonspecific binding, and probed with anti-NuMA

(1:100, A-204, Matritech) or anti-ubiquitin (1:100, U-5379, Sigma) in PBS-milk for 1 hour at room temperature. Membranes were washed in PBS with 0.1% Tween-20, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (τ -chain specific)(1:500, Sigma Chemical Co.) or horseradish peroxidase-conjugated anti-rabbit IgG (1:3000, Promega) as appropriate for 1 hour, and washed with PBS. Detection was carried out by chemiluminescence (BM Chemiluminescence substrate, Boehringer Mannheim). If the same membrane was to be reused, it was stripped by incubation at 50°C for 30 min in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and washed three times for 20 minutes with PBS with 0.1% Tween-20 before probing with another primary antibody.

RESULTS

Relative distribution of NuMA and chromatin

To examine the distribution of NuMA in relation to chromatin by confocal microscopy, samples immunolabelled for NuMA were counterstained with Sytox Green, a DNA specific dye with spectral characteristics similar to fluorescein (Fig. 18). Single sections of NuMA labelling (Fig. 18A-B) and the corresponding sections of Sytox Green-labelled chromatin (Fig. 18A'-B') showed that NuMA labelling was diffuse in normal (Fig. 18A-A') and FC (Fig. 18B-B') nuclei in control samples. Further, the merged images showed that NuMA was nucleoplasmic and excluded from chromatin masses (Fig. 18A", Fig. 18B"). In heat-treated normal (Fig. 18C-C"), PC (Fig. 18D-D"), and FC (Fig. 18E-E") nuclei, NuMA spots were clearly visible within the diffuse labelling in confocal microscopy sections (Fig. 18C-E). The merged images indicated that NuMA spots were also located in the nucleoplasm, lying preferentially adjacent to chromatin masses (Fig. 18C", Fig. 18D", Fig. 18E").

These data confirm the results obtained by conventional fluorescence microscopy of samples labelled for NuMA and counterstained with DAPI (Fig. 3), and by confocal microscopy of apoptotic cells double-labelled for NuMA and DNA strand breaks (Fig. 5).

Relative distribution of NuMA and ubiquitin during apoptosis

In preliminary experiments monitoring the distribution of ubiquitin in heat-treated splenocytes, ubiquitin spots were observed that appeared similar in size and distribution

Figure 18: Confocal fluorescence microscopy of NuMA distribution in relation to chromatin. Control (A-A", B-B") and heat-treated (C-E") samples were fixed at t_2 and labelled with anti-NuMA (A-E), and with Sytox Green (A'-E'); the corresponding merged images are shown in A"-E". Labelling is shown for normal (A-A", C-C"), PC (D-D"), and FC (B-B", E-E") nuclei. Magnification 3000X.

A	A'	A''
B	B'	B''
C	C'	C''
D	D'	D''
E	E'	E''

to NuMA spots (not shown). To determine the spatial relationship of ubiquitin and NuMA labelling, control and heat-treated samples were fixed at t_2 , double-labelled with anti-ubiquitin and anti-NuMA, and examined by conventional and confocal fluorescence microscopy.

In control DAPI-normal nuclei, both conventional (Fig. 19A-A") and confocal microscopy (Fig. 20A) showed that labelling with anti-ubiquitin was diffuse and slightly granular, distributed throughout the nucleoplasm, and excluded from masses of condensed chromatin. A similar distribution of ubiquitin has been previously described in control mouse splenocytes [Roy *et al.*, 1992] and human peripheral blood lymphocytes [Delic *et al.*, 1993].

NuMA labelling showed the usual diffuse pattern described in Chapter 2 and appeared similar overall to the labelling with anti-ubiquitin (Fig. 19A', Fig. 20A'-A"). Moreover, in control FC nuclei as well, labelling with both antibodies was diffusely distributed around (Fig. 19A-A") or between (not shown) masses of collapsed chromatin. NuMA and ubiquitin labelling patterns were also similar in heat-treated nuclei (Fig. 19B-B", Fig. 19C-C"). As described in previous chapters, approximately half of the heat-treated DAPI-normal nuclei exhibited the control diffuse NuMA pattern (Fig. 19B'). In these cells, anti-ubiquitin labelling was also diffuse and was similar in intensity to the controls (Fig. 19B). In the other half of the DAPI-normal population, NuMA spots were visible in addition to the diffuse NuMA pattern (Fig. 19B'). In these nuclei, the diffuse ubiquitin labelling was also punctuated by bright spots (Fig. 19B). Furthermore, by conventional microscopy, it appeared that the ubiquitin spots and NuMA spots in DAPI-normal (Fig. 19B-B") and FC

Figure 19: Distribution of ubiquitin in relation to NuMA. Control (A-A'') and heat-treated (B-C'') samples were fixed at t_2 , labelled with anti-ubiquitin (A-C) and anti-NuMA (A'-C'), and counterstained with DAPI (A''-C''). The bottom cell in each photo is DAPI-normal, nonspotty. A-A'' - FC nucleus with diffuse ubiquitin and NuMA labelling. B-B'' - DAPI-normal nucleus spotty for both ubiquitin and NuMA. C-C'' - FC nucleus spotty for both ubiquitin and NuMA. Magnification 2600X.

Figure 20: Confocal fluorescence microscopy of ubiquitin distribution in relation to NuMA. Control (A-A") and heat-treated (B-C") samples were fixed at t_2 and labelled with anti-NuMA (A-C), and anti-ubiquitin (A'-C"); the corresponding merged images are shown in A"-C". Labelling is shown for DAPI-normal (A-A", B-B"), and FC (C-C") nuclei. Magnification 3100X.

A	A'	A''
B	B'	B''
C	C'	C''

nuclei (Fig. 19C-C") were largely co-localized. Confocal microscopy confirmed that, in heat-treated DAPI-normal and FC nuclei with the control pattern of diffuse NuMA labelling, anti-ubiquitin labelling was also diffuse (Fig. 20B-B", Fig. 20C-C"). However it consistently appeared somewhat more granular than the NuMA staining (Fig. 19B-B", Fig. 20C-C"). In heat-treated spotty nuclei, the NuMA and ubiquitin spots were co-localized in DAPI-normal (Fig. 20B-B") and in apoptotic (Fig. 20C-C") nuclei.

Biochemical analysis of NuMA and ubiquitin

The immunolabelling results above raised the possibility that NuMA is a ubiquitinated protein. As an approach to testing this hypothesis, whole cell lysates were prepared from control and heat-treated samples at intervals from t_0 to t_6 , and were processed for immunoblotting with anti-NuMA and anti-ubiquitin (Fig. 21).

Immunoblotting with anti-NuMA detected two bands at 230 and 210 kDa in all samples (Fig. 21A). A 180 kDa band was observed within 30 min to 1 hour of t_0 in both control and heated samples. This band increased in intensity over the 6 hour period in both samples.

Anti-ubiquitin detected a smear of label in the high molecular weight range in all samples (Fig. 21B). Bands at 230 and 210 kDa were visible within the smear of label in control samples at all time points. In heat-treated samples, on the other hand, the diffuse labelling in this area was too intense to allow individual bands to be visualized. However, in t_4 and t_6 samples, the 210 kDa band was observed. Furthermore, a 180 kDa band was labelled with anti-ubiquitin in control t_1 - t_6 samples and in heat-treated t_2 to t_6 samples. The

Figure 21: Immunoblotting analysis of NuMA and ubiquitin. Immunoblotting anti-NuMA (A) and anti-ubiquitin (B) was carried out on 10 % SDS-PAGE gels loaded with 20 µg of protein per lane. Samples were whole cell lysates prepared from control (lanes 1-6) and heat-treated samples (lanes 7-12) processed after heat treatment (1, 7), or after further culture at 37°C for 30 minutes (2, 8), 1 hour (3, 9), 2 hours (4, 10), 4 hours (5, 11) or 6 hours (6, 12). Molecular weight standards are shown in kDa.

A

B

smear of labelling in the high molecular weight range in control samples may be due to the presence of several high molecular weight ubiquitinated proteins. Alternatively, the smear may be due to the presence of high molecular weight ubiquitin polymers. However, the detection within the smear of bands with the same mobility as NuMA products indicates that NuMA may be one of the ubiquitinated proteins. In addition, the appearance over time in culture of a smaller 180 kDa band, labelled by both anti-NuMA and anti-ubiquitin antibodies, suggests that NuMA remained ubiquitinated during apoptotic degradation.

DISCUSSION

We have previously used confocal microscopy to compare the relative distribution of NuMA and DNA strand breaks labelled with TUNEL (Fig. 5). The results showed that both the diffuse NuMA pattern and NuMA spots were nucleoplasmic and excluded from TUNEL-labelled chromatin. Moreover, NuMA spots lay preferentially adjacent to TUNEL-labelled chromatin. Conventional fluorescence microscopy of samples labelled with anti-NuMA and DAPI suggested a similar distribution of NuMA with respect to intact DNA (Fig. 3). In this study, we have confirmed these observations by confocal microscopy using Sytox Green to monitor the distribution of both intact and fragmented chromatin.

There is only a small number of well-characterized ubiquitin-protein conjugates, including histones and cyclins [reviewed in Jennissen, 1995]. However, immunoblotting with anti-ubiquitin antibodies [Haas and Bright, 1985; Parag *et al.*, 1987], and autoradiographic detection of microinjected radiolabelled ubiquitin [Carlson *et al.*, 1987; Carlson and Rechsteiner, 1987] have indicated the presence of high molecular weight ubiquitin-protein conjugates in rat hepatoma [Parag *et al.*, 1987], HeLa [Carlson *et al.*, 1987; Carlson and Rechsteiner, 1987], human erythroleukemia and African green monkey kidney [Haas and Bright, 1985] cells, but their identity was not established. In this study, immunoblotting of control and heat-treated splenocytes showed similar bands detected by anti-NuMA and anti-ubiquitin antibodies. These data tentatively identify NuMA as a high molecular weight ubiquitinated protein in resting mouse splenocytes.

Immunoprecipitation of NuMA with anti-ubiquitin antibodies, or detection of immunoprecipitated NuMA with anti-ubiquitin antibodies would provide more conclusive evidence for ubiquitination of NuMA in these cells.

If NuMA is proven to be ubiquitinated in resting splenocytes, it will be necessary to determine the purpose of this ubiquitination. Clearly, one function may be to tag the protein for degradation during apoptosis. In this study, immunofluorescence and immunoblotting both suggest that NuMA is ubiquitinated in resting splenocytes, and remains ubiquitinated during apoptosis. Proteolysis of NuMA has been reported to be an early event during apoptosis of thymocytes [Weaver *et al.*, 1996], Jurkat T [Casiano *et al.*, 1996], HL-60 [Hsu and Yeh, 1996; Zweyer *et al.*, 1997], and HeLa cells [Hsu and Yeh, 1996]. However, the specific proteases involved in the degradation of NuMA are unknown [Casiano *et al.*, 1996; Hsu and Yeh, 1996; Weaver *et al.*, 1996; Zweyer *et al.*, 1997]. It is possible that the ubiquitin system is involved in the apoptotic cleavage of NuMA.

If NuMA is indeed ubiquitinated in resting splenocytes, the NuMA-ubiquitin conjugate may be stable in the resting G₀ nucleus. Insight into the role of NuMA ubiquitination will be obtained by determining the number of ubiquitin molecules conjugated to NuMA in freshly isolated and apoptotic lymphocytes. Evidence from other studies suggests that multi-ubiquitinated, but not mono-ubiquitinated proteins become targets for the 26S protease. Histones, for example, are mono-ubiquitinated, but this modification does not target the proteins for degradation. The mechanism for degradation of stable mono-ubiquitinated proteins like histones is unknown [reviewed in Jennisson, 1995]. With

respect to splenocyte apoptosis, it is possible that NuMA exists in a mono-ubiquitinated form in the healthy resting cell, and that it is multi-ubiquitinated and subsequently degraded during apoptosis.

In this study, variations in the fluorescent intensity of ubiquitin labelling were rarely observed during apoptosis, suggesting that spontaneous and heat-induced apoptosis in mouse splenocytes are not accompanied by increased ubiquitin synthesis. Similarly, Northern blotting did not detect an increase in levels of ubiquitin mRNA during glucocorticoid-induced apoptosis in mouse thymocytes or mouse T cell hybridomas [Schwartz *et al.*, 1993]. In contrast, however, increased synthesis of ubiquitin during VM-26-induced apoptosis in resting mouse splenocytes was suggested by intense ubiquitin fluorescence in apoptotic cells [Roy *et al.*, 1992]. Moreover, immunofluorescence and Northern blotting have detected increased levels of ubiquitin and ubiquitin mRNA during radiation-induced apoptosis of human lymphocytes [Delic *et al.*, 1993]. It is possible, therefore, that the expression of ubiquitin during apoptosis varies with the apoptotic stimulus. It has been suggested further that, in certain cell types, levels of free ubiquitin may be sufficient for ubiquitin-protein conjugation and ubiquitin-mediated proteolysis during apoptosis [Haas *et al.*, 1995]. Metabolically inactive cells, such as splenocytes, might utilize the ubiquitin in these pools for ubiquitin-protein conjugation.

NuMA ubiquitination may also serve other purposes in lymphocytes. For instance, protein ubiquitination appears to be involved in developmentally programmed cell death of insect skeletal muscle [Haas *et al.*, 1995]. Also, in the maturing reticulocyte, a cell type undergoing extensive cellular reorganization during the process of becoming an

erythrocyte, substantial activity of the ubiquitin system has been reported [Mayer *et al.*, 1991]. Such studies have led to the hypothesis that ubiquitin-mediated proteolysis functions in various types of developmental situations requiring extensive cellular reorganization [Mayer *et al.*, 1991; Haas *et al.*, 1995].

Resting lymphocytes are poised between apoptosis and stimulation, both of which require extensive remodelling of the cell, and in particular of the nucleus. Activation of resting lymphocytes with mitogens such as Con A results in a greater than 5 fold increase in nuclear volume and a nearly 10-fold increase in the volume of interchromatinic material. The sparse nucleoplasmic regions in the unstimulated cell are replaced by an extensive network of granular and fibrillar material. These changes in morphology are dependent on protein synthesis, and result in the stable incorporation of much newly synthesized protein into a more elaborate nuclear matrix [Setterfield *et al.*, 1983]. However, the mechanism by which the nuclear matrix is remodelled during stimulation is unknown. One possibility is that new matrix is simply built upon the matrix present in resting cells. However, another possibility is that the matrix in resting cells is degraded and that a new matrix is rebuilt during stimulation. Degradation of key structural proteins of the nuclear matrix, like NuMA, may contribute to nuclear destabilization and collapse during apoptosis on the one hand [Weaver *et al.*, 1996], or to nuclear remodelling during stimulation on the other.

Recent evidence supports the involvement of the ubiquitin system in degradation of nuclear matrix proteins during apoptosis. In a study of adenovirus E1A-induced apoptosis in a human epithermoid cell line MA1, apoptotic cell extracts were used to examine Topo II degradation [Nakajima *et al.*, 1996]. Topo II became multi-ubiquitinated in the

extracts, and was degraded in a ubiquitin- and ATP-dependent manner [Nakajima *et al.*, 1996]. Furthermore, protease inhibitors specific for the 26S protease inhibited Topo II degradation in the extracts [Nakajima *et al.*, 1996]. Serine protease inhibitors, on the other hand, did not prevent Topo II degradation [Nakajima *et al.*, 1996]. Serine protease inhibitors were also unable to prevent apoptotic cleavage of NuMA in glucocorticoid-treated thymocytes [Weaver *et al.*, 1996]. It would be interesting to determine whether NuMA degradation could be prevented by inhibitors of the 26S protease.

Yet, other functions for ubiquitination of NuMA are also possible. For instance, ubiquitination of core histones is thought to play a role in transient associations of chromatin with the nuclear matrix [Davie, 1996]. Based on variations in the levels of ubiquitinated histones during the cell cycle, and enrichment of ubiquitinated histones in the *Drosophila* HSP70 gene, it has also been suggested that reversible ubiquitination of histones may influence gene transcription and chromosome condensation [reviewed in Finley and Chau, 1991; Davie, 1996]. With respect to NuMA, recent evidence suggests that phosphorylation may mediate the participation of NuMA in mitotic spindle organization, and in nuclear reassembly following mitosis [Compton and Luo, 1995; Sparks *et al.*, 1995]. It is possible that ubiquitination, like phosphorylation, may be involved in regulating association of NuMA with chromatin, and/or with the mitotic spindle.

According to a recent study, proteolysis of NuMA may occur during mitosis. Hsu and Yeh [1996] recently reported detection of a truncated form of NuMA during mitosis of HeLa cells. The 180 kDa truncated NuMA, which was similar in size to the cleavage

product reported during apoptosis [Casiano *et al.*, 1996; Weaver *et al.*, 1996; Zweyer *et al.*, 1997], disappeared at the end of mitosis. These results led the authors to suggest that NuMA is present in a stable form during interphase, but that a proportion of NuMA is degraded during mitosis [Hsu and Yeh, 1996]. The authors proposed that NuMA cleavage during mitosis and apoptosis may be carried out by a common protease, and may be required for release of NuMA from the nuclear matrix [Hsu and Yeh, 1996]. It is possible that ubiquitin-mediated proteolysis is involved in both of these processes.

The possible functions for ubiquitination of NuMA listed above are purely speculative. It is clear that further experimentation will be required to confirm that NuMA is ubiquitinated in mouse splenocytes. Moreover, it will be necessary to determine whether NuMA is ubiquitinated in other cell types, and if so, to determine the function(s) of NuMA ubiquitination.

CHAPTER 5.

**Complex effect of protein synthesis inhibition on nuclear events during spontaneous
and heat-induced splenocyte apoptosis**

INTRODUCTION

Apoptosis was originally defined biochemically by the detection of DNA fragments, multiples of 200 bp in size, that appeared on agarose gels as a "DNA ladder" [Wyllie, 1980]. These fragments were interpreted to be the result of activation of an endonuclease which digests chromatin in the linker regions between nucleosomes [Wyllie, 1980]. More recently, it has been shown that, during apoptosis, DNA is sequentially cleaved into fragments of 200-300 kbp and 30-50 kbp prior to, and sometimes even in the absence of, internucleosomal DNA cleavage [Walker *et al.*, 1991; Brown *et al.*, 1993; Oberhammer *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994]. The sequential degradation of DNA during apoptosis is thought to reflect the higher order folding of chromatin in the nucleus, and to result from release of loops (50 kb) and rosettes (300 kb) of DNA [Filipski *et al.*, 1990; Walker *et al.*, 1991; Brown *et al.*, 1993; Oberhammer *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994].

The morphological collapse of chromatin during apoptosis also occurs in stages. Chromatin first marginates into smooth masses at the nuclear periphery, then collapses further into a variety of irregular shapes or into a single ball (see Chapter 2). Finally, chromatin may fragment into a number of spherical bodies [Wyllie *et al.*, 1980; Earnshaw, 1995]. The extent of chromatin collapse has been linked to the extent of DNA fragmentation. In thymocytes, partial collapse of chromatin toward the nuclear periphery correlates with the production of large DNA fragments [Cohen *et al.*, 1992; Weaver *et al.*,

1993; Cohen *et al.*, 1994; Sun *et al.*, 1994; Walker *et al.*, 1994]. On the other hand, Hara *et al.* [1996] recently showed that, in HL60 cells, fragmentation of the nucleus into small round chromatin-containing bodies is related to oligonucleosomal DNA fragmentation.

A number of studies have examined whether protein synthesis is necessary for DNA fragmentation during lymphocyte apoptosis. The data suggest that the requirement for protein synthesis depends on the apoptotic stimulus and the developmental stage of the cells. Whereas oligonucleosomal DNA fragmentation has been reported to be protein synthesis-dependent during thymocyte apoptosis induced by radiation or drug treatments [Sellins and Cohen, 1991; Cohen *et al.*, 1992; Migliorati *et al.*, 1992; Perandones *et al.*, 1993; Weaver *et al.*, 1993; Sun *et al.*, 1994; Rinner *et al.*, 1996], it appears to be protein synthesis-independent during spontaneous or heat-induced thymocyte apoptosis [Sellins and Cohen, 1991; Migliorati *et al.*, 1992; Weaver *et al.*, 1993; Rinner *et al.*, 1996]. Moreover, glucocorticoid-treated mouse thymocytes and mature mouse spleen T cells have been reported to differ in their dependence on protein synthesis with respect to DNA fragmentation [Perandones *et al.*, 1993].

We have previously shown that heating splenocytes results in a rapid change in NuMA organization. The immediate appearance of NuMA spots after a 5-30 minute heat treatment suggested that protein synthesis was not required for this response. To examine more closely the role of protein synthesis in the appearance of NuMA spots, the behaviour of NuMA was compared by immunofluorescence microscopy in control and heat-treated splenocytes treated with cycloheximide. Unexpectedly, both the proportion of DAPI-normal cells with NuMA spots in heat-treated populations, and the persistence of the spots

during heat-induced apoptosis increased when protein synthesis was inhibited.

These observations prompted an examination of the effect of protein synthesis inhibition on other nuclear events during spontaneous and heat-induced apoptosis. Splenocyte populations cultured with or without CH were monitored for DNA content by flow cytometry, and for plasma membrane integrity by Trypan Blue staining. In individual cells, DNA fragmentation and chromatin collapse were examined in cells labelled with TUNEL and DAPI respectively, and the behaviour of peripheral nuclear antigens was monitored by immunofluorescence microscopy.

The data show that inhibition of protein synthesis did not prevent either spontaneous or heat-induced cell death. They also indicate that DNA fragmentation, chromatin collapse, and loss of plasma membrane integrity occur independently of protein synthesis during spontaneous apoptosis in mouse splenocytes. In contrast, the results suggest that, during heat-induced splenocyte apoptosis, DNA fragmentation and chromatin collapse do not proceed to completion in the absence of protein synthesis. The behaviour of peripheral nuclear antigens proceeded as previously described, independently of protein synthesis and heat treatment.

MATERIALS AND METHODS

Cell Preparation and Cycloheximide treatment

Splenocytes were isolated from male Balb/c mice in the absence of cycloheximide (CH), and processed as control or heat-treated samples as described in Chapter 2 (see Appendix III). For CH-treated samples, the excised spleen was immediately placed into RPMI containing 5 $\mu\text{g/mL}$ CH (Sigma). Cells were processed as control or heat-treated samples in RPMI with CH, and were cultured for up to 24 hours in complete medium with CH.

Flow cytometry

Control and heat-treated cells with or without CH ($\pm\text{CH}$) were fixed for flow cytometry at t_0 , t_6 , and t_{24} . Flow cytometry of propidium iodide fluorescence was carried out by the methods of [Walker *et al.*, 1991], as described in Chapter 2 (see Appendix IV).

Trypan Blue staining

Control and heat-treated cells $\pm\text{CH}$ were assayed for Trypan Blue exclusion at t_0 , t_6 , and t_{24} . Aliquots of cell suspension (100 μL) were mixed with 100 μL of Trypan Blue solution (1:4, 0.425% Trypan Blue: 4.25% NaCl). The mixture was loaded on a haemocytometer, and the number of stained and unstained cells in the culture was determined by cell counting on a Leitz inverted light microscope. For each sample, the percentage of unstained or stained cells was calculated by dividing the number of

unstained or stained cells by the initial seeding density (2.5×10^6 cells).

Immunofluorescence labelling

Control and heat-treated samples $\pm$ CH were harvested at t_2 or t_6 , layered onto poly-L-lysine coated coverslips, and processed for immunofluorescence staining as previously described [Chaly *et al.*, 1984; 1988] (see Appendix V). For single labelling of NuMA, samples were then incubated sequentially in PBS-diluted primary and secondary antibodies. Primary antibodies were: anti-NuMA, a mouse monoclonal IgG1 (1:25, A-204) (Matritech); anti-W1, a rabbit anti-peptide serum (1:500) [Tang *et al.*, 1993]; anti-NuMA_{whole}, a rabbit serum against full length recombinant human NuMA (1:2500, gift of Duane Compton); and anti-NuMA_{ctb}, a rabbit serum against C-terminal 50 kD of recombinant human NuMA (1:2500, gift of Duane Compton). Secondary antibodies used as appropriate were: CY3-conjugated donkey anti-rabbit IgG (heavy- and light-chain specific) (1:200, Jackson) and CY3-conjugated donkey anti-rabbit IgG (heavy- and light-chain specific) (1:200, Jackson). All samples were counterstained with DAPI, mounted, and viewed by conventional fluorescence microscopy as described in Chapter 2.

Labelling of DNA strand breaks

Control and heat-treated splenocytes $\pm$ CH were fixed at t_6 , assayed for DNA strand breaks by TUNEL [Gavrieli *et al.*, 1992; Weaver *et al.*, 1996], counterstained with DAPI, mounted and viewed by conventional fluorescence microscopy as described in Chapter 2. For samples double-labelled for immunofluorescence and TUNEL, antibody labelling was

carried out first, as described above. The primary antibodies used were antibody PI2 (1:100) [Chaly *et al.*, 1984]; anti-NuMA (1:25, A-204) (Matritech); and anti-lamin B (1:500) [Chaudhary and Courvalin, 1993]. To block non-specific binding, samples were incubated in 0.15% gelatin in PBS for 20 min. Samples were then washed in PBS and processed for TUNEL. Double-labelled samples were counterstained with DAPI, mounted, and viewed by conventional fluorescence microscopy as in Chapter 2.

RESULTS

Protein synthesis inhibition and splenocyte apoptosis

To determine the effect of protein synthesis inhibition on spontaneous and heat-induced apoptosis, splenocytes were isolated in the presence or absence of 5 $\mu\text{g/mL}$ cycloheximide (CH), processed as control (C $\pm$ CH) or heat-treated (HT $\pm$ CH) samples, and cultured with or without CH for up to 24 hours. All samples were monitored for plasma membrane integrity by Trypan Blue staining (Fig. 22A-B), DNA fragmentation in individual cells labelled with TUNEL and DAPI (Fig. 22C), and DNA content by flow cytometry (Fig. 22D-E).

To determine whether protein synthesis inhibition affects cellular viability over time in culture, samples were cultured for up to 24 hours and evaluated for exclusion of the charged dye, Trypan Blue (Fig. 22A-B). Figure 22A shows the number of cells that excluded Trypan Blue as the means from three experiments. In all samples at t_0 , greater than 90% of the cells were unstained. These data indicate that CH treatment did not induce necrosis in control or heat-treated samples. At t_6 , all samples but HT-CH still contained 65-80% unstained cells. In HT-CH samples at t_6 , only 45% of the cells still excluded Trypan Blue. However, by 24 hours, less than 40% of control cells, and less than 24% of heat-treated cells excluded Trypan Blue regardless of the presence of CH.

The percentage of Trypan Blue-stained cells from these experiments was also examined to determine whether cells with impaired plasma membrane integrity were lost over time in culture. The data are shown in Figure 22B. At all time points, the total number of stained

Figure 22: Effect of protein synthesis inhibition on nuclear events during spontaneous and heat-induced apoptosis. Control (C) and heat-treated (HT) splenocytes were cultured in the presence (+CH) or absence (-CH) of 5 $\mu\text{g/mL}$ cycloheximide for up to 24 hours. For all histograms, results are the means from 3 experiments; error bars show the standard deviation from the mean.

- (A-B)** Histograms showing the percentage of cells that were unstained (A) or stained (B) with Trypan Blue in control (open bars), heat-treated (cross-hatched bars), control with CH (solid bars), and heat-treated with CH (hatched bars) samples. At t_6 only, the data for the heat-treated samples were significantly different ($p \leq 0.04$) from the controls and from the heat-treated samples with CH.
- (C)** Histogram showing the percentage TUNEL-positive cells at t_6 in control (open bars), heat-treated (cross-hatched bars), control with CH (solid bars), and heat-treated with CH (hatched bars) samples. The data for control samples were significantly different ($p \leq 0.01$) from the data for heat-treated samples.
- (D)** Histogram showing the percent apoptotic cells detected by flow cytometry in control (open bars), heat-treated (cross-hatched bars), control with CH (solid bars), and heat-treated with CH (hatched bars) samples. At t_6 only, the data for the heat-treated samples were significantly different ($p \leq 0.05$) from the controls and from the heat-treated samples with CH.
- (E)** Flow cytometric profiles of propidium iodide fluorescence from a single experiment for control and heat-treated splenocytes cultured with or without CH.

plus unstained cells was close to the initial number of cell in the culture, indicating that cells that no longer excluded Trypan Blue were retained in culture for up to 24 hours. At t_0 , all samples contained less than 9% Trypan Blue positive cells. At t_6 , 20-24% of the population in C $\pm$ CH and HT+CH samples was Trypan Blue positive. In HT-CH samples, on the other hand, nearly half (45%) of the population was stained with Trypan Blue. By 24 hours in culture, greater than 50% of the population was Trypan blue positive in C $\pm$ CH samples. In HT $\pm$ CH samples, on the other hand, greater than 70% of the population stained with Trypan Blue at 24 hours.

These data show that CH treatment delayed loss of plasma membrane integrity in heat-treated samples cultured for 6 hours. However, over 24 hours in culture, protein synthesis inhibition did not affect cell viability in control or heat-treated samples.

The effect of CH on induction of apoptosis was examined by monitoring the appearance of cells with fragmented DNA by TUNEL. Figure 22C shows the percentage of TUNEL-positive cells at t_6 as the means from three experiments. CH treatment showed no effect on the proportion of labelled cells. In C $\pm$ CH samples, less than 12% of the population was TUNEL-positive at t_6 . HT-CH samples contained more than double the control proportion (30%) of TUNEL-positive cells at t_6 , as expected, and so did the HT+CH population.

To verify these results, cells were processed for flow cytometry (Fig. 22D-E). In Figure 22D, the percentage of cells exhibiting reduced propidium iodide fluorescence are presented as the means from three experiments. Figure 22E shows flow cytometry data for a single representative experiment. In all samples at t_0 , a single peak was detected

corresponding to the DNA content of resting cells (Fig. 22D-E). These data indicate that CH treatment had no immediate effect on cellular DNA content. After 6 hours in culture, approximately 15% of the C±CH populations had a hypodiploid DNA content. Consistent with previous experiments, HT-CH samples showed 39% of the population in the sub-G1 peak, approximately double that in controls. Surprisingly, however, only 14% of the population exhibited a hypodiploid DNA content in HT+CH samples at t_6 , a percentage similar to that detected in controls. By 24 hours, though, greater than 50% of the population had a reduced content of DNA in all samples.

Both the TUNEL and the flow cytometry data suggest that CH had no effect on the level of spontaneous apoptosis. However, with respect to heated samples, the data appear contradictory. Heat hyper-induced apoptosis was protein synthesis-independent according to TUNEL, but appeared protein synthesis-dependent by flow cytometry. This contradiction may reflect the DNA fragmentation events detected by the two methods. The flow cytometric method is based on extraction of low molecular weight fragments of DNA from ethanol-permeabilized cells [Darzynkiewicz *et al.*, 1994; Sgonc and Wick, 1994]. Therefore, a reduction in size of the sub-diploid peak in HT+CH samples may represent reduced oligonucleosomal DNA fragmentation in these cells. TUNEL, on the other hand, detects DNA strand breaks *in situ* and would therefore be expected to detect both high molecular weight and low molecular weight DNA cleavage events during apoptosis. The data suggest, therefore, that protein synthesis may be required for oligonucleosomal DNA fragmentation, but not for high molecular weight DNA fragmentation during heat-induced apoptosis. They also suggest that protein synthesis is

not required for high or low molecular weight DNA fragmentation during spontaneous apoptosis. Moreover, a delay in loss of plasma membrane integrity by Trypan Blue staining appears to correlate with the low proportion of hypodiploid cells in HT+CH samples. It is possible that oligonucleosomal DNA fragmentation and loss of plasma membrane integrity are controlled by the same regulatory pathway.

Protein synthesis inhibition and chromatin morphology

The flow cytometry data indicated that protein synthesis inhibition changed the pattern of DNA fragmentation in heat-treated samples, but not in control samples. To determine whether protein synthesis inhibition also had an effect on chromatin collapse during heat-induced apoptosis, the morphology of chromatin in these cells was examined in samples labelled with TUNEL and DAPI.

As shown above, the proportion of TUNEL-positive cells within the total population at t_6 was unaffected by CH treatment. The morphology of the collapsed chromatin, however, depended on the treatment the cells received. At t_6 , the TUNEL-positive population was made up almost exclusively of FC nuclei in C±CH and HT-CH samples (Fig. 23A-A', Fig. 23B-B', Fig. 23C-C'). These nuclei contained chromatin collapsed into various shapes, ranging from irregular, through near-spherical, to spherical as described in Chapter 2. However, in HT-CH samples, FC nuclei with a single spherical mass of chromatin were observed much more frequently than in any other sample (Fig. 23C-C'), as confirmed by cell counting (Table 2). The proportion of nuclei with a single spherical chromatin mass was less than 5.2% in C±CH samples, and was even lower at $1.2 \pm 0.4\%$

Figure 23: Effect of protein synthesis inhibition on chromatin collapse during spontaneous and heat-induced apoptosis. C-CH (A-A'), C+CH, (B-B'), HT-CH (C-C') and HT+CH (D-D') samples were fixed at t_g and labelled with TUNEL (A-D) and DAPI (A'-D'). Irregularly-shaped FC nuclei are shown in all samples. FC nuclei with a single spherical mass of chromatin (arrowheads) and PC-like nuclei (arrow) are shown for HT-CH and HT+CH samples respectively. Magnification 1600X.

Table 2. Effect of protein synthesis inhibition on chromatin morphology during spontaneous and heat-induced apoptosis.

Samples ^a	% PC nuclei at $t_2^{b,c}$	% PC-like nuclei at $t_6^{b,c}$	% FC nuclei with a single spherical mass of chromatin at $t_6^{b,c}$
C-CH	0.13 ± 0.1	0.1 ± 0.2	5.2 ± 2
C+CH	0.06 ± 0.1	0	5.1 ± 3
HT-CH	1.9 ± 1	0.5 ± 0.6	33 ± 5
HT+CH	5.2 ± 1	15 ± 5	1.2 ± 0.4

^a Splenocytes were cultured at 37°C (C) for 2.5 or 6.5 hours, or were heated at 42°C for 30 min, then cultured at 37°C for 2 or 6h (HT) in the presence (+CH) or absence (-CH) of 5 µg/mL cycloheximide. Samples were fixed and labelled with TUNEL and DAPI.

^b Nuclei were evaluated as DAPI-normal, PC, PC-like or FC based on the morphology of chromatin by DAPI staining.

^c The proportion of PC nuclei within the total cell population was determined at t_2 by evaluating 500 cells. The proportion of PC-like nuclei, and the proportion of FC nuclei with a spherical chromatin within the TUNEL-positive population were determined at t_6 by evaluating 250 cells. Values shown are the means from three experiments and the standard deviation from the mean.

in HT+CH samples at t_6 . However, $33 \pm 5\%$ of the FC nuclei showed this morphology of chromatin in samples treated with heat but not CH (HT-CH).

Moreover, HT+CH samples at t_6 contained apoptotic nuclei with a novel chromatin morphology. The arrangement of chromatin in these cells appeared to be intermediate between PC and FC nuclei (e.g. Fig. 23D-D', Fig. 24A'-A"). In these PC-like cells, chromatin was uniformly stained with DAPI as previously described for PC and FC nuclei. However, PC-like cells were labelled faintly with TUNEL in comparison to FC nuclei, and contained chromatin that was not fully collapsed but that no longer retained the distribution observed in a DAPI-normal cell (Fig. 24A'-A"). The proportion of apoptotic cells with a PC-like chromatin morphology was determined by cell counting (Table 2). PC-like nuclei were rare in C±CH and HT-CH samples, representing less than 0.5% of the TUNEL-positive population. In HT+CH samples, on the other hand, approximately 15% of TUNEL-positive nuclei had a PC-like morphology of chromatin.

Chromatin collapse was also examined in t_2 samples labelled with DAPI, focussing on PC and PC-like nuclei (Table 2). Consistent with previous experiments, PC nuclei were rare in control samples at less than 0.13% of the total cell population whether treated with CH or not. They were somewhat more frequent in HT-CH samples, representing $1.9 \pm 1\%$ of the cell population. However, in HT+CH samples, the proportion of PC nuclei was higher still at $5.2 \pm 1\%$ of the total population. PC-like nuclei were observed too rarely to count in t_2 samples.

These results show that, during spontaneous apoptosis, neither the range of collapsed chromatin morphologies nor the relative proportions of a particular morphology (e.g. PC,

Figure 24: Protein synthesis inhibition and loss of nuclear antigens during heat-induced apoptosis. HT+CH samples were fixed at t_6 , and labelled with anti-NuMA (A), antibody PI2 (B), or anti-lamin B (C). All samples were then labelled with TUNEL (A'-C') and DAPI (A''-C''). A-A'' - spotty NuMA labelling is shown for FC (small arrowhead) and PC-like (large arrowhead) nuclei. B-B'' - discontinuous PI2 labelling is shown for FC (small arrowhead) and PC-like (large arrowhead) nuclei. C-C'' - discontinuous lamin B labelling is shown for FC (small arrowhead) and PC-like (large arrowhead) nuclei. Magnification 2600X.

spherical FC) was affected by inhibition of protein synthesis. In contrast, heat treatment in the presence and in the absence of protein synthesis both affected the morphology of collapsed chromatin, though in different ways. In HT-CH samples, cells exhibited the control range of collapsed chromatin morphologies, but single spherical chromatin masses were several fold more prevalent than in control samples. This may be a reflection of the acceleration in the rate of apoptosis induced by heat treatment (see Chapter 2). On the other hand, the proportion of FC nuclei with single spherical masses fell below control levels in HT+CH samples, which also contained cells with a novel collapsed chromatin morphology intermediate between PC and FC. These data indicate that, during heat-induced apoptosis, chromatin collapse was inhibited in the absence of protein synthesis. Furthermore, the very low proportion of FC nuclei with spherical chromatin masses in HT+CH samples appears to be correlated with the unexpectedly low proportion of cells sorting to the hypodiploid apoptotic peak during flow cytometry of these samples. These data suggest that PC-like nuclei contain incompletely fragmented DNA, and, conversely, that complete collapse of chromatin during heat-induced apoptosis requires the formation of low MW fragments of DNA.

Protein synthesis inhibition and loss of nuclear antigens

1) NuMA

To determine the effect of protein synthesis inhibition on the distribution of NuMA, samples at t_0 were labelled with anti-NuMA, processed for TUNEL, and counterstained with DAPI. Samples labelled with anti-NuMA and DAPI at t_0 and t_2 were also examined.

The NuMA labelling patterns were then quantified by cell counting (Table 3).

The distribution of NuMA in DAPI-normal (Fig. 25A-A', Fig. 25B-B') and FC (not shown) nuclei was identical in C±CH samples. Both types of samples showed the diffuse pattern of NuMA expected for control nuclei. As previously observed for C-CH samples (see Fig. 13), DAPI-normal nuclei with NuMA spots were rare in C+CH samples at t_0 , representing $3.0 \pm 2\%$ of the DAPI-normal population (Table 3). Furthermore, similar proportions ($<5.3\%$) of FC nuclei in C-CH and C+CH samples contained NuMA spots at t_2 and t_6 (Table 3).

As well, the NuMA labelling pattern in HT-CH samples was similar to that in HT+CH samples. A proportion of DAPI-normal nuclei in both types of samples contained NuMA spots in addition to diffuse NuMA labelling (Fig. 25C-C', Fig. 25D-D'). NuMA spots were retained in many FC nuclei from both samples (Fig. 25E-E', Fig. 25F-F'). However, cell counting showed that an average of $75 \pm 15\%$ of DAPI-normal nuclei contained NuMA spots in HT+CH samples at t_0 , a much higher proportion than the $46 \pm 10\%$ observed with heat treatment alone (see Fig. 13). Moreover, in HT+CH samples, all PC-like nuclei contained NuMA spots (Table 3), and the proportion of FC nuclei with NuMA spots at both t_2 and t_6 was much greater ($83 \pm 4\%$) in HT+CH samples than in HT-CH samples ($49 \pm 9\%$) (Fig. 24A-A", Table 3).

These results show that inhibition of protein synthesis had no effect on the distribution of NuMA during spontaneous apoptosis. These results further show that the development of NuMA spots in heat-treated nuclei did not require protein synthesis. In fact, nuclei with NuMA spots were more common in the absence of protein synthesis, and a higher

Figure 25: Protein synthesis inhibition and NuMA distribution. C-CH (A-A'), C+CH (B-B'), HT-CH (C-C', E-E'), and HT+CH (D-D', F-F') samples were fixed at t_2 and labelled with anti-NuMA (A-F) and DAPI (A'-F'). Diffuse NuMA labelling is shown for C-CH and C+CH DAPI-normal nuclei. In HT-CH and HT+CH samples, NuMA labelling is diffuse or spotty in DAPI-normal or FC (arrowheads) nuclei. Magnification 1600X.

Table 3. Effect of protein synthesis inhibition on the proportion of nuclei with NuMA spots.

Samples ^a	% nuclei with NuMA spots ^{b,c}			
	DAPI-normal nuclei at t ₀	FC nuclei at t ₂	PC-like nuclei at t ₆	FC nuclei at t ₆
C-CH	ND ^e	2.3 ± 1	N/A ^d	3.2 ± 1
C+CH	3.0 ± 2	5.3 ± 4	N/A	4.1 ± 2
HT-CH	ND	55 ± 5	N/A	48 ± 9
HT+CH	75 ± 15	84 ± 4	100	83 ± 4

^a Splenocytes were cultured at 37°C (C) for 0.5, 2.5 or 6.5 hours, or were heated at 42°C for 30 min, then cultured at 37°C for 0, 2 or 6h (HT) in the presence (+CH) or absence (-CH) of 5 µg/mL cycloheximide. Samples were fixed and labelled with anti-NuMA at t₀ and t₂, and TUNEL and anti-NuMA at t₆.

^b Nuclei were evaluated as DAPI-normal, PC, PC-like or FC based on the morphology of chromatin by DAPI staining.

^c 500 DAPI-normal, 50 PC-like nuclei, 100 FC nuclei at t₂, and 250 FC nuclei at t₆ were evaluated for the presence or absence of NuMA spots. Values shown are the means from three separate experiments and the standard deviation from the mean.

^d N/A, not applicable. PC-like nuclei were observed only in HT+CH samples.

^e ND, not done; refer to Figure 13.

proportion of nuclei retained NuMA spots late in apoptosis.

2) Immunofluorescence labelling with other anti-NuMA antibodies

To examine further the behaviour of NuMA, immunofluorescence labelling was carried out with several different anti-NuMA antibody preparations including the monoclonal anti-NuMA from Matritech used routinely in our studies; anti-W1, a rabbit anti-peptide serum [Tang *et al.*, 1993]; anti-NuMA_{whole}, a rabbit serum against full length recombinant human NuMA (gift of Duane Compton); and anti-NuMA_{tail}, a rabbit serum against C-terminal 50 kD of recombinant human NuMA (gift of Duane Compton).

As in all previous experiments, a diffuse nucleoplasmic NuMA pattern was observed in control nuclei labelled with the Matritech anti-NuMA (Fig. 26A-A'), and bright spots were observed within the diffuse pattern in about half of the heat-treated nuclei (Fig. 26B-B'). Labelling with the other antibodies showed similarities and differences to these basic patterns. All antibodies produced diffuse nucleoplasmic labelling in all nuclei in both control and heated samples, as did the Matritech antibody (Fig. 26A-H). However, both the anti-NuMA_{whole} (Fig. 26E-E', Fig. 26F-F') and anti-NuMA_{tail} (Fig. 26G-G', Fig. 26H-H') also labelled bright spots not only in the nuclei of heated samples, but also in all nuclei in controls. The anti-W1 antibody gave an intermediate result; with spots visible in all control nuclei (Fig. 26C-C'), and much brighter spots in about half of the heat treated nuclei (Fig. 26D-D').

Figure 26: NuMA distribution with various anti-NuMA antibodies. Control (A-A', C-C', E-E', G-G') and heat-treated (B-B', D-D', F-F', H-H') samples were fixed at t_2 and labelled with anti-NuMA (A-204, Matritech) (A, B), anti-W1 (C, D), anti-NuMA_{whole} (E, F), or anti-NuMA_{tail} (G, H). All samples were counterstained with DAPI (A'-H'). Magnification 2600X.

A	A'
B	B'
C	C'
D	D'
E	E'
F	F'
G	G'
H	H'

3) PI2 and lamin B

To determine the effect of protein synthesis inhibition on the behaviour of peripheral nuclear antigens, samples at t_2 and t_6 were labelled with antibodies to the nuclear envelope antigens PI2 or lamin B, processed for TUNEL, and counterstained with DAPI. The PI2 and lamin B labelling patterns at t_2 and t_6 were then quantified by cell counting (Tables 4-5).

As in previous experiments, both anti-lamin B and PI2 labelled the entire nuclear periphery of both control and heat-treated DAPI-normal TUNEL-negative cells (not shown). These labelling patterns were unaffected by cycloheximide treatment (e.g. Fig. 24B-B"). Furthermore, the labelling patterns of PC and FC nuclei were identical in control and heat-treated samples, whether treated with CH or not. In t_6 samples, quantitative evaluation of FC nuclei found that labelling with both antibodies was discontinuous or absent in >95% of FC nuclei in both control and heat-treated samples, in the presence or absence of CH (Table 4, Table 5, Fig. 24B-B", Fig. 24C-C"). Loss of lamin B and PI2 immunolabelling was also examined in PC nuclei in heat-treated t_2 samples (Tables 4-5). In HT-CH samples, greater than 80% of PC nuclei exhibited continuous labelling with both antibodies, as we have previously reported in Chapter 2. Moreover, labelling was also continuous in greater than 80% of PC nuclei from heat-treated samples cultured in the presence of CH. However, in PC-like nuclei, which were observed only in HT+CH samples at t_6 , greater than 98% showed discontinuities or lacked labelling with both antibodies (Fig. 24B-B", Fig. 24C-C").

These data show that inhibition of protein synthesis did not prevent loss of peripheral

Table 4. Effect of protein synthesis inhibition on loss of lamin B labelling during spontaneous and heat-induced apoptosis.

Samples ^a	% nuclei with continuous anti-lamin B labelling ^{b,c}		
	PC nuclei at t_2	PC-like nuclei at t_6	FC nuclei at t_6
C-CH	N/A ^d	N/A ^d	2.7 ± 1
C+CH	N/A	N/A	1.6
HT-CH	84, 90	N/A	2.5 ± 0.5
HT+CH	88, 84	1.3 ± 1	1.6 ± 1

^a Splenocytes were cultured at 37°C (C) for 2.5 or 6.5 hours, or were heated at 42°C for 30 min, then cultured at 37°C for 2 or 6h (HT) in the presence (+CH) or absence (-CH) of 5 µg/mL cycloheximide. Samples were fixed and labelled with TUNEL and anti-lamin B.

^b Nuclei were evaluated as DAPI-normal, PC, PC-like or FC based on the morphology of chromatin by DAPI staining.

^c 10-25 PC-like nuclei, and 100-300 FC nuclei were evaluated as having continuous or partial labelling with anti-lamin B for each sample at t_6 . Values shown are the means from three separate experiments and the standard deviation from the mean. In two of the experiments, 10-50 PC nuclei were evaluated as having continuous or partial labelling with anti-lamin B for each sample at t_2 . Values from both experiments are shown.

^d N/A, not applicable. PC nuclei were too rare to count in t_2 samples, and PC-like nuclei were observed only in HT+CH samples at t_6 .

Table 5. Effect of protein synthesis inhibition on loss of PI2 labelling during spontaneous and heat-induced apoptosis.

Samples ^a	% nuclei with continuous PI2 labelling ^{b,c}		
	PC nuclei at t_2	PC-like nuclei at t_6	FC nuclei at t_6
C-CH	N/A ^d	N/A ^d	1.5 ± 1
C+CH	N/A	N/A	1.3 ± 0.3
HT-CH	95, 80	N/A	2.6 ± 1
HT+CH	80, 90	2.0 ± 2	1.7 ± 0.8

^a Splenocytes were cultured at 37°C (C) for 2.5 or 6.5 hours, or were heated at 42°C for 30 min, then cultured at 37°C for 2 or 6h (HT) in the presence (+CH) or absence (-CH) of 5 µg/mL cycloheximide. Samples were fixed and labelled with TUNEL and antibody PI2.

^b Nuclei were evaluated as DAPI-normal, PC, PC-like or FC based on the morphology of chromatin by DAPI staining.

^c 10-25 PC-like nuclei, and 100-250 FC nuclei were evaluated as having continuous or partial labelling with antibody PI2 for each sample at t_6 . Values shown are the means from three experiments and the standard deviation from the mean. In two of the experiments, 20 PC nuclei were evaluated as having continuous or partial labelling with antibody PI2 for each sample at t_2 . Values from both experiments are shown.

^d N/A, not applicable. PC nuclei were too rare to count in t_2 samples, and PC-like nuclei were observed only in HT+CH samples at t_6 .

nuclear antigens during spontaneous and heat-induced apoptosis. Moreover, these data further confirm that loss of peripheral nuclear antigens is not sufficient for complete collapse of chromatin.

DISCUSSION

Protein synthesis inhibition and spontaneous apoptosis

In this study, I have examined the effect of inhibiting protein synthesis on the nuclear morphology and the rate of death during spontaneous and heat-induced apoptosis in resting mouse splenocytes. The data show that the rate of spontaneous apoptosis, as detected by both TUNEL and flow cytometry, was not affected by protein synthesis inhibition. Moreover, Trypan Blue exclusion data showed that treatment with CH had no effect on loss of plasma membrane integrity during spontaneous apoptosis. Furthermore, the range and relative proportions of collapsed chromatin morphologies, and the behaviour of nuclear antigens by immunofluorescence microscopy were identical in samples cultured with or without CH. These data demonstrate that protein synthesis is not required for spontaneous apoptosis in resting mouse splenocytes.

Protein synthesis inhibition and heat-induced apoptosis

Similarly, in heated samples, CH treatment had no effect on the number of TUNEL-positive cells at t_6 . Moreover, by 24 hours, the proportion of apoptotic cells detected by flow cytometry, and the proportion of cells that stained with Trypan Blue were essentially the same in heated samples cultured with or without CH. These data show that protein synthesis is not required for hyper-induction of apoptosis by heat. In contrast, during glucocorticoid- or VM-26-apoptosis in thymocytes, treatment with CH has been shown to inhibit all parameters associated with apoptosis, including DNA fragmentation as

measured by pulse field gel electrophoresis [Weaver *et al.*, 1993], reduction in cell size, and loss of plasma membrane integrity [Walker *et al.*, 1991]. These results suggest that heat-induced apoptosis may involve a different regulatory pathway than glucocorticoid- or VM-26-induced apoptosis.

Protein synthesis inhibition and DNA fragmentation

Although the TUNEL data clearly showed that treatment with CH did not reduce the proportion of cells with fragmented DNA, flow cytometric analysis of heated samples showed a reduction in the percentage of hypodiploid cells in the presence of CH to control levels. We interpret these data to mean that the lower proportion of hypodiploid cells by flow cytometry in HT+CH samples is due to a reduction in oligonucleosomal DNA fragmentation in these samples. Although the TUNEL technique provides no indication of the DNA fragment sizes being produced, TUNEL detects DNA fragmentation *in situ*, and is expected to reveal both high and low molecular weight DNA cleavage events during apoptosis. Together with the reduced DNA solubilization implied by flow cytometry, the results imply that the DNA cleavage events detected by TUNEL in HT+CH samples represent high molecular weight DNA fragmentation. This hypothesis could be tested using pulse field gel electrophoresis to examine DNA fragmentation patterns of heated samples prepared in the presence and absence of CH.

Flow cytometric analysis has demonstrated a similar reduction in the number of cells with hypodiploid DNA upon inhibition of protein synthesis during glucocorticoid-induced thymocyte apoptosis [Perandones *et al.*, 1993; Rinner *et al.*, 1996]. Furthermore,

spectrophotometric analysis of diphenylamine (DPA)-reactive DNA in the supernatant of lysed cells [Sellins and Cohen, 1991; Sun *et al.*, 1994] and agarose/pulse field gel electrophoresis [Weaver *et al.*, 1993; Sun *et al.*, 1994] have directly demonstrated that CH inhibited DNA fragmentation in radiation- or drug-treated samples. Flow cytometry, DPA analysis, and gel electrophoresis also showed that oligonucleosomal DNA fragmentation proceeds independently of protein synthesis during spontaneous apoptosis of thymocytes [Sellins and Cohen, 1991; Perandones *et al.*, 1993; Weaver *et al.*, 1993; Sun *et al.*, 1994; Rinner *et al.*, 1996].

However, in contrast to the results of this study, internucleosomal DNA cleavage analyzed by flow cytometry [Migliorati *et al.*, 1992] or by DPA analysis [Sellins and Cohen, 1991] has been reported to proceed independently of protein synthesis during heat-induced apoptosis in thymocytes. These results have led to the proposal that heat may induce apoptosis by a protein synthesis-independent mechanism, by altering DNA topology and allowing access of nucleases to DNA, for example [Migliorati *et al.*, 1992]. One possible explanation for the contradiction between the above results and the results of this study may involve the timing of addition of CH, as has been previously suggested [Sellins and Cohen, 1991]. In both of the above studies, CH was added at the end of the thymocyte isolation protocol, whereas in this study, CH was added immediately after removal of the spleen from the animal. Therefore, it is possible protein synthesis during the isolation procedure may have provided the components required for DNA fragmentation after heat treatment. Furthermore, unlike thymocytes, freshly isolated mouse splenocytes are a non-cycling population of mature B and T cells, fully arrested in

G₀. It is also possible, therefore, that the differential response of spleen T cells and thymocytes to CH reflects the developmental stage of the cells [Perandones *et al.*, 1993]. In keeping with this hypothesis, Perandones *et al.* [1993] showed that glucocorticoid-induced DNA fragmentation was protein synthesis-dependent in mouse thymocytes but not in mature mouse spleen T cells.

Protein synthesis inhibition and chromatin collapse

The results of my study discussed above show that, during spontaneous apoptosis, chromatin collapse proceeded independently of protein synthesis. In contrast, protein synthesis inhibition during heat-induced apoptosis resulted in a higher proportion of PC nuclei, the appearance of nuclei with a novel collapsed chromatin morphology intermediate between PC and FC, and a reduction in the proportion of FC cells with a single spherical chromatin mass. These data indicate that, during heat-induced apoptosis, the completion of chromatin collapse was inhibited in the absence of protein synthesis.

Moreover, the range of collapsed chromatin morphologies, and the relative proportion of cells with a particular morphology, appeared correlated with the proportion of cells that sorted to the sub-diploid peak. Control samples cultured in the presence or absence of CH showed identical patterns of chromatin collapse, and similar proportions of hypodiploid cells. In HT-CH samples, the full range of chromatin morphologies was also observed, but single spherical chromatin masses were prominent; these samples also showed the highest number of cells in the sub-diploid peak. HT+CH samples, on the other hand, showed a low proportion of cells with spherical chromatin masses and lower proportion of

hypodiploid cells. These results indicate a direct relationship between the extent of DNA fragmentation and the extent of chromatin collapse, as has been previously suggested for apoptotic thymocytes [Cohen *et al.*, 1992; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun *et al.*, 1994; Walker *et al.*, 1994] and HL60 cells [Hara *et al.*, 1996]. Specifically, these results indicate that oligonucleosomal DNA fragmentation is required for complete collapse of chromatin during heat-induced apoptosis.

In addition to inhibiting chromatin collapse in heated samples, CH treatment also delayed loss of plasma membrane integrity in heated samples. It is possible that loss of plasma membrane integrity and oligonucleosomal DNA fragmentation are controlled by the same regulatory pathway during apoptosis.

Protein synthesis inhibition and the behaviour of nuclear antigens

1) peripheral nuclear antigens

Immunofluorescence labelling showed that loss of peripheral nuclear antigens occurred in an identical fashion, independently of protein synthesis during both spontaneous and heat-induced apoptosis. These data suggest that proteolysis of lamins may also be protein synthesis independent in all of the apoptotic populations examined. To confirm these hypotheses, immunoblotting could be used to compare protein degradation patterns in the presence or absence of CH.

Previous studies have shown that lamins are early targets for proteolysis [Oberhammer *et al.*, 1994; Lazebnik *et al.*, 1995b; Neamati *et al.*, 1995; Weaver *et al.*, 1996]. These results have led to the conclusion that chromatin collapse and the formation of apoptotic

bodies resulted from disruption of the nuclear lamina [Lazebnik *et al.*, 1995b; Neamati *et al.*, 1995]. However, in this study, PC-like nuclei, in which chromatin collapse is clearly incomplete, rarely showed extensive staining for lamins. These data provide further evidence that disassembly of the lamina is not sufficient to result in complete chromatin collapse (see Chapter 2).

2) NuMA

In this study, CH treatment did not prevent the appearance of NuMA spots in heat-treated DAPI-normal nuclei, but in fact increased the proportion of cells with NuMA spots. The significance of this increase is unknown. However, we have shown that a higher proportion of cells develop NuMA spots with a short heat treatment of 10 minutes than with longer heat treatments of 20 to 30 minutes (see Chapter 3). These data led to the suggestion that, with longer heat treatments, some cells which had developed NuMA spots may return to the diffuse pattern. It is possible that protein synthesis may be required for recovery of diffuse NuMA labelling after heat treatment.

The significance of the NuMA spots themselves is also unknown. Previous data showed that NuMA spots developed after a short heat treatment of 5 minutes, suggesting that biosynthesis was not required for the development of NuMA spots. In this study we have confirmed that the development of NuMA spots does not require protein synthesis. Labelling with other anti-NuMA antibodies is also in keeping with this conclusion. In contrast to the labelling patterns observed with the Matritech antibody, all other anti-NuMA antibodies detected NuMA spots in both control and heat-treated nuclei. These

results suggest that the nuclear substructures visualized as NuMA spots are present in all splenocytes at all times, but are detected by the Matritech antibody only after heat treatment. The differential accessibility of the Matritech antibody to the spots suggests a heat-induced change in the conformation of NuMA, or of components interacting with NuMA. The different labelling patterns for NuMA may be due to recognition of different epitopes by various anti-NuMA antibodies, as has been previously suggested [Compton *et al.*, 1992; He *et al.*, 1995]. All four anti-NuMA preparations bind to the carboxyl half of the protein [Duane Compton, personal communication; Tang *et al.*, 1993]. However, the exact epitope is known only for anti-W1 and is located in the rod domain near the carboxyl terminus [Tang *et al.*, 1993].

Protein synthesis inhibition also resulted in a higher proportion of FC nuclei with NuMA spots in heat-treated samples. Although no conclusions about the fragmentation state of NuMA can be made from these data, it is possible that the retention of NuMA spots through late stages of apoptosis may be the result of inhibition of NuMA degradation in the presence of CH. This hypothesis could be confirmed by immunoblotting of heat-treated samples prepared in the presence or absence of CH.

In summary, these data suggest that different pathways may be involved in DNA fragmentation during spontaneous and heat-induced apoptosis. Furthermore, these results suggest that proteolysis of NuMA and lamin B may differ in the requirement for protein synthesis. However, further experimentation examining DNA and protein degradation at the biochemical level will be required to confirm these interpretations.

SUMMARY

This study was originally based on the general hypothesis that the nucleus is assembled in an orderly fashion and, conversely, would be disassembled in an orderly, stepwise manner during apoptotic nuclear collapse. In general, the results bear out this hypothesis (Chapter 2). In addition, the observation of NuMA spots led to another series of studies that effectively constituted a comparison of spontaneous and heat-induced apoptosis (Chapters 3-5).

In current models of the nucleus, the 10 nm fibre of DNA is folded into loops that are anchored at the nuclear periphery and in the nucleoplasm by interaction with the nuclear matrix [reviewed in Davie, 1996]. During apoptosis, the sequential cleavage of DNA into loop-sized fragments, then into oligonucleosomal DNA fragments, is believed to result in the release of DNA loops [Filipski *et al.*, 1990; Walker *et al.*, 1991; Cohen *et al.*, 1992; Roy *et al.*, 1992; Brown *et al.*, 1993; Oberhammer *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun and Cohen, 1994; Walker *et al.*, 1994]. The results in this study are in agreement with this hypothesis, in that chromatin collapse during apoptosis occurred in stages, reflecting apparently the progressive destruction of chromatin loop organization. Chromatin collapse was not observed in TUNEL-normal nuclei, which on the basis of the faint TUNEL signal in these nuclei, most likely contain relatively few DNA strand breaks. According to pulse field gel electrophoresis data, these nuclei would contain only high molecular weight DNA fragments. On the other hand, nuclei with partially collapsed chromatin showed a much brighter TUNEL signal, indicating that DNA fragmentation was more extensive in these cells. However, the similarity of these nuclei to

the pre-apoptotic nuclei isolated by Cohen *et al.* [1994] suggests that they also are likely to contain high molecular weight DNA fragments only. Once chromatin collapses fully to the nuclear periphery, a number of morphologies were observed, as has been previously described [Wyllie *et al.*, 1980; Earnshaw, 1995]. Once chromatin collapses fully, however, the morphology of the chromatin masses was quite variable, forming rings, irregular bodies or a single spherical mass. Similar chromatin morphologies have been previously reported in apoptotic nuclei by Wyllie *et al.* [1980] and Earnshaw [1995]. FC nuclei with a single spherical mass appear to represent the morphological and biochemical endpoint for apoptotic lymphocytes in this *in vitro* system. On the one hand, these FC cells consistently exhibited no staining for NuMA, lamin B or PI2, indicating that the nuclear matrix has been fully disassembled. On the other hand, whereas such nuclei and hypodiploid cells were very prominent in heat-treated samples at t_6 , both FC nuclei with a single spherical chromatin body and hypodiploid cells were much less common in samples treated with both heat and CH. This correlation between the frequency of such FC nuclei and the extent of DNA solubilization suggests that chromatin collapse into a single spherical mass requires very extensive DNA fragmentation, including the formation of oligonucleosomal sized DNA fragments. However, the rearrangement and/or loss of nuclear matrix antigens was only partial in FC nuclei with a ring-shaped or irregular collapsed chromatin. This suggests that such FC nuclei are both morphologically and biochemically intermediate between PC nuclei and FC nuclei with a single spherical chromatin body. These data corroborate previous studies that suggested a direct relationship between the extent of DNA fragmentation and the extent of chromatin collapse [Weaver *et al.*, 1993; Cohen *et al.*, 1994; Walker *et al.*, 1994; Hara *et al.*, 1996].

I have also examined the organization of the nuclear envelope antigens lamin B and PI2, and of the nucleoplasmic antigen NuMA in nuclei in progressive stages of chromatin collapse. The data show that disassembly of nuclear envelope antigens during splenocyte apoptosis is related both temporally and spatially to DNA fragmentation and chromatin collapse. As with previous studies of thymocytes [Weaver *et al.*, 1996], DNA fragmentation was the first detectable nuclear change, occurring before chromatin collapse or loss of peripheral nuclear antigen labelling. Collapse of chromatin towards the nuclear periphery then proceeded without significant changes in the morphology of nuclear envelope antigens. Extensive loss of nuclear envelope antigens and further collapse of chromatin then proceeded in parallel, with discontinuities in both lamin B and PI2 labelling corresponding to regions where chromatin had pulled away from the nuclear periphery. Moreover, PI2 and lamin B labelling was largely continuous in FC nuclei with a ring of chromatin at the nuclear periphery, but the antigens were not detected in FC nuclei with single spherical chromatin masses. Therefore, the data from this study indicate that disassembly of the nuclear lamina is required to release chromatin from the nuclear periphery, allowing for complete chromatin collapse, as has been suggested in previous studies [Lazebnik *et al.*, 1995a]. They are also consistent with the proposed role of lamins in attaching chromatin loops at the nuclear envelope. That is, chromatin loops, or their fragmented remnants, can, apparently become detached from the envelope only once their anchoring to the lamina is lost as lamins are solubilized.

On the other hand, PC-like nuclei in HT+CH samples exhibited no lamin B or PI2 labelling, yet their chromatin was only partially collapsed. These data demonstrate that disassembly of the lamina is not sufficient of itself for complete chromatin collapse.

Detachment of chromatin from the envelope would appear to require further apoptotic processing of the chromatin or of the non-lamin nuclear envelope components.

The morphological and biochemical changes observed during spontaneous and heat-induced apoptosis were identical other than for the induction of NuMA spots by heat treatment. This is particularly remarkable in view of evidence that these processes differ as to the cell types affected and in their dependence on protein synthesis. Mouse splenocytes underwent considerable apoptosis in culture within 6 hours, with approximately half of the population dead by 24 hours. These data are consistent with previous studies of spontaneous apoptosis in mouse lymphocytes [Migliorati *et al.*, 1992; Clarke *et al.*, 1993; Illera *et al.*, 1993; Perandones *et al.*, 1993; Reap *et al.*, 1995; Zhang *et al.*, 1995]. The results from surface IgG labelling showed that it was primarily the B cell subset that underwent spontaneous apoptosis, as previously observed in total mouse splenocyte populations by flow cytometry [Reap *et al.*, 1995]. My study has also shown that heat treatment accelerates the rate of splenocyte apoptosis within the first 6 hours of culture, apparently by inducing apoptosis in T cells. To my knowledge, this is the first study to demonstrate that heat can induce apoptosis in different lymphocyte subsets within a mixed lymphocyte population.

Furthermore, the data from this study indicate that despite the similarities between the cells during the execution phase, lymphocytes undergoing spontaneous and heat-induced apoptosis are following different regulatory pathways through the commitment phase. This is manifested by the different requirements for protein synthesis during spontaneous and heat-induced apoptosis. DNA solubilization and complete chromatin collapse were protein synthesis-dependent during heat-induced, but not spontaneous apoptosis. Since it

is the oligonucleosomal DNA which is solubilized, my data imply that, as suggested by previous studies [Sellins and Cohen, 1991; Perandones *et al.*, 1993; Weaver *et al.*, 1993; Sun *et al.*, 1994; Rinner *et al.*, 1996] oligonucleosomal DNA fragmentation is not dependent on protein synthesis during spontaneous lymphocyte apoptosis. However, in contrast to analyses of heat-induced thymocyte apoptosis [Sellins and Cohen, 1991; Migliorati *et al.*, 1992], my data also imply that oligonucleosomal DNA fragmentation is dependent on protein synthesis when splenocyte apoptosis is induced by heat. It will be necessary to verify these conclusions in further experiments. Specifically, the patterns of DNA fragmentation during heat-induced apoptosis in the presence and absence of cycloheximide must be examined directly using pulse field gel electrophoresis.

In contrast to peripheral nuclear antigens, the behaviour of NuMA was different, morphologically, in control and heated samples. In control cultures, NuMA labelling was exclusively diffuse both in healthy and apoptotic cells as previously described [Miller *et al.*, 1993; He *et al.*, 1995; Weaver *et al.*, 1996]. However, heat treatment for as little as 10 minutes resulted immediately in the development of NuMA spots in approximately half of the splenocyte population, and the spots were observed in the majority of PC nuclei, but were rarer in FC nuclei. We interpret this decline of spot frequency in FC nuclei as resulting from disassembly of NuMA organization due to proteolytic cleavage. The spots are not a general feature of exogenously-induced apoptosis, as they were absent from splenocytes in VM-26-treated cultures (see Appendix II). NuMA spots were observed primarily in T cells, and were detected in all heat-treated nuclei in early stages of apoptotic DNA fragmentation. It appears, therefore, that NuMA spots may be an early marker for heat-induced T cell apoptosis.

However, the morphological and functional significance of NuMA spots is not clear. Although spots were observed only in a subset of the heat treated population when the Matritech anti-NuMA antibody was used in immunolabelling, apparently identical spots were observed in all cells of both control and heated samples when other anti-NuMA preparations were employed. Moreover, the spots appeared even if protein synthesis was inhibited in the heat-treated samples. Also, their appearance within 5 minutes of application of heat, particularly in the light of the very low metabolic rate of resting splenocytes, argues against a biosynthetic basis for their formation. We have postulated, therefore, that the spots may represent an underlying NuMA distribution that is revealed by a change in accessibility of epitopes for the Matritech anti-NuMA brought about by heat treatment. This change in accessibility may represent conformational changes in NuMA itself or in NuMA-associated proteins. One approach to testing this hypothesis, would be to use immunoprecipitation to determine whether there are differences in the proteins associated with NuMA before and after heat treatment. In addition, a determination of the epitopes for the different anti-NuMA antibodies may aid in explaining the disparate immunolabelling patterns. Studies of NuMA distribution in intact nuclei and in isolated nuclear matrices by immunogold electron microscopy may also aid in elucidating the significance of the NuMA spots. It will also be necessary to determine more fully whether NuMA spots are specific to heat-treated apoptotic resting splenocytes, or are a more general phenomenon. Immunofluorescence labelling studies with the Matritech anti-NuMA and other anti-NuMA antibodies could be performed after heat treatment using lymphocytes from other sources, for example mouse thymocytes, mitogen stimulated mouse splenocytes, and cell lines such as mouse 3T3 fibroblasts.

However, though the organization of NuMA during cell death was altered by heat treatment, it was degraded to the same sized fragments in control and heat-treated samples, with degradation visible earlier in heat-induced populations. Moreover, the fragment sizes and the degradation kinetics were similar for both NuMA and lamin B to those previously reported for thymocytes [Weaver *et al.*, 1996] and other cell types [Oberhammer *et al.*, 1994; Lazebnik *et al.*, 1995b; Casiano *et al.*, 1996; Hsu and Yeh, 1996; Rao *et al.*, 1996; Weaver *et al.*, 1996; Zweyer *et al.*, 1997]. Degradation of these proteins, therefore, appears to be a conserved process during apoptosis, regardless of the cell type and the apoptotic stimulus.

Whether these proteins are also degraded in CH-treated samples was not determined for lack of time. However, NuMA spots remained prominent in PC-like and FC nuclei in these samples. This is in contrast to the reduced proportion of FC nuclei with NuMA spots observed in samples that were heated but not treated with CH (see above). If our interpretation of the latter results is correct, it implies that NuMA cleavage may be inhibited in heated samples in the absence of protein synthesis. Moreover, since loss of lamin B labelling in these samples proceeded identically in the presence or absence of cycloheximide, the data raise the possibility that NuMA and lamin B may be degraded by different pathways, with degradation of NuMA, but not of lamin B, requiring protein synthesis. To test this hypothesis, lamin B and NuMA degradation patterns must be compared during heat-induced apoptosis in the presence or absence of cycloheximide.

Previous studies using serine protease inhibitors such as TLCK in apoptotic cell extracts have implicated a serine protease in lamin degradation during apoptosis [Lazebnik *et al.*, 1995a]. However, the protease(s) involved in NuMA degradation during apoptosis

is presently unknown. Immunolabelling and immunoblotting results in my study suggest that NuMA is ubiquitinated during splenocyte apoptosis, implying that NuMA may be degraded by the ubiquitin system during apoptosis. Since NuMA degradation appears to be a conserved apoptotic process, it will be necessary to determine if NuMA is ubiquitinated in other cell types undergoing apoptosis. This could be achieved by immunoblotting and immunoprecipitation with different apoptotic systems. If NuMA is proven to be ubiquitinated in other cell types undergoing apoptosis, apoptotic cell extracts could then be used to study NuMA degradation, as has been done for proteins like lamin B, and Topo II [Lazebnik *et al.*, 1993, 1995b; Nakajima *et al.*, 1996]. Apoptotic cell extracts could be used to determine whether NuMA degradation occurs in an ATP- and ubiquitin-dependent manner, as has been reported for Topo II, and also to see whether inhibitors of the 26S protease would inhibit NuMA degradation [Nakajima *et al.*, 1996].

However, as speculated in Chapter 4, if NuMA is proven to be ubiquitinated, there may be other roles for this modification, for example degradation of NuMA during lymphocyte stimulation, or during the cell cycle. To determine whether NuMA degradation occurs during the cell cycle, immunoblotting of synchronized populations, sampled at different stages of the cycle, could be used. This technique could also be used to determine whether NuMA is degraded during stimulation, by sampling mitogen-activated lymphocytes for immunoblotting at various times post-stimulation. If NuMA degradation is detected during either of these processes, immunoprecipitation with anti-NuMA antibodies followed by immunoblotting with anti-ubiquitin antibodies would then determine whether NuMA ubiquitination is associated with NuMA degradation. Inhibitors of the 26S protease could then be used to determine if NuMA degradation occurs by the

ubiquitin pathway. Moreover, if NuMA degradation occurs during stimulation, NuMA synthesis would be necessary to replace the degraded NuMA. Experiments in which proliferating cells are cultured in the presence of radiolabelled amino acids and then processed for autoradiography, and for immunoblotting with anti-NuMA antibodies of samples separated by SDS-PAGE, could be used to examine NuMA synthesis during mitogen stimulation, and during the cell cycle in general.

CONCLUSIONS

- 1) With the exception of NuMA spots, all of the morphological and biochemical parameters changed identically during spontaneous and heat-induced apoptosis.
- 2) Spontaneous apoptosis affects mainly the B cell subset, whereas heat induces apoptosis in T cells.
- 3) NuMA may be ubiquitinated in resting and apoptotic splenocytes.
- 4) NuMA spots are a marker for T cell death induced by heat.
- 5) Heat-induced, but not spontaneous, apoptosis is dependent on protein synthesis for DNA solubilization and completion of chromatin collapse.

APPENDIX I: Immunofluorescence staining of heat shock proteins

INTRODUCTION

The stress response is characterized by a rapid increase in the expression of a specific group of proteins collectively known as heat shock proteins, accompanied by a general decrease in synthesis of other cellular proteins. In mammalian cells, the proteins whose synthesis increases after heat shock have apparent molecular masses of 8, 28, 58, 72, 73, 90, and 110 kDa. The 72 kDa protein, also known as HSP 70, is the most highly inducible member of the stress protein family. The 73 kDa protein, on the other hand, is found in the nucleus and cytoplasm of normal unstressed cells, and is referred to as the constitutive HSP 70, or HSC 70 [reviewed in Welch, 1992].

Immunofluorescence labelling studies have been carried out to determine the subcellular localization of HSP 70 in gerbil fibroma, normal rat kidney, baby hamster kidney, rat embryo fibroblast, and HeLa cells subjected to a 42–45°C heat treatment [Welch and Feramisco, 1984; Welch and Suhan, 1985; 1986; Milarski *et al.*, 1989]. In interphase cells grown at 37°C, nuclear HSP 70 fluorescence was faint or absent [Welch and Suhan, 1985; Milarski *et al.*, 1989]. Bright nucleoplasmic and nucleolar HSP 70 labelling appeared within 1–4 hours of sustained heat shock, but decreased after 2–4 hours of recovery at 37°C [Welch and Feramisco, 1984; Welch and Suhan, 1985; 1986; Milarski *et al.*, 1989].

I have used conventional fluorescence microscopy to examine the cellular localization

of HSP 70 and HSC 70 in resting mouse splenocytes subjected to a 30 minute heat treatment at 42°C. These experiments were carried out to attempt to verify that the heat treatment was inducing the characteristic stress response. Although HSP 70 was never detected in resting cells, it was detected in stimulated lymphocytes and in mouse 3T3 cells. These results suggest that immunofluorescence staining may not be a sensitive enough technique to detect heat shock proteins being synthesized in resting mouse splenocytes.

MATERIALS AND METHODS

Preparation of cells

Splenocytes were isolated from male Balb/c mice as previously described [Davis *et al.*, 1993] (see Appendix III). Control unstimulated samples were cultured at 37°C and 5% CO₂. For stimulation, Con A was added to the cell suspension at a final concentration of 4 µg/ml, as in Daev *et al.* [1994], and the samples cultured at 37°C and 5% CO₂ for up to 30.5 hours.

Mouse 3T3 cells were grown in α MEM supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml Fungizone. On the day before the experiment, 0.25×10^6 were seeded onto half coverslips in 35 mm tissue culture dishes.

Heat Treatment

Resting splenocytes and splenocytes stimulated with Con A for 24 hours were heat-treated for 30 minutes at 42°C as described in Chapter 2. At the end of the heat treatment, some samples were fixed for immunofluorescence labelling. The remaining samples were cultured at 37°C for up to 6 hours prior to processing.

For heat treatment of 3T3 cells, the 35 mm dishes were immersed in the water bath for 90 minutes at 43°C. The cells were cultured at 37°C for 4 hours prior to fixation for immunofluorescence.

Immunofluorescence Staining

Fixation, permeabilization, and immunofluorescence staining were carried out as previously described [Chaly *et al.*, 1984, 1988] (see Appendix IV). Samples were then incubated sequentially in PBS-diluted primary and secondary antibodies. Primary antibodies were: anti-HSC70, a rat monoclonal IgG (1:100) (StressGen) and anti-HSP70, a mouse monoclonal IgG (1:100) (StressGen). Secondary antibodies were: CY3-conjugated goat anti-mouse IgG (Fc fragment specific)(1:200) (Jackson Immunochemicals) and FITC-conjugated goat anti-rat IgG (1:100) (Cappel). All samples were counterstained with DAPI, mounted, and viewed by conventional fluorescence microscopy as described in Chapter 2.

RESULTS

As a preliminary test of the anti-HSC and anti-HSP antibodies, mouse 3T3 cells were heat-treated for 90 minutes at 43°C, cultured for a further 4 hours at 37°C, then processed for immunofluorescence labelling. This heat treatment has been previously shown to result in an increase in HSP 70 synthesis by immunoblotting and immunofluorescence labelling in HeLa cells [Milarski *et al.*, 1989], and a redistribution of HSC 70 fluorescence in rat fibroblasts [Welch and Mizzen, 1988]. In both control and heat-treated 3T3 cells, HSC 70 fluorescence was detected in the nucleus, but was intense in the nucleoli of heat-treated cells (not shown). However, HSP 70 fluorescence was observed only after heat treatment. Many of the heat-treated cells showed bright labelling of the nucleoli (Fig. 27F). In other cells labelling was diffuse and nucleoplasmic, or was undetectable (Fig. 27F).

To examine the distribution of HSPs in resting mouse splenocytes, control and heat-treated samples were fixed and labelled with anti-HSC 70 or anti-HSP 70 immediately after heat treatment, or following a further 2, 4, or 6 hours of culture at 37°C. In all control samples, diffuse nucleoplasmic HSC 70 fluorescence was observed as well as bright nucleolar staining in DAPI-normal nuclei (Fig. 27A). In heat-treated samples, bright nucleolar HSC 70 fluorescence was also observed in DAPI-normal nuclei but the diffuse nucleoplasmic labelling was no longer apparent (Fig. 27B). HSP 70 fluorescence, on the other hand, was not observed in any of the control (not shown) or heat-treated DAPI-normal or apoptotic cells at any time point after heat treatment (Fig. 27C).

Figure 27: Immunofluorescence staining of heat shock proteins. Control (A-A') and heat-treated (B-B', C-C') resting splenocytes and heat-treated stimulated splenocytes (D-D', E-E') or 3T3 fibroblasts (F-F') were labelled with anti-HSC (A, B) or anti-HSP (C-F) and counterstained with DAPI (A'-F'). A-A' - nucleolar and nucleoplasmic HSC fluorescence is shown for control DAPI-normal nuclei. B-B' - nucleolar HSC fluorescence is shown for heat-treated DAPI-normal nuclei. C-C' - HSP fluorescence is absent from heat-treated DAPI-normal and apoptotic (small arrowhead) nuclei. D-D' - nucleolar HSP fluorescence is shown in heat-treated stimulated nuclei. E-E' - fully stimulated nuclei show nucleolar fluorescence in heat-treated stimulated samples, but not morphotype I nuclei (large arrowhead). F-F' - nucleolar and nucleoplasmic HSP fluorescence is shown for heat-treated 3T3 nuclei. Magnification A-C' 2500X, D-E' 2000X, F-F' 1400X.

To determine whether the HSP 70 antibodies would label the nuclei of activated mouse splenocytes, cells were stimulated with Con A for 24 hours, processed as control or heat-treated samples, and then fixed and immunolabelled for HSP 70 immediately after heat treatment, or after a further 2, 4, or 6 hour of culture. HSP 70 fluorescence was not observed in any of the control samples, in heat-treated samples fixed directly after heat-treatment, or in heat-treated samples cultured for 4 or 6 hours (not shown). However, bright nucleolar labelling was observed in many of the heat-treated cells after 2 hours in culture and the labelling intensity/extent appeared to be related to the extent of splenocyte stimulation (Fig. 27D-D'). Lymphocytes in stimulated populations have been previously categorized into three morphotypes based on the size of the nuclei: resting cells (morphotype I); fully stimulated cells (morphotype III) and partially stimulated cells, intermediate between resting and stimulated (morphotype II) [Setterfield *et al.*, 1983]. The morphotypes are an indication of the level of physiological activity of the cells [Setterfield *et al.*, 1983]. In the 24 stimulated samples, all three morphotypes were observed (Fig. 27D, Fig. 27E). However, only fully stimulated cells exhibited nucleolar labelling with anti-HSP 70 after heat treatment (Fig. 27D-D', Fig. 27E-E'). The resting morphotype I cells remained unlabelled (Fig. 27E-E').

DISCUSSION

Freshly isolated mouse splenocytes are a non-cycling population of mature B and T cells, fully arrested in G₀. They are essentially metabolically inactive and exhibit very low levels of transcription and protein synthesis. Upon addition of a mitogen such as concanavalin A (Con A), splenocytes become stimulated and enter G1 asynchronously. There is massive upregulation of cell metabolism and remodelling of nuclear structure in the first 24 hours, at which time DNA replication begins. By 48 hours, approximately 5% of the population is in mitosis [Setterfield *et al.* 1983, 1985; Chaly *et al.*, 1988; Chaly and Brown, 1988; Davis *et al.*, 1993].

In this study, immunofluorescence microscopy was used to examine synthesis of HSP 70 in resting mouse splenocytes subjected to heat treatment. As an internal control, HSC 70 fluorescence was also monitored. As expected, HSC 70 was detected in the nucleus of all control and heat treated cells. In resting splenocytes, HSP 70 was not detectable by immunofluorescence microscopy at any time up to 6 hours after heat treatment. On the other hand, in mouse 3T3 cells and in splenocytes stimulated with mitogen for 24 hours HSP 70 fluorescence was readily detected within 2-4 hours of heat treatment. Furthermore, in stimulated cultures, HSP 70 fluorescence was detected in partially and fully stimulated cells, but not in morphotype I, resting cells. Similarly, earlier studies have detected HSP 70 in heat-treated mouse thymocytes and cytotoxic T lymphocytes by Northern Blotting, immunoprecipitation and SDS-PAGE of [35S]-methionine labelled cells [Knox *et al.*, 1991; Migliorati *et al.*, 1992]. Both of these cell types represent

lymphocyte populations containing activated cells.

The data presented here suggest that resting splenocytes may not be capable of responding to stress with a rapid increase in expression of heat shock proteins because of their low metabolic rate. However, it is possible that heat shock proteins are synthesized in the heat-treated resting splenocytes, but at levels undetectable by immunofluorescence microscopy. Immunoblotting would provide a more sensitive method to assay for heat shock protein synthesis in resting cells. Once stimulated, the cells acquire the metabolic machinery that may allow for a rapid increase in the synthesis of heat shock proteins. An inability to synthesize high levels of heat shock proteins upon heat treatment may contribute to the susceptibility of resting splenocytes to heat-induced cell death.

APPENDIX II: Behaviour of NuMA during VM-26-induced apoptosis of mouse splenocytes

INTRODUCTION

VM-26 is a cancer chemotherapeutic drug that inhibits Topo II by forming a drug-stabilized enzyme-DNA complex which prevents the resealing activity of the enzyme [Osheroff *et al.*, 1991]. Such drugs have been used to study the role of Topo II in regulating chromatin structure [Filipski *et al.*, 1990; Iarovaia *et al.*, 1995]. However, treatment of thymocytes with VM-26 or other Topo II inhibitors has been shown to result in cell death with the morphological and biochemical features of apoptosis [Walker *et al.*, 1991; Brown *et al.*, 1993; Weaver *et al.*, 1993; Cohen *et al.*, 1994; Sun *et al.*, 1994; Walker *et al.*, 1994]. Treatment of freshly isolated mouse splenocytes with VM-26 has also been reported to result in a rapid increase in the proportion of apoptotic cells detected by flow cytometry [Roy *et al.*, 1992; Daev *et al.*, 1994].

In the current study, VM-26 was used to induce apoptosis in resting mouse splenocytes. These experiments were carried out to determine whether the appearance of NuMA spots was specific to apoptosis induced by heat, or a phenomenon common to apoptosis induced by a variety of agents. The results of these experiments confirm that VM-26 rapidly induces apoptosis in resting lymphocytes, but does not result in a spotty NuMA labelling pattern.

MATERIALS AND METHODS

Splenocyte isolation and culture

Splenocytes were isolated from male Balb/c mice as previously described [Davis *et al.*, 1993] (see Appendix III). Control samples were cultured at 37°C and 5% CO₂ in complete RPMI. Cells were incubated for 2h at 37°C in complete RPMI 1640 containing 5 uM VM-26 (Bristol Laboratories), as in Daev *et al.* [1994]. At the end of the treatment, some samples were fixed for flow cytometry or immunofluorescence staining. The remaining samples were pooled, resuspended in fresh complete medium, and cultured at 37°C for a further 2, 4, or 6 hours prior to fixation.

Immunofluorescence staining

Fixation, permeabilization, and immunofluorescence staining of samples were carried out as previously described [Chaly *et al.*, 1984, 1988] (see Appendix IV). Samples were incubated sequentially in anti-NuMA (1:25 in PBS, A-204) (Matritech) and CY3-conjugated goat anti-mouse IgG (Fc fragment specific)(1:200 in PBS) (Jackson Immunochemicals). All samples were counterstained with DAPI, mounted, and viewed by conventional fluorescence microscopy as described in Chapter 2.

Flow cytometry

Flow cytometry of propidium iodide fluorescence was carried out by the methods of [Walker *et al.*, 1991], as described in Chapter 2 (see Appendix IV).

RESULTS

Flow cytometry confirmed that treatment of cells with VM-26 induced apoptosis, as previously reported [Roy *et al.*, 1992] (Fig. 28A). Control and VM-26-treated samples fixed at the end of the VM-26 treatment produced a single DNA peak corresponding to the DNA content of resting cells (not shown). VM-26 had little effect on the proportion of apoptotic cells within the first 2 hours. However, by 6 hours, greater than 50% of the treated population but less than 15% of the control population was apoptotic.

The behaviour of NuMA during VM-26-induced apoptosis was examined by immunofluorescence labelling (Fig. 28B-B"). In all samples, DAPI-normal and apoptotic nuclei showed the diffuse pattern for NuMA previously described for control samples (Fig. 28B-B'). The proportion of DAPI-normal nuclei with NuMA spots was also determined, by cell counting for two separate experiments. NuMA spots were observed in less than 3% of DAPI-normal cells in both control and VM-26-treated samples at t_0 . Furthermore, in both types of samples, spots were observed in less than 3% of DAPI-normal cells, in less than 8% of PC cells, and in less than 3% of FC cells at t_2 . These results show that NuMA spots are not a feature of VM-26-induced apoptosis. Since NuMA spots are also not observed during spontaneous apoptosis in resting splenocytes, they may be related specifically to the induction of apoptosis by heat.

Figure 28: NuMA distribution during VM-26-induced apoptosis in resting mouse splenocytes.

(A) Histogram showing the percent apoptotic cells detected by flow cytometry after isolation (0_{isol}), at the end of VM-26 treatment (0_{VM}), or after a further 2 or 4 hours in culture in control (open bars) and VM-26-treated (solid bars) samples.

Results are the average of 2 experiments. At 0_{isol} , bars are less than 0.1%.

(B) NuMA labelling in VM-26-treated samples. Splenocytes were treated with 5 μM VM-26 for two hours, cultured in medium without VM-26 for a further 2 hours, then fixed and labelled with anti-NuMA (B) and DAPI (B'). Samples showed diffuse NuMA labelling. Magnification 2600X.

(A)

APPENDIX III: Protocol for isolation of lymphocytes from mouse spleen.

This protocol, adapted from the Master of Science Thesis of Lynn Davis [1993], was used to isolate splenocytes from male Balb/c mice that were at least 6 weeks old.

1. Sacrifice the mouse by cervical dislocation. Lay the mouse on its right side, swab the left side of the mouse with 70 % ethanol, and place it in the tissue culture hood.
2. Using sterile forceps and scissors, remove a piece of skin the size of a dime from below the ribcage. With a second set of sterile scissors, cut open the peritoneum to expose the spleen. Place the spleen on a sterile metal screen in a sterile 60 mm glass petri dish.
3. Add 4 mL of RPMI 1640 to the dish. Using the flat end of a sterile plastic 10 mL syringe plunger, push the spleen through the screen without grinding the tissue. Add a further 4 mL of RPMI 1640 to the dish.
4. Draw the splenocyte suspension into a sterile 5 mL syringe using a 20 gauge needle. Repeat until no clumps are observed in the cell suspension.
5. Transfer the cell suspension to a 15 mL conical centrifuge tube using a sterile Pasteur pipette. Underlay the cell suspension with 4 mL ice cold cool calf serum, and let stand on ice for 5 minutes to allow tissue fragments to settle out of the cell suspension.
6. Using a Pasteur pipette, transfer the cell suspension to another 15 mL conical centrifuge tube, and centrifuge in a benchtop centrifuge at 1500 rpm for 5 minutes.

7. Discard the supernatant, resuspend the pellet in ice cold 0.83 % NH_4Cl , and let stand on ice for 5 minutes to lyse the erythrocytes.
8. Underlay the suspension with 4 mL of ice cold fetal bovine or cool calf serum (Sigma), and centrifuge at 1500 rpm for 5 minutes. Discard the supernatant, resuspend the cells in 10 mL of complete RPMI 1640 containing CPSR-2 and antibiotics, determine the cell density, and dilute the cell suspension to 2.5×10^6 cells/mL in complete RPMI 1640.

APPENDIX IV: Propidium iodide (PI) staining for flow cytometry

This protocol was obtained from Christine Carson (Apoptosis Research Group, National Research Council of Canada), and is adapted from Walker *et al.* [1991].

1. Centrifuge 1 mL of cell suspension (2.5×10^6 cells) at 1500 rpm for 5 min in a bench top centrifuge.
2. Discard the supernatant and resuspend cells in 1 mL of PBS by vortexing at approximately 3/4 maximum speed.
3. Add 4 mL of -20°C absolute ethanol while continuing to vortex.
4. Store cells at -20°C in 4 mL snap cap culture tubes.
5. To process cells for PI staining, centrifuge at 1500 rpm for 5 minutes as above.
6. Aspirate the ethanol/PBS, and resuspend cells in 1 mL of PBS by vortexing.
7. Add 100 µL of heat-treated, DNase-free 0.1 mg/mL stock RNase solution.
8. Incubate cells at 37°C for 30 minutes.
9. Add 100 µL of 1 mg/mL PI solution, incubate for at least 10 minutes at room temperature in the dark before analyzing fluorescence by flow cytometry.

APPENDIX V: Immunofluorescence staining of nuclear antigens

The protocol for fixation, permeabilization, and immunofluorescence staining was adapted from Chaly *et al.* [1984; 1988].

Preparation of poly-L-lysine-coated coverslips

1. On a mesh screen in a humid chamber, coat each coverslip with poly-L-lysine solution (MW 40,000-70,000, 0.2 mg/mL in distilled water) and let stand for 5 minutes.
2. Rinse each coverslip two times by dipping rapidly in distilled water and allow to air dry before use.

Processing of splenocytes for immunofluorescence staining

1. Centrifuge 1 mL of cell suspension in a benchtop centrifuge for 5 minutes at 1500 rpm.
2. Remove 800 μ L of the supernatant using a 1 mL Pipetman, and resuspend the pellet in the remaining 200 μ L of medium by tapping the bottom of the culture tube.
3. Spread cell suspension onto poly-L-lysine-coated coverslips in a 6-well plate and let stand for 10 minutes to allow cells to settle and attach to the coverslip.
4. Add 3% PBS-buffered paraformaldehyde (PFA) to each well and fix for 5 minutes.
5. Remove PFA by aspiration and wash coverslips 3 times for 4 minutes with PBS .
6. Incubate cells for 20 minutes in 0.2 % Triton X-100 in 1X PBS to permeabilize cells.
7. Wash cells 3 times for 4 minutes in PBS.

Note: For immunofluorescence labelling of 3T3 cells, 0.5×10^6 cells were seeded onto half coverslips in a 60 mm culture dish on the day before the experiment, and were fixed, permeabilized and labelled as described above.

Immunofluorescence staining

1. Pipette 50 μ L of PBS-diluted primary antibody onto parafilm in a humid chamber.
2. Place coverslips face down onto the drop of antibody and incubate for 45 minutes.
3. Flood the coverslips with PBS, transfer coverslips to a 6 well plate and wash 3 times for 4 minutes with PBS.
4. Incubate coverslips in secondary antibody as above.
5. Wash coverslips 3 times for 4 minutes with PBS.
6. For single-labelled samples, stain with DAPI as described below.
7. For double-labelled samples, incubate cells in 0.15% gelatin in PBS for 20 minutes to block non-specific binding, wash twice for 30 minutes with PBS, and incubate with primary and secondary antibodies as described above.
6. Stain coverslips for 5 minutes with 0.2 μ g/ml DAPI, wash three times for 30 seconds each in PBS, and mount using 6 μ L of Vectashield mounting medium.

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