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DISRUPTION OF MURINE LIMB DEVELOPMENT *IN VITRO* BY A TERATOGEN: ROLE OF P53 DEPENDENT AND INDEPENDENT CELL DEATH

by

Seyed Adel Moallem, Pharm.D.

A thesis submitted to the Faculty of Graduate Studies and Research
of McGill University in partial fulfilment of the requirements
for the Degree of Doctor of Philosophy.

May, 1998

Department of Pharmacology and Therapeutics
McGill University
Montréal, Québec
Canada

THIS THESIS IS DEDICATED WITH LOVE TO MY FAMILY:

MY WIFE AND MY

TWO LITTLE BLOSSOMS

My deepest thanks to my wife Lida who has always been by my side in these difficult years, her support, sacrifice and care earned me what I have today. I am indebted to her kindness and love.

My love and passion to my two sweethearts Yasaman and Mohammad Hassan. Sorry I have missed taking you to parks, brushing your teeth, and reading you bedtime stories so many times! I promise I'll be a better dad and friend!

My respect and appreciation to my parents. My education has always been their top priority.

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ABSTRACT

In this thesis, the effects of an active analog of cyclophosphamide (4-hydroperoxycyclophosphamide, 4OOH-CPA), an alkylating agent and teratogen, on mouse limb development, cell death and the role of the p53 gene in this process were studied. A mouse limb bud culture system was used for this purpose. Limbs developed normally in this system and showed physiological apoptosis in a spatio-temporal-controlled manner. Drug treatment caused limb reduction malformations, increased the amount of apoptosis and triggered its occurrence prematurely, primarily in areas with physiological apoptosis. This indicated that drug-treatment might have engaged the already available program of apoptosis in the developing limb, and suggested this might be the cause of limb malformations. Next we examined the induction pattern of three proteins, cathepsin D, transglutaminase and clusterin, known to be involved in the molecular mechanism of apoptosis. The pattern of their induction suggested that cathepsin D was involved in the digestion of apoptotic debris, transglutaminase was active in preserving the cellular integrity during apoptosis, and clusterin played a role in later phases of apoptosis. Since 4OOH-CPA causes DNA damage, we studied the role of the p53 gene, a DNA-damage response gene involved in regulating cell cycle and apoptosis, in a p53-transgenic mouse model. Absence of p53 led to more severe limb malformations in the treated limbs. To investigate the reason(s) for this higher sensitivity to drug insult, cell death and cell cycle patterns were studied. While wild-type limbs showed an induction of apoptosis by drug treatment, which might be crucial in eliminating damaged cells , the null limbs were resistant to this effect. Furthermore, in response to 4OOH-CPA-treatment, the cell cycle was not

arrested in p53 -/- limbs, in contrast to wild-type limbs; this arrest is necessary for DNA repair. Further investigation revealed the occurrence of necrosis in p53 -/- limbs. Thus, we suggest that the combination of the failure of cell cycle arrest and induction of necrosis may play an important role in the higher sensitivity of the p53-homozygous limbs in response to the teratogenic insult. The p53 +/- limbs showed an intermediate response in all experiments, indicating a "dose-effect" of the p53 gene. In this study we examined the role of apoptosis and its regulation and the role of the p53 gene during limb abnormal development in response to a DNA-damaging agent, thus helping shed light on an important mechanism in teratology.

ABRÉGÉ

Dans cette thèse, les effets d'un analogue actif de la cyclophosphamide (4-hydroxyperoxycyclophosphamide, 4OOH-CPA), un tératogène et agent alkylant, sur le développement des membres de la souris, la mort cellulaire et le rôle du gène p53 dans ce processus, ont été étudiés. Un système de culture de membres de souris a été utilisé à cet effet. Les membres de souris se sont développé normalement dans ce système et une apoptose physiologique été observée suivant un contrôle spatio-temporel spécifique. Le traitement avec l'analogue a provoqué des malformations et une réduction de la taille des membres, a augmenté l'induction de l'apoptose et a déclenché son occurrence prématurément, principalement dans les régions d'apoptose physiologique. Ceci indique que le traitement avec l'analogue pourrait avoir engagé le programme d'apoptose déjà disponible dans le membre en développement, et suggère que ceci pourrait être la cause des malformations observées dans les membres. Nous avons ensuite examiné les patrons d'induction de trois protéines, la cathepsine D, la transglutaminase et la clusterine, que l'on sait être impliquées dans le mécanisme moléculaire de l'apoptose. Le patron de leur induction suggère que la cathepsine D est impliquée dans la digestion des débris apoptotiques, que la transglutaminase a un rôle actif dans la préservation de l'intégrité cellulaire lors de l'apoptose et que la clusterine joue un rôle dans les phases tardives de l'apoptose. Puisque le 4OOH-CPA cause des dommage à l'ADN, nous avons étudié le rôle du gène p53, un gène qui répond aux dommages causés à l'ADN et impliqué dans la régulation du cycle cellulaire et de l'apoptose, dans un

modèle de souris transgénique pour le gène p53. L'absence de p53 a conduit à des malformations encore plus sévères dans les membres traités avec l'analogue. Afin d'étudier les raisons possibles de cette hypersensibilité, la mort cellulaire et le cycle cellulaire ont été examinés. Alors que les membres normaux montraient une induction de l'apoptose par le traitement à l'analogue, qui pourrait être cruciale pour l'élimination des cellules endommagées, les membres mutés (sans p53: -/- p53) montraient une résistance et ne démontraient aucune induction. De plus, à l'inverse des membres normaux, le cycle cellulaire n'était pas arrêté dans les membres -/- p53 en réponse au traitement avec le 4OOH-CPA, un processus nécessaire pour la réparation de l'ADN. Une analyse subséquente a montré la présence de nécrose dans les membres -/- p53. Ainsi, nous suggérons que le fait que le cycle cellulaire ne soit pas arrêté combiné à l'induction de la nécrose pourraient jouer un rôle important dans l'hypersensibilité des membres -/- p53 en réponse à un dommage tératogénique. Les membres hétérozygotes -/+ p53 ont démontrés une réponse intermédiaire dans toutes les expériences, indiquant un effet "lié à la dose" de la présence du gène p53. Dans cette étude nous avons examiné le rôle de l'apoptose et sa régulation et le rôle du gène p53 durant le développement anormal de membres en réponse à un agent causant des dommages à l'ADN, aidant ainsi à faire la lumière sur un important mécanisme en tératologie.

LIST OF FIGURES

PREFACE

Format of the Thesis

This thesis comprises three data chapters. All of which are manuscripts that are included almost entirely in the form in which they were submitted for publication. Connecting texts are provided in accordance with section B.2/ of the "Guidelines Concerning Thesis Preparation" of the Faculty of Graduate Studies and Research, McGill University, which states the following:

> "Manuscripts and Authorship:
>
> Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform of all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary. Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to all a clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defence. Since the task of examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of the co-authored papers."

The Introduction, Chapter One, includes a general review of the background literature regarding cell death, particularly apoptosis, limb development, cyclophosphamide and p53 and provides the rationale for the studies presented in

this dissertation. The *in vitro* model, hypotheses and the research objectives are also presented in Chapter One. Chapter Two has been published in Teratology (52: 3-14, 1995). Chapter Three has been published in Biology of Reproduction (55: 281-290, 1996). Chapter Four has been published in Development (125: 3225-3234, 1998). A general discussion of the results is presented in Chapter Five. A List of Original Contributions, that summarises the major findings of Chapters Two through Four, is included at the end of Chapter Five.

Contribution of Authors

All experiments and measurements presented in this thesis were performed by the candidate. The post-fixation, embedding, cutting and staining of limb samples for electron microscopy and immunogold electron microscopy were done by Ms. Marie Ballak, the technician in the Electron Microscopy Laboratory in the Department of Pharmacology and Therapeutics at McGill University.

ACKNOWLEDGMENTS

First, and foremost, I should like to thank my supervisor **Dr. Barbara Hales** for her guidance, encouragement, and kindness throughout my studies. She has been instrumental in teaching me scientific reasoning. I became what I am today only because of her advice.

My great thanks to **Dr. Bernard Robaire** for his helpful and constructive criticism and discussion of my work and publications. His great grasp in critical thinking, and research administration have been inspiring.

I would like to thank my adviser, **Dr. A. Claudio Cuello** for his wisdom, insights and concern for my academic and personal status.

I thank the **Department of Pharmacology and Therapeutics** for the opportunity to study in an excellent teaching and research environment.

I would like to acknowledge the financial support of the following groups at different stages of my studies: the **Department of Pharmacology and Therapeutics**, the **School of Medicine**, and the **Faculty of Graduate Studies and Research** at McGill University.

My thanks to **Dr. Jacquetta Trasler** for her excellent and constructive critique of my presentations and especially for allowing me to use her office to write this thesis.

My appreciation and regard to **Dr. Daya Varma** for his wisdom and truthful discussion of everything with me!, to **Dr. Yves De Koninck** for his excellent and efficient management of the departmental computer network and the secret pocket! and to **Dr. Alfredo Ribeiro-da-Silva** for his friendship and generosity in computer software and hardware; I enjoyed the countless computer discussions we had.

My greatest thanks to **Dr. Chunwei Huang** for her outstanding technical assistance and excellent lab management, to **Dr. Sara Austin** who taught me limb bud culture and to

Dr. Beiyun Chen for her patience to my endless questions about Western blot, immunohistochemistry and etc.

My thanks and regards to **Dr. Terence Ozolins** (TAZ The Devil) for his inspiring commitment to perfection, to Ms. **Wafa Harrouk** for her strong character and persistence, to Mr. **Robert Vinson** for sharing his computer knowledge with me, and to Ms. **Jennifer (J.J.) Manley**, **Ms. Sepideh Khatabakhsh**, **and Ms. Anna Liachenko** for being good friends and lab partners.

I am thankful to **Ms. Emmanuelle Roux** for translating my Abstract into French.

I am thankful to **Mr. Alan Forster** for his photographic help and the daily chat, and to **Ms. Marie Ballak** for her outstanding technical assistance in my electron microscopic studies.

Thanks to the office staff for their excellent work and also for never saying to me: go today and come tomorrow. They are **Catherine Anley**, **Naomi Belinski**, **Hélène Duplessis**, **Pam Moore**, **Sid Parkinson** and **Genny Snider.**

My gratitude to my wonderful classmates: **Dr. Maria Diaz Perez**, **Ms. Sue Grant**, **Dr. Keli Hu**, and **Dr. Kylie Ann Scoggan**.

Finally, I would like to say that I am honoured and privileged to know and work with many people throughout this study, they are: **Dr. Zohreh Banakar, Dr. Yannick Blanchard**, **Dr. Lu Cai**, **Dr. Dan Cyr**, **Dr. Xingfei Deng**, **Ms. Tonia Doerkson**, **Mr. Xueping Fan**, **Mr. Mohammad Ghahremani**, **Ms. Kathleen Jue**, **Ms. Carmen Mertineit**, **Ms. Valérie Serre,** and **Dr. Robert Viger**.

To everybody I mentioned in this section:

I WILL MISS YOU ALL!

CHAPTER ONE

INTRODUCTION

1. Cell death

1.1 Overview and Description

Over the past three decades, two fundamentally different forms of cell death, apoptosis and necrosis, have been defined in terms of morphology, biochemistry and incidence. Few topics have generated as much interest as the apoptotic process. Apoptosis, the mechanism of programmed cell death in multicellular organisms, once wallowed in relative obscurity, but is now intensely studied by cancer biologists, developmental biologists, immunologists, neurologists and countless others. This is largely justified by the presence of either inappropriate apoptosis or its pathologic absence in a wide range of diseases (Barr and Tomei, 1994; Thompson, 1995; Rudin and Thompson, 1997). It is a difficult task to cover all aspects of apoptosis comprehensively in this chapter. However, an overview of apoptosis, its mechanisms and role during development will be discussed.

1.1.1 History

The concept of cell death dates back to 1858, when cell death at the gross level was discussed in Virchow's Cellular Pathology as degeneration, mortification, and necrosis equivalent to the term "gangrene" (reviewed in Clarke and Clarke, 1996). Later in 1892, Strobe studied breast cancer cells and described "chromatolysis" and the nuclear pathology, which is considered a modern

description of apoptosis (reviewed in Gupta, 1996). In 1914 Grapher postulated that to counterbalance mitosis there should be an "amitotic" mechanism; he concluded that physiological elimination of cells occurred by chromatolysis during the shrinkage of organs where a sister cell engulfed a neighbouring cell that broke down (reviewed in Clarke and Clarke, 1996). Glucksmann understood the significance of chromatolysis in morphogenetic mechanisms. In a landmark paper, he described physiological cell death in the embryo (Glucksmann, 1951). In 1972, Kerr, Wyllie and Currie (Kerr et al., 1972) discovered a form of cell death that was morphologically distinct from necrosis, had features suggesting an intrinsically controlled active process that occurred under both physiological and pathological circumstances, was important in normal embryonic morphogenesis, and was of great kinetic significance in tissues and tumours. These investigators recognized that *necrosis* was an inappropriate term to describe active cell death occurring under physiological conditions; they proposed the term *apoptosis* for this process, emphasizing its role opposite that of mitosis in regulating tissue size. The word *apoptosis* meant "falling off" or "dropping off" and had been used to describe the dropping off of leaves from trees in autumn. They published their work in a landmark paper in the same year (Kerr et al., 1972). For more than a decade, their 1972 paper was largely ignored. Wyllie continued his study on the molecular biology of apoptosis; in 1980 he showed that chromatin in apoptotic cells is cleaved at internucleosomal sites by an endogenous nuclease (Wyllie, 1980). Since the late 1980s, exploitation of molecular biological techniques in particular has led to

rapid advances in knowledge about apoptosis.

1.1.2 Incidence

Under physiological conditions, apoptosis is involved in the programmed focal elimination of cells that accompanies embryonic and fetal development (see subsection 1.3 in this chapter) and in regulating the size of organs and tissues in adult life. Pathologically, it accounts for deletion of cells in many types of atrophy and in the regression of hyperplasia.

The continuous spontaneous apoptotic deletion of cells is a consistent feature of rapidly and slowly proliferating mammalian cell populations, e.g. intestinal crypts and the basal compartment of seminiferous tubules (Hinsul and Bellamy, 1981; Bhathal and Gall, 1985; Columbano et al., 1985; Walker, 1987). The size of many cell populations is known to be under the control of growth factors or hormones, with rapid diminution in cell number occurring after withdrawal or addition of the relevant trophic substances. The mode of cell deletion in these circumstances appears invariably to be by apoptosis. Thus, a physiological fall in trophic hormonal stimulation is accompanied by a wave of apoptosis in the human premenstrual endometrium (Hopwood and Levison, 1976) and in the human breast towards the end of the end of the menstrual cycle (Ferguson and Anderson, 1981). Pathological atrophy of the rat prostate induced by castration is associated with extensive apoptosis of epithelial cells (Sandford et al., 1984). Also, moderate doses of ionizing radiation and ultraviolet light increase apoptosis in normal proliferating

populations (Duncan et al., 1983; Ijiri and Potten, 1984). Mild hyperthermia results in apoptosis (Allan and Harmon, 1986) and many cancer-chemotherapeutic substances enhance apoptosis in normal proliferating and neoplastic cell populations (Ijiri and Potten, 1983). Another important occurrence of apoptosis is during cell-mediated immunity. There is a substantial body of evidence demonstrating that cell-mediated immune reactions induce apoptosis (Berke, 1994).

Cell death with the ultrastructural features of necrosis is observed in tissues subjected to severe hypoxia and ischaemia (Borgers et al., 1987) and in tissues undergoing autolysis *in vitro* (Finlay-Jones and Padadimitriou, 1973). *In vitro* exposure to reactive oxygen metabolites can cause necrosis (Bishop et al., 1987). Cell death following severe hyperthermia is known to take the form of necrosis (Buckley, 1972).

1.1.3 Morphology

The recognition of apoptosis and necrosis is based primarily on the distinctive changes that take place within the affected cells. However, when these two processes occur *in vivo*, they also differ in their distribution and in the tissue reactions that are associated with them (reviewed in Kerr and Harmon, 1991). Thus, apoptosis typically involves scattered individual cells in a tissue, whereas necrosis involves groups of adjoining cells. Further, necrosis is usually accompanied by an acute inflammatory response; this event is characteristically absent in apoptosis. At the electron microscopic level, the earliest unequivocal

morphological evidence of the onset of apoptosis is found in the nucleus. The chromatin condenses and becomes aggregated in sharply delineated, finely granular masses of uniform texture that abut the inner surface of the nuclear envelope. Then, the nuclear convolution becomes extreme and is followed by budding to produce discrete nuclear fragments of varying size and chromatin content. A series of equally dramatic events takes place in the cytoplasm concurrently with the nuclear changes just described. Overall condensation occurs with protruding or blebbing of the cell surface (Kerr et al., 1972). Clear vacuoles may be numerous in the condensing cytoplasm (Robertson et al., 1978). The blisters that have formed on the cell surface soon separate to produce membrane-bounded apoptotic bodies of varying size and composition. The cytoplasmic membrane and organelles in newly formed apoptotic bodies are still intact. Most apoptotic bodies formed within tissues are rapidly phagocytosed by resident macrophages or by other nearby cells. After their phagocytosis, apoptotic bodies are degraded by lysosomal enzymes derived from the cells in which they lie (reviewed in Wyllie et al., 1980).

Under certain circumstances, such as exposure of cells to very high temperatures or to concentrated solutions of corrosive chemicals, cell death is instantaneous. In most cases of cellular injury, however, the damage is less catastrophic, so the development of necrosis is preceded by the appearance of morphological abnormalities that are indicative of grossly disturbed cellular homeostasis. These features include swelling of the cell sap, surface blebbing, shrinkage followed by swelling of the inner compartment of mitochondria, dispersal

of ribosomes and mild clumping of nuclear chromatin (Trump et al., 1973; Trump et al., 1981; Farber, 1982). Then, gross swelling of all cytoplasmic compartments, rupture and dissolution of plasma, organelle and nuclear membranes can lead to cellular disintegration.

1.1.4 Biochemistry

The most widely publicised biochemical events associated with apoptosis are endonuclease activation. This results in the cleavage of DNA at the nucleosomal linker regions eventually giving rise to regular oligonucleotide runs of DNA that are multiples of approximately 180-200 base pairs (Wyllie, 1980; Umansky, 1982; Arends et al., 1990). Such runs, when separated on an agarose gel give rise to a characteristic "ladder". DNA laddering, associated with endogenous endonuclease activation, has been used as a "marker" for apoptosis (Compton, 1992). Wyllie (1980) showed that in glucocorticoid-treated thymocytes endogenous endonuclease activation was associated with apoptotic chromosome condensation. Wyllie et al. (1984) reported that this preexisting or endogenous neutral endonuclease was activated by Ca^{2+} and Mg^{2+} but inhibited by Zn^{2+}. Subsequently endonuclease induced DNA laddering was used as a "reliable" marker for apoptosis (Walker et al., 1988; McConkey et al., 1989). Later, it was found that the DNA is cleaved initially into large (i.e., 300 and/or 50 kb) fragments prior to its cleavage into oligonucleosomal-sized fragments (Oberhammer et al., 1993; Walker et al., 1993). Another interesting discovery was that there are cell surface alterations during

apoptosis, such as the exposure of molecules normally confined to the interior of the cell, e.g. the exposure of phosphatidylserine on the outer surface; this is believed to play a role in the recognition of apoptotic bodies by the neighbouring cells for phagocytosis (Fadok et al., 1992; Martin et al., 1995; Martin et al., 1996; Vangs et al., 1996).

A breakthrough came in the recognition that in all known instances of apoptosis, a family of cysteine proteases related to the IL-1-β-converting enzyme (ICE) becomes activated; these proteases execute apoptosis through specific cleavage of substrates (reviewed in Martin and Green, 1995). One of the first recognized and commonly cleaved death substrates is the enzyme poly-ADP-ribose-polymerase (PARP) whose proteolytic cleavage during apoptosis is one of the first detectable events (Lazebnik et al., 1994). The biochemical identification of an enzyme capable of cleaving PARP yielded a new member of the ICE family called CPP32 (Nicholson et al., 1995). Importantly, specific peptide-based inhibitors of CPP32 prevented both cleavage of PARP and apoptosis (Nicholson et al., 1995).

The development of necrosis is characterized by the occurrence of an irreversible, marked increase in the permeability of the mitochondrial and plasma membranes (Trump et al., 1984). This is reflected by classical tests for cell death that demonstrate penetration into the cell of colloidal, electron-dense material (Hoffstein, et al., 1975) or vital dyes, and accelerated loss of previously cell-bound radioactive chromium (Wyllie et al., 1980). At least in many cases, the genesis of the irreversible increase in membrane permeability is believed to involve enzymatic degradation of membrane phospholipids. Following direct injury to the cell

membrane by trauma or membrane-active toxins (Schanne et al., 1979; Trump et al., 1981) or as a result of failure of membrane pumps secondary to cellular energy depletion (Jennings and Reimer, 1981; Trump et al., 1981), cations move across the membrane along concentration gradients. The accompanying fluid shifts cause cellular swelling. Where the disturbances are severe, the increased concentration of free cytosolic calcium results in extensive activation of membrane-bound phospholipases (Farber et al., 1981; Trump et al., 1981), which degrade membrane phospholipids. The collapse of cell volume homeostasis following loss of membrane integrity leads to cessation of all ordered biochemical activity, and the release of hydrolases from ruptured lysosomes causes a rapid acceleration of cellular disintegration in the late stages of the evolution of necrosis (Hawkins et al., 1972). DNA exposed by proteolytic digestion of histones is cleaved by lysosomal deoxyribonuclease into fragments displaying a continuous spectrum of sizes (Afanas'ev et al., 1986; Duvall and Wyllie, 1986).

1.2 Role of cell death in development

1.2.1 Normal development

Development of all multicellular organisms depends on mitosis, differentiation, maturation and cell death. An elegant balance among these processes results in the formation of a viable individual. The term programmed cell death (PCD) was introduced by Lockshin and Williams (1965) to describe the developmentally regulated loss of specific larval muscles following the emergence

of adult moths at the end of metamorphosis. During this transition cells are lost in a spatially and temporally reproducible manner in response to physiological signals as part of a developmental or homeostatic process. More recently, the term PCD has been used in a wider context to refer to any cell death that is dependent on a gene-mediated "program", independent of the signal(s) that initiates the process. Therefore, while a developmental biologist might not consider the irradiation-induced death of thymocytes an example of PCD, an immunologist might. Another issue is the use of the terms PCD and apoptosis. PCD is a process, while apoptosis describes a cellular morphology. In most cases, the loss of cells during PCD occurs with an apoptotic morphology but this is not always the case. Conversely, many cells that are induced to die in response to pathological stimuli do so with an apoptotic morphology.

The role of PCD in development was first appreciated by Hamburger and Levi-Montalcini (1949). Quantitatively they demonstrated that approximately 50% of the sympathetic and motor neurons produced during embryogenesis in chicks died during a discrete developmental period prior to birth. Since then, many examples of PCD have been identified in different tissues, organs and organisms. Some examples include: the nervous system (reviewed in Oppenheim, 1991), glia cells in rat (Barres et al., 1992), lens cells in vertebrates (Ishizaki et al., 1993), limbs in birds and mammals (reviewed in Hinchliffe, 1981), muscle cells in humans (Webb, 1974), kidney in mammals (Coles et al., 1993), palate epithelium in rats and mice (Mori et al., 1994), germ cells in mice (Coucouvanis et al., 1993), skin cells in humans (Polokowska et al., 1994), intestinal epithelium in rats (Harmon et al.,

1984), thymocytes in birds and mammals (MacDonald and Lees, 1990), and tail regression of humans (Fallon and Simandl, 1978). The occurrence of PCD in *Caenorhabditis elegans* (*C. elegans*) has proven to be an ideal system in which to study PCD during development. A well-characterized set of cell lineages results in the appearance of 1090 somatic cells. The nematode is transparent and 131 cells can be identified that undergo PCD (Driscoll and Chalfie, 1992). Genetic analysis of *C. elegans* has revealed genes that function in normal developmental cell death, as well as genes that mutate to cause inappropriate cell death (reviewed in Driscoll, 1992). Molecular characterization of these "death" genes in *C. elegans* has provided insight into normal and abnormal mechanisms of cell death in this system, which in many cases clearly are applicable to vertebrates (Driscoll and Chalfie, 1992; reviewed in Hengartner and Horvitz, 1994).

PCD functions in animal development for different purposes. First, it plays an essential role in sculpting parts of the body. The formation of digits in some higher vertebrates is a well-studied example, where PCD eliminates the cells between developing digits (reviewed in Hinchliffe, 1981). If the cell death is inhibited by treatment with a peptide caspase inhibitor, digit formation is blocked (Milligan et al., 1995; Jacobson et al., 1996). Similarly, PCD is involved in hollowing out solid structures to create lumina. For example, in early mouse embryos the preamniotic cavity is formed by the death of the ectodermal cells in the core of the developing embryo (Coucouvanis and Martin, 1995). Second, in the course of animal development, various structures are formed that are later removed by PCD. For example, during metamorphosis in amphibians and insects, structures required

for larval life, such as the tadpole tail, are selectively lost during adult development (Tata, 1966). Third, in many organs, cells are overproduced and then culled by PCD to adjust their numbers. For example, as has been mentioned in the above paragraphs, in the vertebrate nervous system, both neurons and oligodendrocytes are generated in excess, and up to half or more are eliminated by PCD, apparently to match their numbers to the number of target cells that they innervate (reviewed in Oppenheim, 1991). Fourth, PCD also functions as part of a quality-control process in animal development, eliminating cells that are abnormal, misplaced, nonfunctional, or potentially dangerous to the organism. A striking example is that during T cell maturation large numbers of immature thymocytes are generated that bear self-reactive T cell receptors. Should these auto-reactive cells mature and exit the thymus, they have the potential to initiate an auto-immune response. This catastrophic outcome is prevented by inducing these self-reactive cells to die during the process of negative selection (Murphy et al., 1990). There are many cell deaths that occur during animal development for which the function of death is unknown. This is the case for the inner cell mass of early mammalian embryos (El Shershaby and Hinchliffe, 1974), and in developing germ cells in mammalian spermatogenesis (Allan et al., 1987).

1.2.2 Teratogenesis

Programmed cell death in the embryo is under tight regulation and strongly influences the morphology of the embryo. Interference with embryonic programmed

cell death results in embryo malformations; in fact it is believed that several chemical and physical agents are teratogenic to the developing embryo because they induce extensive cell death in various tissue types (Russell and Russell, 1954; Knudsen et al., 1992; Thayer and Mirkes, 1995). Specifically, it has been shown that 4-hydroperoxycyclophosphamide (Little and Mirkes, 1992) and cyclophosphamide (Nomura et al., 1996), pentistatin (Knudsen et al., 1992), adenosine deaminase (Gao et al. 1994), the DNA alkylating agent N-acetoxy-2-acetyl aminofluorene (Thayer and Mirkes, 1995), and recently diabetes (Fallen et al., 1997) induce cell death in developing tissues. Several teratogens affect the developing limb if administered during a specific stage of embryonic development (Kochhar 1973; Kochhar, 1977). Jiang and Kochhar (1992) have reported the induction of apoptosis in limb buds in areas where the programmed cell death occur, after exposure to retinoic acid.

On the other hand, reduction of the rate of programmed cell death during embryogenesis can lead to abnormal morphogenesis. For example maternal exposure to bromodeoxyuridine, which prevents programmed cell death in certain areas of the limb, causes embryonic preaxial polydactylism (Scott, 1981). Also, antiproliferative agents, such as cytosine arabinoside (Scott et al., 1977), 5-fluorodeoxyuridine (Scott et al., 1977) and 6-mercaptopurine riboside (Scott et al., 1980), which all cause preaxial polydactylism, delay programmed cell death in the apical ectodermal ridge region of the limb.

Therefore, there is ample evidence indicating that interference with physiological cell death during development can have teratogenic consequences.

1.3 Gene expression during apoptosis

During the last decade many genes involved in the process of apoptosis have been identified (reviewed in Hale et al., 1996). We describe some of the genes that have been used in this study as markers for the occurrence of apoptosis.

1.3.1 Cathepsin D

Cathepsin D is an aspartyl endopeptidase that accounts for a major part of the proteolytic activity in lysosomes and endosomes (Barrett, 1977; Diment et al., 1988; Bowser and Murphy, 1990; Bohley and Seglen, 1992). Early evidence of protease activation in apoptosis came from observations made by Lockshin (Lockshin, 1969; Lockshin, 1981) and Bowen (Bowen, 1984) during programmed cell death in the intersegmental muscles of insects undergoing metamorphosis. In these studies, the authors observed an elevation of autophagic lysosomal activity in areas of active cell death. It is now generally thought that the release of proteases from lysosomes plays a secondary role in cell death, since in several different experimental systems lysosomes appear intact until the final stages of cell disruption (reviewed in Nixon and Cataldo, 1993). The specific activity of cathepsin D, an important lysosomal protease, has been shown to increase significantly during prostate involution (Tanabe et al., 1982). Sensibar et al. (1990) showed that castration in rats induces the induction of prostatic cathepsin D protein in apoptotic cells. Cathepsin D expression is induced under both physiological (development,

tissue remodelling) and pathological (tumour invasion and metastasis) conditions (Rochefort et al., 1990; Garcia et al., 1996).

1.3.2 Transglutaminase

The transglutaminase multigene family includes intracellular and extracellular enzymes that catalyse Ca^{2+}-dependent cross-linking reactions in which γ-carboxamide groups of glutamine residues serve as acyl donors and primary amino groups of protein-bound lysine, or small molecular weight amines function as acceptor substrates (Folk, 1980; Fesus et al., 1985). The reaction results in posttranslational modification of proteins by establishing ε(γ-glutamyl)lysine cross-linkage and/or covalent incorporation of biogenic amines (di- and polyamines and histamines) into proteins. Diamines and polyamines also participate in cross-linking reactions through the formation of N,N-bis(γ-glutamyl)polyamine cross-bridges (Folk, 1980). Transglutaminase-dependent cross-linking of proteins leads to protein polymerization that confers stability as well as resistance to mechanical disruption and chemical attack (reviewed in Greenberg et al., 1992). In mammals, five transglutaminase genes have been identified so far, encoding biochemically and immunologically distinct transglutaminases: blood coagulation Factor XIIIa, epidermal, keratinocyte, prostate and tissue type transglutaminases (Piacentini et al., 1994). The various transglutaminases have been implicated in a wide range of biological phenomena occurring in both extracellular and intracellular compartments and inducing blood coagulation, wound healing, terminal differentiation and cell

death. Although these functions appear divergent, they all converge toward protection of cell and tissue integrity (Greenberg, 1992).

The first reports of the participation of a transglutaminase in the process of apoptosis were published in 1987 (Fesus et al., 1987). It was suggested that transglutaminase helps in the formation of a cross-linked protein scaffold in apoptotic cells. These detergent-insoluble protein nets join cytoplasmic and membrane proteins which maintain cellular integrity during the formation of apoptotic bodies and prevent the dead cells from breaking into fragments prior to their digestion (Fesus and Thomazy, 1988; Piacentini et al., 1991; Jiang and Kochhar, 1992; Knight et al., 1993). There is strong evidence that tissue transglutaminase is a specific marker for apoptosis in many tissues (Fesus and Thomazy, 1988; Piacentini et al., 1991; Jiang and Kochhar, 1992; Knight et al., 1993). Jiang and Kochhar (1992) have correlated the induction of tissue transglutaminase activity in limb buds with apoptosis induced by exposure to retinoic acid. Cell transfection with a tissue transglutaminase cDNA resulted in a significant reduction in proliferative rate and a large increase in the number of apoptotic cells (Melino et al., 1994); transfection with a cDNA segment in the antisense orientation led to a dramatic decrease in both spontaneous and retinoic acid-induced apoptosis. Thus, the regulation of tissue transglutaminase is important in tissues undergoing apoptosis (Melino et al., 1994).

1.3.3 Clusterin

Clusterin, a glycosylated and sulfated glycoprotein, was first isolated from ram rete testis fluid as a protein that elicited clustering of Sertoli cells (Fritz et al., 1983). The rat homolog of clusterin, sulfated glycoprotein-2 or SGP-2, was subsequently isolated from Sertoli cells and detected in several sites of the rat reproductive system (Collard and Griswold, 1987). The expression of clusterin in the testis, epididymis, ovary, uterus, and a wide variety of tissue types during embryogenesis suggests an important role in reproduction and development (Ahuja et al., 1994). Clusterin expression has been associated with programmed cell death or apoptosis in the prostate (Buttyan et al., 1989; Grima et al., 1990), the interdigital areas of mouse limb buds (Buttyan et al., 1989), thymocytes (Bettuzi et al., 1991) and the kidney (Gobe et al., 1995). The role of clusterin during apoptosis is not clear. One of the proposed roles for clusterin is in the transport of lipid between cells; the induction of clusterin in apoptosis or tissue injury may provide a mechanism to eliminate excess lipid derived from dead or injured cells or to scavenge toxic lipid byproducts (reviewed in Rosenberg et al., 1993). Alternatively, clusterin may act as an inhibitor of complement-mediated cytolysis by binding to the soluble C5b-7 to form a cytolytically inactive complex; this would protect both the apoptotic cell and its vital neighbours from complement attack (reviewed in Jenne and Tschopp, 1992 and Rosenberg et al., 1993). Recently, it has been suggested that clusterin expression in some tissues is not enhanced, but rather is down-regulated in the cells undergoing apoptosis, and that its expression in the apoptotic tissue is restricted to the vital neighbouring cells (French et al., 1994; Little and Mirkes, 1995; Arai et al., 1996). Thus, it is speculated that clusterin is a cell survival

gene, exerting a protective function on the surviving cells (reviewed in Koch-Brandt and Morgan, 1996).

2. Limb Development

Embryonic development depends, at the cellular and molecular levels, on a large number of interdependent processes including cellular proliferation, migration, differentiation and death that cooperate to affect tissue morphology and physiology. Historically, vertebrate limb development has been used as an excellent model to study these processes in normal development and during insult with a teratogen, especially since this system has reached a level of investigation permitting the initiation of a meaningful commentary on abnormal development (Fallon and Caplan, 1983). Limb development, due to its unique features, has been utilized in this study as our model system to investigate the role of apoptosis and the p53 gene in drug-induced teratogenesis.

2.1 Developmental biology of the vertebrate limb

The vertebrate limb originates from a dual contribution of lateral plate and somitic mesoderm (Chevallier et al., 1977; Christ et al., 1977). Through differential proliferation of the flank, specific regions of the lateral plate form buds at presumptive limb levels (Searls and Janners, 1971). Shortly thereafter, cells from the lateral edges of nearby somites migrate into the limb. All adult limb muscle derives from these migratory cells. Limb muscle, nerve, and vasculature have their

origins in extra-limb regions, while all other limb tissues, including skeletogenic mesenchyme, cartilage, and tendons, derive from lateral plate mesoderm. The limb bud is enveloped by an overlying ectodermal jacket, whose distal tip, in most tetrapods, forms a specialized epithelial structure, the apical ectodermal ridge (AER) running along its anterior-posterior axis at the interface of dorsal and ventral territories (Fallon and Kelly, 1977; Todt and Fallon, 1984). Cells directly under the AER remain undifferentiated in the so-called "progress zone," while condensation initiates in proximal limb regions, so that the humeral anlage forms first, followed by the radius and ulna, and lastly the digits. The net result of these developmental processes is the establishment of familiar prototypical tetrapod limb features. The first clues as to how this complex shape and form is achieved came from the work of experimental embryologists who identified specific regions of the developing chick limb bud that are essential in directing growth and patterning.

From several studies it has been indicated that the intermediate mesoderm is required for limb bud initiation (Stephens and McNaulty, 1981; Strecker and Stephens, 1983). It has been suggested that the intermediate mesoderm produces a factor that maintains proliferation of the flank in presumptive limb regions. The expression pattern of chick fibroblast growth factor 8 (FGF-8), as well as its morphogenetic abilities, make it an excellent candidate for playing a role in this process (Vogel et al., 1996). It has been observed that when beads soaked in FGF-8 or other FGF family members are placed in interlimb regions, new ectopic limb buds form from the lateral plate mesoderm (Cohn et al., 1995; Vogel et al., 1996). The experimentally induced limb buds form an AER at their tip and develop quite

normally, and at 10 days of gestation their morphology is typical of normal limbs. Hence, FGF-8 is sufficient to induce an entire program of limb bud initiation and subsequent patterning.

Once limb buds form, their continued proliferation depends on the AER. If the AER is removed, outgrowth is affected, resulting in distal truncations (Saunders, 1948; Summerball, 1974; Rowe and Fallon, 1982). At least three FGFs are expressed in limb ectoderm: FGF-2, -4 and-8 (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). Once the ridge has fully formed, FGF-8 expression is restricted to the AER (Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996).

Heterotopic grafting of small blocks of posterior mesenchyme into ectopic anterior locations provided important information about the anterior posterior positional specification (Saunders and Gasseling, 1968). It has been found that in response to these grafts, whole mirror-image duplications of the distal limb were readily obtained. Because the graft reorganized the anterior posterior order of structures within the limb, this region was termed the zone of polarizing activity (ZPA), or polarizing region. It has been proposed that ZPA cells secrete a morphogen which sets the identity of cells along the anterior posterior axis (Wolpert, 1969). The molecule responsible for the morphogenetic properties of the ZPA is Sonic hedgehog (Shh; Echelard et al., 1993; Riddle et al., 1993). Shh's expression colocalizes with ZPA activity in the chick limb bud, and its expression can be induced by application of retinoic acid, an agent known to induce polarizing activity (Riddle et al., 1993). Transplantation of the ZPA, or application of Shh to ectopic

locations can reorganize anterior posterior limb pattern and can, concurrently, induce ectopic proliferation of limb bud mesenchyme. Therefore it has been inferred that the normal function of ZPA is to promote proliferation of posterior limb bud mesenchyme and to specify the identity of the limb along the anterior posterior axis (Wolpert, 1969; Tickle et al., 1975).

Wnt-7a has been identified as a signalling molecule in dorsal ventral patterning (Dealey et al., 1993; Parr et al., 1993). Transcripts of Wnt-7a are strikingly confined to dorsal limb bud ectoderm in both mouse and chicken embryos (Dealey et al., 1993; Parr et al., 1993). Parr and McMahon (1995) have produced a transgenic mouse in which Wnt-7a is functionally inactivated. The paws of these mice show a marked change in dorsal ventral pattern. For example, special features of the skin found normally on the sole (pads ets.) are now found on both sole and upper paw surface.

Morphologically, programmed cell death in given areas seems to play a significant role in limb bud shaping, at least in the avian embryo. The patterns of cell death are not the same for all species. In the chick wing bud there are initially three necrotic (as were called prior to the introduction of the term apoptosis) zones: two along the anterior and the posterior border, in a more proximal position in earlier than in older buds, and a patch in an axial position (Saunders et al., 1962; Hinchliffe and Thorogood, 1974). In chick mutants, an increase (*talpid*) or decrease (*wingless*) in the number of digits seems to be related to the absence or to the increase above normal of one or two necrotic zones (Hinchliffe and Ede, 1967; Hinchliffe and Ede, 1973). Later-appearing areas of cell death in the interdigital

mesenchyme of the developing foot plate in many amniotes, from reptiles to man, play a morphogenetic role in separating and shaping the digits (Kieny et al., 1976; Cameron and Fallon, 1977). There are two lines of observation that lead to this conclusion. First, in amniotes with completely free digits (e.g., chicken or human), zones of cell death seem to eliminate most or all interdigital mesodemal cells. However, in amniotes with webbing between the digits the death pattern is modified so that only the part of the interdigital area that is actually free in the adult shows massive cell death in the embryo or fetus (Saunders and Fallon, 1967). A second line of evidence on the utility of cell death for embryonic development comes from preventing the cell death by injecting the dye Janus green B (Menkes and Deleanu, 1964). If this material is injected into the chicken embryo at 6 1/2 days of incubation, interdigital cell death is prevented and the digits of both wing and leg show soft-tissue syndactyly.

Another area which shows cell death is the AER. Two well delineated, though unequal, areas of cell death appear in the early AER of rat and mouse limb buds, as soon as it starts forming in the ventromarginal territory (reviewed in Milaire and Rooze, 1983). It is interesting to notice that these early degenerative phenomena appear in the AER before this structure has acquired its maximal thickness, and therefore have nothing to do with the later involution of the ectodermal thickening (reviewed in Milaire and Rooze, 1983).

2.2 Limb malformations

Many chemical agents have been shown to cause limb malformations. However, most of them are not specific to the limbs alone. Only some of teratogens can selectively produce limb defects if administered to pregnant females over a limited time span (reviewed in Kochhar, 1977). It is evident that limbs are not equally susceptible to teratogens during all stages of morphogenesis. Susceptibility begins as limb bud elongation is initiated and ends by the time the limb skeleton is formed as cartilage. Early experiments to induce limb malformations in mammalian embryos were done by exposing pregnant rats to X-rays which resulted in clubbing of the feet and absence of toes in the offspring (Murphy and DeRenyi, 1930).

In the 50s and 60s the thalidomide tragedy forced many countries to institute birth defects monitoring systems. Thalidomide was marketed as sedative. When it was taken by pregnant women, it caused thousands of limb defect embryopathies in Europe and Australia (Fraser, 1988). Since then, many chemical and physical agents were discovered to be teratogenic. Some aspects of limb teratogenesis were discussed under subsection 1.2.2.

3. Cyclophosphamide

The model teratogen used in this study was 4-hydroperoxycyclophosphamide, an activated analog of the alkylating agent cyclophosphamide. Cyclophosphamide, a widely used antineoplastic drug, is teratogenic in rats (Chaube et al., 1967) and mice (Gibson and Becker, 1968; Gebhardt, 1970). Cleft palate, exencephaly, digital defects, kinky tail, polydactyly,

syndactyly, ectrodactyly, adactyly, fusion of the long bones, curvature of the long bones, missing ribs, open eyes, aphakia, microphakia, hydronephrosis and hydrocephalus have been produced in mice fetuses (Gibson and Becker, 1968; Gebhardt, 1970; Hales, 1981; Torchinsky et al., 1995). Murine limbs cultured during organogenesis *in vitro* with 4-hydroperoxycyclophosphamide exhibit growth retardation and dysmorphogenesis and a decrease in the DNA, RNA, and protein contents (Hales and Jain, 1986).

Cyclophosphamide is activated by a cytochrome P-450 monooxygenase to 4-hydroxycyclophosphamide. This compound, as well as its pre-activated analog, 4-hydroperoxycyclophosphamide, tautomerizes with aldophosphamide which spontaneously breaks down to form the active metabolites, phosphoramide mustard and acrolein (Foley et al., 1961; Takamizawa et al., 1975; Hales, 1982).

It is thought that phosphoramide mustard is the active metabolite responsible for the antitumour properties of cyclophosphamide (Connors et al., 1974). This is substantiated by the observation that phosphoramide mustard cross-links DNA and the residual, irreparable DNA cross-links eventually kill their target cells (Murnane and Byfield, 1981). In addition, it has been demonstrated that phosphoramide mustard readily alkylates the N7 position of guanosine and deoxyguanosine (Mehta et al., 1980). Acrolein is another important cyclophosphamide metabolite. It is well known that acrolein is cytotoxic (Alarcon and Meinhofer, 1971) and contributes to the haemorrhagic cystitis caused by cyclophosphamide therapy (Wrabetz et al., 1980).

More recent studies provided evidence that cyclophosphamide leads to

unrepairable DNA single-strand breaks and DNA crosslinks (Hengstler et al., 1997), although Wang et al. (1993) have proposed that the cytotoxic effect of cyclophosphamide is mediated mostly by DNA-DNA interstrand cross-links and DNA-protein cross-links. Bubley et al. (1994) reported an interesting study in which it was suggested that cyclophosphamide (and some other alkylating agents) exert some DNA sequence-specifity in their DNA targeting.

The teratogenic action of cyclophosphamide is also mediated by its metabolites (Fantel et al., 1979); as both phosphoramide mustard (Mirkes et al., 1981) and acrolein (Hales, 1982; Mirkes et al., 1984) have been proven to be teratogenic. It is thought that alkylating and damaging embryonic macromolecules (DNA, RNA and protein), especially DNA, is the main mechanism for cyclophosphamide teratogenicity (Short, 1972; Murthy, 1973).

There have been several reports showing the effect of cyclophosphamide on cell death. Manson et al. (1982) studied the interaction between cell death and DNA damage in mice limbs induced by cyclophosphamide and found that some limb cells died while other survived with DNA damage, although no attempt was made to examine the type of cell death. In other studies, cyclophosphamide induced malformations in different organs, resorption and growth retardation in mice and was found to be associated with cell death induction in various tissues (Peiffer et al., 1991; Torchinsky et al., 1995). Again, the type of cell death and its time course were not investigated. These reports point to a cell death induction by cyclophosphamide, however this effect was not well characterized.

Cyclophosphamide is also a potent mutagen and carcinogen, producing gene

mutations, chromosome aberrations, micronuclei and sister chromatid exchanges in a variety of cultured cells (reviewed in Anderson et al., 1995). The cyclophosphamide-induced cellular damage, if it can not be repaired, eventually leads to death of the damaged cell which is thought will induce embryo malformations (Scott, 1977; Manson et al., 1982; Peiffer et al., 1991; Torchinsky et al., 1995; Nomura et al., 1996).

4. The p53 gene

Since the main molecular effect of cyclophosphamide is the induction of DNA damage, we intended to examine the p53 gene involvement in this process; a gene that has been known to play a central role in protecting cells against DNA damage, and hence preserving the cellular genome.

The p53 tumour suppressor protein, that has a fundamental role in growth and neoplasia, has been one of the most extensively studied proteins in recent years. It has been subjected to many extensive and excellent reviews (Gottlieb and Oren, 1996; Ko and Prives, 1996; Velculescu and El-Deiry, 1996; Almog and Rotter, 1997; Levine, 1997). It is beyond the scope of this thesis to present a comprehensive review of p53 functions and actions. However, a brief overview of its biology and its involvement in development and teratogenesis will be discussed.

p53 was first identified in 1979 by virtue of its association with simian virus 40 (SV40) large T-antigen and by its apparently high expression in chemically induced tumours or spontaneously transformed cells (DeLeo et al., 1979; Lane and

Crawford, 1979; Linzer and Levine, 1979). For the next decade, p53 was thought to be a transforming oncogene whose expression resulted in increased DNA synthesis, a necessary feature of cellular proliferation. By 1989 several lines of evidence were beginning to suggest that the wild-type p53 gene was actually a tumour suppressor gene, instead of an oncogene (Finlay et al., 1989; Nigro et al., 1989). Over the past few years, studies have begun to clarify the molecular basis for tumour suppression by p53, as well as provide insight into the role of p53 in normal growth control (reviewed in Gottlieb and Oren, 1996).

The p53 gene is the most commonly mutated gene in human cancer (Chang et al., 1995). Genetic alterations of p53 in human tumours include allelic losses, missense and frameshift mutations, intragenic deletions and epigenetic changes. Analysis of the spectrum of p53 alterations in different tumour types, exposure risks, or geographic locations has led to a new understanding of cancer pathogenesis based on molecular epidemiology. Thus, new risk-assessment and intervention strategies are being developed (reviewed in Harris and Hollstein, 1993).

4.1 Biology of p53

The nuclear phosphoprotein p53 functions in cell-cycle arrest, apoptotic cell death, inhibition of tumour growth and preservation of genetic stability. It performs these functions through involvement in several biochemical pathways, including transcriptional activation, transcriptional suppression, and inhibition of DNA replication (reviewed in Gottlieb and Oren, 1996).

The p53 protein can be divided into three domains with known-functions, that are highly conserved at the aminoacid level throughout evolution (reviewed in Gottlieb and Oren, 1996). The amino-terminal domain contains the activation domain, the central core contains its sequence-specific DNA-binding domain, and then there is the multifunctional carboxy-terminal domain (Unger et al., 1992; reviewed in Gottlieb and Oren, 1996).

Wild-type p53 is present in extremely small quantities in most cells and displays a rapid turnover rate that is in the order of minutes. It is generally agreed that the inductive response is post-transcriptional (Kastan et al., 1991) and appears to be cell-type dependent (Midgley et al., 1995). Irradiation of cells with either ionizing radiation or UV light or exposure to other DNA-damaging agents induces p53; the presence of DNA strand breaks is critical for this induction (Kastan et al., 1991; Nelson and Kastan, 1994). It has been predicted that even a single double-stranded break is enough to induce p53 (DiLeonardo et al., 1994).

Wild-type p53 protein can transcriptionally transactivate genes involved in cell arrest (e.g., $p21^{waf1}$, a potent inhibitor of most cyclin-dependent kinases; El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993) and interact either with the DNA repair and synthetic machinery (e.g., proliferating cellular nuclear antigen (PCNA), GADD45 and $p21^{waf1}$; Li et al; 1994; Smith et al, 1994) or proteins modulating apoptosis (e.g., Bax; Miyashita et al., 1995). p53 can also inhibit DNA synthesis by a transcription-independent mechanism, binding to putative origins of DNA replication (Cox et al., 1995). p53 forms protein-protein complexes with cellular proteins involved in DNA synthesis, such as replicating protein antigen (Dutta et al.,

1993), or DNA repair, such as topoisomerase I (Gobert et al., 1996). It seems that cellular context determines whether p53 can induce apoptosis independent of or dependent on its transcription-transactivation function and in the absence of RNA and protein synthesis (Caelles et al., 1994; Sakamuro et al., 1995).

It has been suggested that p53-mediated cell cycle arrest provides more time for DNA repair to take place. However, recent findings suggest that p53 is also involved actively in the DNA repair process (Smith et al., 1995; Li et al., 1996). It is not clear how a cell decides whether to arrest growth and repair DNA or to induce apoptosis in response to increased p53. Rowan et al. (1996) reported that in a p53 mutant cell line, p53 had a direct role in mediating cell-cycle arrest despite the loss of the ability to induce apoptosis. The process of apoptosis may be p53-dependent or independent. Thymocytes from p53-deficient mice are resistant to the induction of apoptosis by radiation, but not by anti-CD3 antibody or glucocorticoids; this observation suggests that the induction of apoptosis by radiation, but not other insults, is p53-dependent (Clarke et al., 1993; Lowe et al., 1993a). Also, treatment of embryonic fibroblasts expressing E1A with irradiation or chemotherapeutic agents induces apoptosis, whereas there are no effects on the viability of p53-deficient E1A-expressing cells (Lowe et al, 1993b). Therefore, apoptosis caused by DNA damaging agents has a clearly established dependency on p53.

4.2 Role in development and teratogenesis

A study by Mora et al. in 1980 found that p53 is expressed in primary cell

cultures obtained from 12- to 14-day-old mouse embryos, but not in those from 16-day old embryos; this suggested that the expression of p53 correlates with specific stages in embryonic development (Mora et al., 1980). Later, it was reported that the level of p53 decreases significantly during embryogenesis (Chandrasekaran et al., 1981) and also that the level of p53 mRNA in embryo tissues from day 11 of gestation is reduced (Rogel et al., 1985). A more detailed examination of the pattern of p53 expression was reported in 1991, when Schmid et al. showed by in situ hybridization that the levels of p53 mRNA decline during embryonic development of the mouse (Schmid et al. 1991). In contrast to the high expression of p53 in all tissues in the early embryo, during organogenesis the pattern of expression becomes tissue-type dependent with p53 mRNA levels declining in tissues that undergo terminal differentiation. This differential regulation of p53 during embryogenesis supports the idea that p53 may have an important role in the regulation of cellular proliferation and differentiation, and raises the possibility that p53 might be involved in controlling specific steps during embryogenesis.

Transgenic p53 "knockout" mice survive to birth, however they develop a high frequency of lymphomas and sarcomas at an early age (Donehower et al., 1992). Initially it was reported that p53-null mice seem to develop normally, however more detailed studies pointed to the conclusion that p53-null mice suffer, at certain frequencies, from developmental abnormalities. Sah et al. (1995) reported that a fraction of the p53-deficient embryos displayed defects in neural tube closure and, subsequently, exencephaly. p53 may be involved in arresting the cell cycle or inducing cell differentiation, both of which are involved in the closure process.

Studies by Armstrong et al. (1995) revealed that these defects were found predominantly in the female embryos.

The role of p53 in mediating drug teratogenicity is controversial. With benzo[a]pyrene as the teratogen, p53 had a "teratological suppressor" function, perhaps by protecting the embryo from DNA-damaging chemicals and developmental oxidative stress (Nicol et al., 1995). In contrast, the presence of p53 was necessary for 2-chloro-2'deoxyadenosine exposure to induce eye defects (Wubah et al., 1996); p53-dependent apoptosis played a critical role in these eye defects as wild-type (+/+) embryos are more susceptible than the homozygote (-/-) knockout embryos lacking p53.

5. Rationale and formulation of project

There have been some studies in which cell death has been examined during normal development and during drug-induced teratogenesis (reviewed in Hinchliffe, 1980; Manson et al., 1982; Gao et al., 1994; Torchinsky et al., 1995; reviewed in Sanders and Wride, 1995). However, there has been no study to date on the relationship between cell death and the p53 gene in normal embryogenesis and after cyclophosphamide (or any of its analogues)-induced malformations.

The purpose of this dissertation was to test the hypothesis that **(1) cyclophosphamide induces apoptosis at the morphological, biochemical and molecular levels which is associated with limb malformations and (2) p53 plays an important role in protecting against limb malformations and**

mediating this apoptotic cell death. For this aim, several specific objectives have been formulated. They were:

1. To characterise the 4OOH-CPA effect on mouse limb bud development and the time course and concentration factors in this process.
2. To examine apoptosis occurrence during normal development and after 4OOH-CPA treatment and its regulation at the level of cathepsin D, transglutaminase and clusterin expression.
3. To evaluate whether p53 is involved in mediating 4OOH-CPA-induced limb malformations and apoptosis.

To test the hypothesis, a mouse limb bud culture system was used (Kochhar, 1983; Kwasigroch et al., 1984). Limb development has been used extensively to study growth, differentiation, patterning and cell death during development (Kochhar, 1983 ;Zakeri and Ahuja, 1994; Hurle et al, 1996). In this system, limb buds are dissected on day 12 of gestation and cultured in a chemically-defined medium in a roller bottle system for up to 144 hours. During this critical period, limbs grow tremendously and any effect on gross morphology can be detected. *In vitro* limb bud development closely resembles that *in vivo* during the same period time (Neubert and Barrach, 1977; Kwasigroch et al., 1986). However, there are important advantages of the *in vitro* culture system over an *in vivo* system. For instance, they include excluding the interfering maternal factors, close access to the organ under investigation, precise control on the time of culture and drug-exposure, facilitated observation of the limb buds, precise control on the composition of the

culture medium and reliable control on the concentration of the drug during the treatment period.

The following experimental approaches were used to achieve the above objectives:

1. Treatment of developing limbs with 4OOH-CPA at two concentrations and culturing for 144 hours. Limbs were stained and morphological criteria were assessed.
2. Fluorescence and electron microscopic examination of limbs after drug treatment to evaluate the localization of apoptosis.
3. Whole-mount in situ DNA labelling of cultured limbs and light microscopic and confocal laser microscopic evaluation of the occurrence of apoptosis.
4. DNA fragmentation analysis after drug administration to evaluate the rate and time course of the occurrence of apoptosis.
5. Cathepsin D, transglutaminase and clusterin expression in control and 4OOH-CPA-treated limbs: Western blot analysis, immunohistochemical localization, and immunogold labelling followed by electron microscopic examination to study the induction, the cellular localization, and the subcellular localization of these proteins, respectively.
6. Limbs from p53-transgenic knockout, heterozygous, and wild type mice were cultured in the absence or presence of 4OOH-CPA; the morphology of stained limbs were assessed and quantified by image analysis.
7. Limbs from different p53 genotypes were examined for the occurrence of apoptosis in control or 4OOH-CPA-treated medium by electrophoresis and

autoradiography of radioactive DNA

8. Light and microscopic evaluation of limbs to study the role of p53 gene in the occurrence of cell death.
9. Western blot analysis of the poly(ADP-ribose) polymerase protein to investigate the fragmentation pattern which is indicative of the occurrence of apoptosis or necrosis.
10. Cell cycle analysis of limb cells to investigate the effect of 4OOH-CPA-treatment on the cell cycle pattern.

Overall, these studies provided valuable information on the effect of a 4OOH-CPA on limb morphogenesis, the occurrence and regulation of apoptosis during this process, the crucial role of p53 in protecting against malformations, the dependence of 4OOH-CPA-induced cell cycle arrest and apoptosis on the presence of p53, and the switch of cell death from apoptosis to necrosis in the absence of p53. Although these studies were performed in a limb culture system utilizing 4OOH-CPA, they might be extended as generally to show the role of DNA damage and p53 during development and apoptosis.

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CHAPTER TWO

The Induction of Apoptosis and Cathepsin D in Limbs Exposed in vitro to an Activated Analog of Cyclophosphamide

S. Adel Moallem and Barbara F. Hales

Status: published in *Teratology* 52:3-14 (1995)

ABSTRACT

Apoptosis, a form of active cell death, plays a role during normal limb development. The present study was done to test the hypothesis that the teratogen cyclophosphamide, an alkylating agent and commonly used anticancer drug, produces malformations by disturbing the regulation of apoptosis in the limb. The effects of a pre-activated analog of cyclophosphamide, 4-hydroperoxycyclophosphamide, on limb development and on apoptosis in the limb were determined in vitro. Cathepsin D is a lysosomal protease which is induced in tissues undergoing destruction by apoptosis. To further examine the process of apoptosis in the limb, the effects of 4-hydroperoxycyclophosphamide exposure on cathepsin D protein concentrations and the immunolocalization of cathepsin D in limb buds were assessed. Limb buds from gestational day 12 mice were excised and cultured in roller bottles in a chemically defined medium for up to 6 days. The addition of 4-hydroperoxycyclophosphamide (1 or 10 µg/ml) to the culture medium produced time and concentration dependent malformations in the limbs. Electrophoresis of the DNA extracted from both control and treated limbs revealed a DNA fragmentation pattern characteristic of apoptosis. Limbs cultured in the control medium showed a "DNA ladder" only after 72 hours in vitro; however, those in the treated groups showed fragmentation within 12 hours of drug exposure. Examination of cell ultrastructure in the interdigital areas with the electron microscope further confirmed the apoptotic type of cell death, and its acceleration in drug exposed limbs. The relative abundance of cathepsin D in limbs exposed to 4-hydroperoxycyclophosphamide for 24 hours was increased compared to control

limbs. Using immunohistochemical staining at the light microscope level, the cathepsin D protein in control limbs was localized mainly to the interdigital and apical ectodermal ridge areas; staining in these areas was increased in limbs exposed to 4-hydroperoxycyclophosphamide. Using immunogold electron microscopy, cathepsin D immunoreactivity was found to be localized in phagocytosed apoptotic bodies; this observation suggests that it is the process of phagocytosis which induces cathepsin D. Thus, exposure to 4-hydroperoxycyclophosphamide increased apoptosis in the interdigital areas and apical ectodermal ridge of mouse limb buds in vitro. Moreover, cathepsin D may play an important role in mediating the phagocytosis of apoptotic bodies in the teratogen-exposed limbs.

INTRODUCTION

Apoptosis is a form of active cell death which is distinct from necrosis or passive cell death (Kerr et al., '72; Schwartzman and Cidlowski, '93). Apoptosis is characterized morphologically by chromatin condensation, cytoplasmic shrinkage and membrane blebbing. During apoptosis, an endogenous endonuclease is activated which causes internucleosomal DNA fragmentation to 180-200 bp multiple fragments (English et al., '89; Wyllie et al., '80). Apoptotic cell death can be initiated by a number of external signals including cytotoxic anti-cancer drugs (Hickman, '92), glucocorticoids, irradiation, heat shock and the withdrawal of some growth factors or hormones (Sen, '92). When the regulation of apoptosis is disturbed, either by inhibition or inappropriate induction, disease can result (Bursch et al., '92).

Apoptosis, or "physiological" cell death, is the most common form of eukaryotic cell death during embryogenesis, tissue turnover and metamorphosis (Glucksmann, '51; Saunders, '66). In the developing embryo, the spatio-temporal occurrence of apoptosis is precisely controlled; apoptosis occurs in the neural tube, heart, palate, duodenal mucosa, and limb bud (Gerschenson and Rotello, '92; Wyllie, '93). Exposure of an embryo during organogenesis to a teratogen, such as retinoic acid or 5-azacytidine, increases cell death in regions where programmed cell death normally occurs (Alles and Sulik, '89; Jiang and Kochhar, '92; Kochhar, '77; Kochhar et al., '93; Kurishita, '89). Limbs are one of the organs in which the pathogenesis of malformations has a primary association with the phenomenon of apoptosis (Alles and Sulik, '89; Kurishita, '89; Ritter et al., '73). Interference with apoptosis may be an important mechanism of action of teratogens that cause limb

malformations.

Cyclophosphamide, a commonly used anticancer drug, has frequently been studied as a model teratogen (Mirkes, '85). Exposure to cyclophosphamide during organogenesis results in a spectrum of malformations that includes exencephaly or hydrocephaly, open eyes, cleft palate, phocomelia, adactyly, oligodactyly, syndactyly, polydactyly, and kinky tails, as well as disturbances in skeletal ossification (Gibson and Becker, '68; Hales, '81). Cyclophosphamide is activated by a cytochrome P-450 monooxygenase to 4-hydroxycyclophosphamide. This compound, as well as its pre-activated analog, 4-hydroperoxycyclophosphamide, tautomerizes with aldophosphamide which spontaneously breaks down to form the active metabolites, phosphoramide mustard and acrolein (Foley et al., '61; Hales, '82; Takamizawa et al., '75; Voelker et al., '74). The addition of 4-hydroperoxycyclophosphamide causes dramatic limb malformations and decreases the DNA, RNA, and protein contents of mouse limb buds in culture (Hales and Jain, '86).

Apoptosis has been associated with active protein synthesis in different systems; some of the proteins which are induced during apoptosis include tissue transglutaminase, clusterin and cathepsin D (Buttyan et al., '89; Fesus et al., '87; Lee et al., '90). During development, apoptosis has been associated with the induction of both transglutaminase and clusterin (Chen et al., '94; Jiang and Kochhar, '92), but little is known about the role of cathepsin D. Cathepsin D is an aspartyl endopeptidase that accounts for a major part of the proteolytic activity in lysosomes and endosomes (Barrett, '77; Bohley and Seglen, '92; Bowser and

Murphy, '90; Diment et al., '88). This protease has been implicated in the destruction of tissues such as the prostate in which apoptosis is induced by androgen withdrawal (Lee et al., '90; Sensibar et al., '90). Cathepsin D expression is induced under both physiological (development, tissue remodelling) and pathological (tumor invasion and metastasis) conditions (Rochefort et al., '90). Although the regulation of cathepsin D expression during the process of apoptosis is not well understood, cathepsin D transcription can be initiated at multiple sites by a mixed promoter with the features of both a housekeeping and a hormonally regulated gene (Cavaillès et al., '93).

The purpose of the present study is to investigate the effect of 4-hydroperoxycyclophosphamide on the regulation of apoptosis and of cathepsin D expression in the day 12 mouse limb bud in culture. Apoptosis was assessed using biochemical and morphological criteria. The relative amounts of cathepsin D protein were determined and its immunolocalization was studied both at the light and electron microscope levels.

MATERIALS AND METHODS

Limb bud culture and drug treatments

Timed pregnant CD1 mice (20-25g), mated between 8 and 10 AM (on day 0 of gestation), were purchased from Charles River Canada Inc. (St. Constant, QC) and housed at the McIntyre Animal Centre (McGill University, Montreal, QC). On day 12 of gestation, females were sacrificed by cervical dislocation, uteri were removed, and the embryos were dissected in sterile Tyrode's saline solution; the

embryos at this time were 5-6 mm in length with about 40 somite pairs. Forelimbs were excised just lateral to the somites, pooled and cultured in a chemically-defined medium as previously described (Kwasigroch et al., '86). 4-Hydroperoxycyclophosphamide (1 µg/ml or 10 µg/ml, a gift from Dr. N. Brock, Asta-Werke, Germany) or vehicle control (water) was added to the designated cultures after one hour. The cultures were terminated 2, 6, 12, 24, 36, 48, 72, or 144 hours later. Three separate replicates were done for each experiment.

Limb morphology

At the designated time points, limbs (six to seven per bottle) were fixed and stained as previously described (Hales and Jain, '86). The morphology of the limbs was examined with a dissecting microscope.

Ultrastructural morphology

Limbs were fixed with 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours, then washed with buffer and postfixed with 1% osmium tetroxide in cacodylate buffer for 1 hour at 4°C. The samples were dehydrated with ethanol and then embedded in Epon at 55°C for 24 hours. Ultrathin sections were cut and examined using a Philips EM410 electron microscope.

DNA fragmentation analysis

At the specified times, three to four limbs per group were placed in liquid N_2 and stored in -80°C. Limbs were homogenized and digested in 100 µl of digestion

buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 25 mM EDTA, pH 8, 0.5% sodium dodecyl sulphate, and freshly added proteinase K, 0.1 mg/ml) with shaking at 50°C for 15 hours. RNA was degraded by incubation of the samples with 1 µg/ml DNase-free RNase (Boehringer Mannheim Ltd, Laval, QC) for 1 hour at 37°C. DNA was extracted, as described by Strauss ('90), with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged for 10 minutes at 1700 x g. The DNA was precipitated by adding 1/2 volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol to the aqueous layer; samples were left overnight at -20°C and then separated by centrifugation at 1700 x g for 2 minutes, rinsed with 70% ethanol and air-dried. The pellet was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

10 µg DNA, as measured by UV absorbance at 260 nm, were added with loading buffer (0.2% SDS, 20 mM EDTA, 50% glycerol, 0.02% bromophenol blue), heated at 65°C for 10 minutes and electrophoresed in 1.6% agarose at 25 V in 10 mM Tris, 1 mM EDTA. The 123 bp and 1kb DNA ladders (Gibco BRL, Burlington, ON) were included in the electrophoresed gel for size calibration. After electrophoresis, the gel was soaked in 0.5% ethidium bromide and the DNA was visualized under UV light.

Vital fluorochrome staining

Control and treated limbs cultured for 24 hours were stained with acridine orange (Gao et al.,'94). After terminating the culture, limbs were incubated in acridine orange (ICN Biochemicals, Cleveland, OH, USA) 0.005 mg/ml in HBSS for

5 minutes at 37 °C and they were rinsed in HBSS for 5 minutes at room temperature. Stained limbs were examined immediately with a Leitz Laborlux D fluorescence microscope. I3 cube (EX 450-490 and LP 520) was used to examine the limbs.

Western blot analysis

At the designated times, three to four limbs per group were placed in liquid N_2 and stored at -80°C. Limbs were homogenized in the presence of liquid N_2, dissolved in sample loading buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% ß-mercaptoethanol) and boiled for 5 minutes. Samples (50 µg protein per lane) were fractionated by SDS-PAGE using 10% acrylamide gels (Studier, '73). Biotinylated SDS-PAGE broad range standards (Bio-Rad Laboratories, Mississauga, ON) were used as molecular weight markers.

The fractionated proteins were transferred to a nitrocellulose sheet (Towbin et al., '79). The blots were blocked with 5% nonfat dried milk in TBS-T (137 mM NaCl; 20 mM Tris, pH 7.4; 0.1% Tween 20) at room temperature for one hour and then incubated with cathepsin D antibody (1:500 in 5% milk/TBS) at room temperature for 18 hours. The rabbit cathepsin D antiserum was kindly provided by Dr. J. A. Sensibar (Sensibar et al., '90). Rabbit serum was used as a control for non-specific binding. The blots were washed three times in TBS-T and incubated with biotinylated anti-rabbit antibody (1:500 in 5% milk/TBS) for 30 minutes. The antibody binding was visualized with streptavidin alkaline phosphatase conjugate (1:3000 in TBS) using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue

tetrazolium as substrates (Amersham Canada Ltd., Oakville, ON). Blots were photographed and bands were scanned using laser densitometry (Pharmacia, Montreal, QC). The relative absorbance of the relevant bands was calculated and normalized to the total absorbance. Three separate replicates were done for this experiment; values represent mean ± S.E.M. Data were analyzed by ANOVA and Bonferroni T-test.

Cathepsin D immunohistochemistry - Light microscopy

At the end of the culture period, limbs were fixed with Bouin's fixative at 4°C for 3 hours and dehydrated with ascending ethanol concentrations (50%, 70%, 95% and 100%) for 20 minutes, with two washes each. The limbs were cleared with xylene for 10 minutes twice and embedded in paraffin. Sections of 5 µm were cut, mounted on poly-l-lysine-coated slides, and rehydrated with xylene and descending concentrations of ethanol and water.

The sections were washed with phosphate buffered saline (PBS), treated with 0.3% H_2O_2 in PBS for 30 minutes, washed with PBS, blocked with 1.5% normal goat serum in PBS for 30 minutes and then incubated with cathepsin D antibody (1:500) overnight. After washing with PBS, sections were stained using the avidin-biotin-peroxidase complex (ABC) method with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Each section was then reacted with DAB solution containing 0.05% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, USA) and 0.01% hydrogen peroxide in 0.05 M Tris buffer, pH 7.2. Sections were counterstained briefly with haematoxylin solution. Normal rabbit serum replaced the cathepsin D antibody in control sections and

these sections were counterstained with methylene blue.

Cathepsin D immunohistochemistry - Electron microscopy

At the end of the culture period, limbs were fixed with 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours. After washing with buffer and dehydration with ethanol, tissue blocks were embedded in LR White acrylic resin (MECA Ltd, Montreal, QC). Tissue blocks were polymerized in gelatin capsules for 24 hours in a vacuum oven at 48°C. Ultrathin sections were cut and mounted on 200 mesh nickel grids. The grids were blocked with 5% normal goat serum for 15 minutes and incubated overnight with cathepsin D antibody (1:200). After washing with buffer, the grids were incubated with colloidal gold-labelled goat anti-rabbit IgG (gold particles: 10 nm in diameter; 1:20 dilution; British BioCell Int., Cardiff, UK). As a control, the cathepsin D antibody was replaced by normal rabbit serum. The grids were counterstained with uranyl acetate and lead citrate and examined in a Philips EM410 electron microscope.

RESULTS

Limb morphology after exposure to 4-hydroperoxycyclophosphamide

Control and 4-hydroperoxycyclophosphamide treated limbs (t_1, 1 µg/ml or t_2, 10 µg/ml) cultured for 6, 24, 72, or 144 hours are shown in Figure 1. These limbs are representative of those stained in each of the three experiments. The control limbs showed development of the anlagen of the long bones and digits during the six day culture period. A scapula, humerus, radius, ulna, carpals, metacarpals, and

phalanges were all present in the control limbs after 144 hours of culture. After exposure to the low (t_1) or high (t_2) concentration of 4-hydroperoxycyclophosphamide, the limbs did not appear to be growth retarded (on the basis of visual observation) after 6 (Fig.1) or 12 hours in culture (data not shown). After 24 hours of culture with the lower concentration of 4-hydroperoxycyclophosphamide, the limbs had begun to show some growth retardation, while after exposure for 36, 48 (data not shown), or for 72 or 144 hours, both the long bone and paw structure anlagen were reduced markedly. Exposure to the higher concentration of 4-hydroperoxycyclophosphamide resulted in a dramatic reduction in limb size from 24 hours onward; most bone anlagen were severely reduced in size and could not be visualized at all subsequent times. These observations indicate a significant limb dysmorphogenesis during the first day of culture.

Internucleosomal DNA cleavage in limbs exposed to 4-hydroperoxycyclophosphamide

Internucleosomal DNA cleavage was assessed in the control and 4-hydroperoxycyclophosphamide treated limbs after 2, 6, 12, 24, 36, 48, 72, or 144 hours of culture (Fig. 2). Using 1.6% agarose gels, apoptotic DNA fragmentation was not evident in the controls until after 72 hours of culture and in the limbs exposed to either concentration of 4-hydroperoxycyclophosphamide for the first 2 or 6 hours of culture. This internucleosomal DNA cleavage was first evident in drug-exposed limbs (1 or 10 µg/ml 4-hydroperoxycyclophosphamide) after culture for 12

hours. The DNA "laddering" persisted until 144 hours after exposure to the lower concentration of 4-hydroperoxycyclophosphamide, and 72 hours after exposure to the higher concentration. The DNA extracted from limbs after 144 hours of culture in the presence of 10 µg/ml of 4-hydroperoxycyclophosphamide was not detectable; it may have been degraded into small fragments and eluted off the end of the gel.

Ultrastructural morphology and fluorometric analysis of the dying cells in the limbs

In Fig. 3 limbs cultured for 24 hours in control (A) or in the presence of 1 µg/ml (B) or 10 µg/ml (C) 4-hydroperoxycyclophosphamide and stained with acridine orange are shown. Acridine orange, a fluorochrome which is reported to be specific for apoptotic forms of cell death, does not significantly label necrotic cells (Abrams et al.,'93). It is clear that the treatment enhanced the labeling of acridine orange in the interdigital area and few cells in the apical ectodermal ridge. Also, cells in the interdigital and apical ectodermal ridge areas of control and 4-hydroperoxycyclophosphamide treated limbs cultured for 24 hours were examined with the electron microscope (Fig.4). Only a few cells in these areas in control limbs appeared phagocytotic and in the process of digesting apoptotic bodies (Fig. 4A). However, limbs cultured in the presence of 1 µg/ml 4-hydroperoxycyclophosphamide for 24 hours showed more dying cells with an apoptotic morphology exhibiting chromatin condensation, cytoplasmic shrinkage and the formation of apoptotic bodies (Fig. 4B). These observations would suggest an acceleration in the cell death program in these cells and/or a decrease in the

phagocytic ability of the neighbouring cells. Exposure to the higher concentration of the drug and to longer culture times resulted in even more cells with a morphology characteristic of cells undergoing apoptosis and phagocytosis as could be detected by the electron microscope (data not shown).

Cathepsin D concentrations in limbs exposed to 4-hydroperoxycyclophosphamide

The relative abundance of cathepsin D was determined by immunoblot analysis of control and 4-hydroperoxycyclophosphamide-treated limbs after 6, 24 and 48 hours in culture (Fig. 5). A 44 $x10^3$ M_r band of immunoreactive cathepsin D was found in all cultured limbs (Fig. 5A). This value is consistent with that reported by Sensibar et al., ('90), for purified rat cathepsin D. In control limbs, a steady increase in the cathepsin D concentration was observed with time in culture. Exposure to either concentration of 4-hydroperoxycyclophosphamide for 6 hours did not significantly alter the relative amounts of cathepsin D in the limb. In contrast, 24 hours of drug exposure increased cathepsin D concentrations in the treated limbs two to three fold above those in the controls. By 48 hours, the relative amounts of cathepsin D in the drug-treated limbs were not different than those in the controls.

Immunohistochemical localization of cathepsin D in the limb

No positive immunohistochemical reaction product was observed with control rabbit serum in control or drug-treated limbs cultured for 24 hours (Fig. 6). Control

and 4-hydroperoxycyclophosphamide-treated limbs cultured for 12 hours showed only very light cathepsin D immunostaining (data not shown). After culture of control limbs for 24 hours, a few cells in the interdigital areas and apical ectodermal ridge stained positively for cathepsin D (Fig. 7A and B). However, in limbs exposed to 1 µg/ml 4-hydroperoxycyclophosphamide (Fig. 7C and D) or 10 µg/ml 4-hydroperoxycyclophosphamide (Fig. 7E and F) for 24 hours, a strong immunostaining reaction was detected. The increase in cathepsin D immunostaining was localized primarily to the interdigital areas after exposure to the lower drug concentration, while staining was increased in both the interdigital and apical ectodermal ridge areas after treatment with the higher concentration (Fig. 7). After 48 hours of culture, the same pattern of immunostaining was found in control limbs; staining remained in the interdigital areas of drug-treated limbs while some increase was observed in the apical ectodermal ridge areas (data not shown).

Localization of cathepsin D in apoptotic bodies undergoing phagocytosis

To determine the localization of cathepsin D in limb mesenchymal cells undergoing apoptosis, immunogold electron microscopy was done on sections from limbs exposed in culture to 1 or 10 µg/ml of 4-hydroperoxycyclophosphamide for 6 or 24 hours. Low cathepsin D immunolabelling was observed in unphagocytosed apoptotic bodies found in the interdigital areas of limbs cultured for 6 hours in the presence of 1 µg/ml 4-hydroperoxycyclophosphamide (Fig. 8A and B); in contrast, phagocytosed apoptotic bodies in drug-treated limbs at this time showed an increase in cathepsin D immunolabelling (Fig. 8A and C). Cathepsin D

immunolabelling was even more intense in the engulfed apoptotic bodies found in the interdigital areas of limbs cultured for 24 hr with 10 μg/ml of 4-hydroperoxycyclophosphamide (Fig. 8D). Thus, 4-hydroperoxycyclophosphamide exposure increased the relative amount of cathepsin D immunogold labelling in those apoptotic bodies undergoing phagocytosis.

DISCUSSION

Exposure of mouse limbs in culture to 4-hydroperoxycyclophosphamide induced limb growth retardation in a concentration and time-dependent manner. In addition, exposure of limbs to 4-hydroperoxycyclophosphamide for only 12 hours, resulted in the production of an apoptotic DNA fragmentation pattern and cell ultrastructural morphology characteristic of apoptosis. These observations suggest that apoptosis is involved in mediating 4-hydroperoxycyclophosphamide-induced cell death in the limb. This drug-induced increase in apoptotic cell death in the limbs was localized to the apical ectodermal ridge and interdigital areas, both regions of the limb in which cell death occurs during normal development (Hurle, '88; Saunders, '66). Since the apical ectodermal ridge is known to promote the outgrowth of the limb by maintaining high mitotic rates in the underlying mesenchyme (Saunders, '77), a cyclophosphamide-induced reduction in the number of healthy cells along the anterioposterior extent of the ridge would be expected to limit outward mesenchymal extension. As well, the interdigital cells die in a controlled fashion to separate the digits during normal development. Accordingly, drug-induced excessive cell death in the interdigital areas could result

in severe digit defects.

We did not detect an apoptotic DNA ladder in the control limbs until after 72 hours of culture. Thus, drug treatment resulted in a dramatic increase in apoptosis at a much earlier stage in limb development. In previous studies in which internucleosomal DNA cleavage in murine limbs was examined by DNA laddering on gel electrophoresis, no cleavage was detected in the limb, either during normal development or after treatment with a potent teratogen such as retinoic acid (Lockshin et al., '91; Zakeri et al., '90). In a more recent study, single stranded DNA was detected in the interdigital zone of the control mouse limb plate on day 14 of gestation (Naruse et al., 1994). Moreover, in embryonic chick interdigital tissue, Garcia-Martinez and co-workers ('93) reported endonucleolytic cleavage; these investigators confirmed the apoptotic nature of this cell death by electron microscopy. It is likely that the differences between these findings are due to variations induced by the limb bud culture system and/or to the sensitivity of the methods used to detect internucleosomal DNA cleavage.

The observation that by using 1.6% gel electrophoresis, apoptotic DNA fragmentation was not found after 144 hours of culture in the limbs exposed to 10 µg/ml 4-hydroperoxycyclophosphamide would suggest that other mechanism(s) of cell death, such as necrosis, may be involved by this time. The type of cell death induced by hyperthermia or drug exposure in other systems has been demonstrated to be both dose and time-dependent (Harmon et al., '90; Lennon et al., '91). Apoptosis may be only one endpoint in the cascade of biochemical and/or molecular alterations induced by a teratogen such as cyclophosphamide.

With the identification of the mammalian equivalent of the *C. elegans* cell death gene, *ced-3*, as interleukin-1β converting enzyme (Yuan et al., '93), there is increasing interest in the role of proteases in mediating apoptosis. During normal development, when apoptosis occurs in a few cells and in a controlled fashion, the expression of the lysosomal protease, cathepsin D, may parallel apoptosis. Only weak cathepsin D immunoreactivity was observed in control limbs. The increase in the relative abundance of cathepsin D in drug-treated limbs was localized by immunohistochemical staining to the interdigital areas and the apical ectodermal ridge areas. After drug treatment, when the rate of apoptotic cell death was increased dramatically, enhanced cathepsin D expression may be important to facilitate digestion of the debris of apoptotic cells. Interestingly, exposure to either concentration of 4-hydroperoxycyclophosphamide increased the cathepsin D immunoreactivity to the same extent. There may be a limit to the extent to which the expression of cathepsin D can respond to this exogenous stimulus.

Weaver and co-workers ('93) reported that serine proteases are involved in the induction of apoptosis at a very early stage; inhibition of these proteases in dexamethasone or tenoposide VM-26-treated thymocytes suppressed the formation of a DNA ladder as well as apoptotic cell shrinkage and nuclear condensation. However, cathepsin D appears to play its role during later phases of apoptosis, mainly in the digestion of apoptotic bodies during the process of phagocytosis. Interestingly, the increase in cathepsin D activity in the prostate following castration was also a relatively late event, peaking at 5 days post-castration (Sensibar et al., '90). Thus, cathepsin D does not appear to initiate apoptosis, but rather appears

to be involved later, mainly when apoptotic bodies are engulfed and digested during the process of phagocytosis. This conclusion is supported by the observation that the cathepsin D immunogold labelling in drug-treated limbs was concentrated in apoptotic bodies undergoing phagocytosis.

The relatively late expression of genes such as cathepsin D may be under complex regulation during apoptosis. The cathepsin D gene is controlled by a promoter with a mixed structure; consequently, it can be regulated like other lysosomal enzymes as a housekeeping gene (high G+C content and several Sp1-binding sites) or as a steroid or hormone regulated gene with a TATAA sequence (Cavaillès et al., '93). Overexpression of cathepsin D under some physiological (normal development) or pathological conditions (breast tumor, teratogen exposure) may result in the production of an alternate transcript, depending on the site at which transcription is initiated (Cavaillès et al., '93). Interestingly, cathepsin D gene regulation is tissue-specific (Touitou et al., '89).

In summary, exposure to a teratogen such as 4-hydroperoxycyclophosphamide appears to result in the disruption of the normal process of apoptosis in the limb, specifically in the apical ectodermal ridge and the interdigital areas. In addition, it is clear that one of the consequences of exposure of the limb to 4-hydroperoxycyclophosphamide is an altered regulation of cathepsin D. The control of the expression of this protease during development deserves further study.

ACKNOWLEDGMENTS

The authors would like to thank Dr. J. A. Sensibar for providing the cathepsin D antibody and Ms. Marie Ballak for her assistance in the electron microscope studies. This study was supported by the Medical Research Council of Canada. S. A. Moallem is the recipient of a studentship award from the Faculty of Medicine of McGill University.

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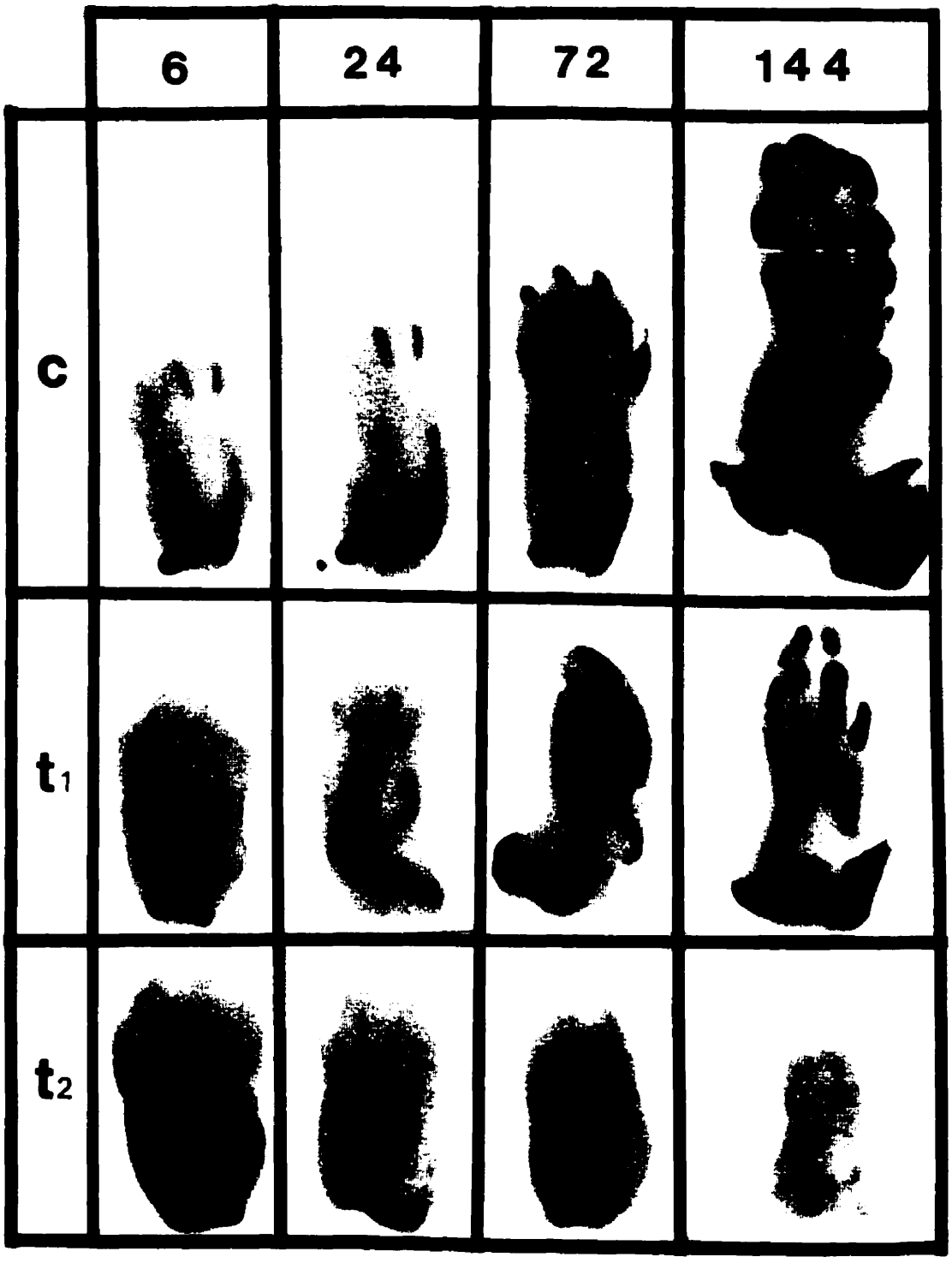

6
24
72
144
c
t1
t2

Figure 1: Toluidine blue-stained mouse limb buds. Forelimbs were excised from embryos on day 12 of gestation and grown in roller bottle cultures for 6, 24, 72, or 144 hours in control medium (c) or in medium containing 4-hydroperoxycyclophosphamide (t_1: 1 µg/ml or t_2: 10 µg/ml). X 9.5

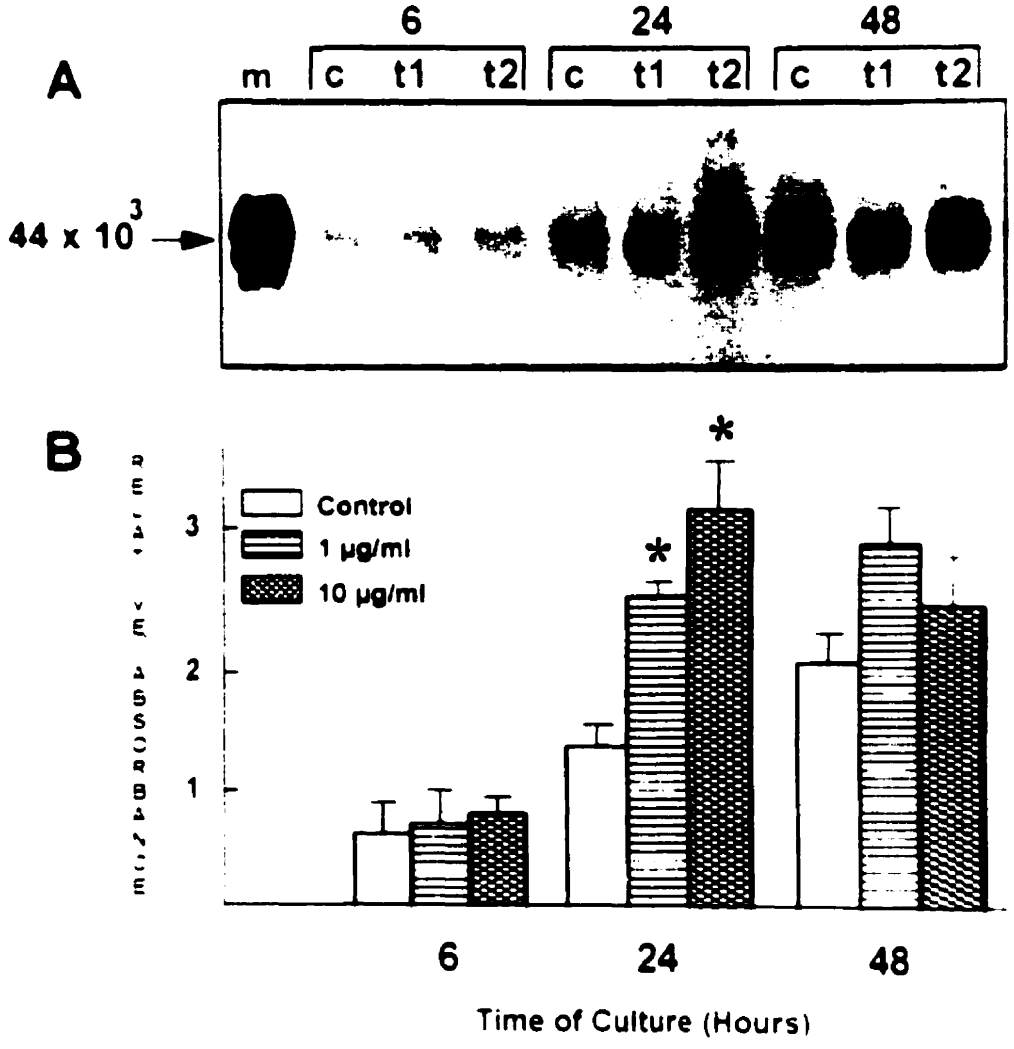
Figure 2: DNA gel electrophoresis of control (c) or 4-hydroperoxycyclophosphamide-exposed (t_1: 1 µg/ml or t_2: 10 µg/ml) limbs cultured for varying periods of time from 2 to 144 hours. The DNA was extracted from the limbs, electrophoresed in 1.6% agarose and stained with ethidium bromide. Lanes m_1 (123 bp markers) and m_2 (1 kb markers) are the DNA standards. Bp sizes (615 and 246) are indicated with arrows on the left.

A
B
C

Figure 3: Vital fluorochrome staining of limbs cultured for 24 hours in control medium (A) or with 1 µg/ml (B) or 10 µg/ml (C) 4-hydroperoxycyclophosphamide. Apoptotic labelled cells in the interdigital area of B and C are indicated by arrow. Bar = 25 µm.


A
B

Figure 4: Cell ultrastructural morphology in the interdigital areas of cultured forelimbs. Forelimbs were cultured for 24 hours in the control medium (A) or with 1 µg/ml 4-hydroperoxycyclophosphamide (B). Cells undergoing apoptosis and apoptotic bodies are indicated by arrowheads. Bar = 5 µm.


A
6
24
48
m
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c
t1
t2
44 x 10^3
B
Control
1 µg/ml
10 µg/ml
*
*
3
2
1
6
24
48
Time of Culture (Hours)

Figure 5: (A) Western blot analysis of cathepsin D. Protein samples from limbs cultured for 6, 24 or 48 hours in control medium (c) or in the presence of 4-hydroperoxycyclophosphamide (t_1: 1 µg/ml or t_2: 10 µg/ml) were electrophoresed, blotted and immunostained with the cathepsin D antibody. Lane m depicts the molecular weight marker. (B) Plot of the densitometric analysis of the cathepsin D protein band. Data are expressed as means ± s. e. m. (n = 3).

A
B

Figure 6: Control immunohistochemical staining in the forelimb. The limb (A) shown was cultured for 24 hours in the presence of 1 µg/ml 4-hydroperoxycyclophosphamide. Normal rabbit serum was used as the primary antibody. Panel (B) represents a higher magnification of the box in panel A. Bar= 25 µM.

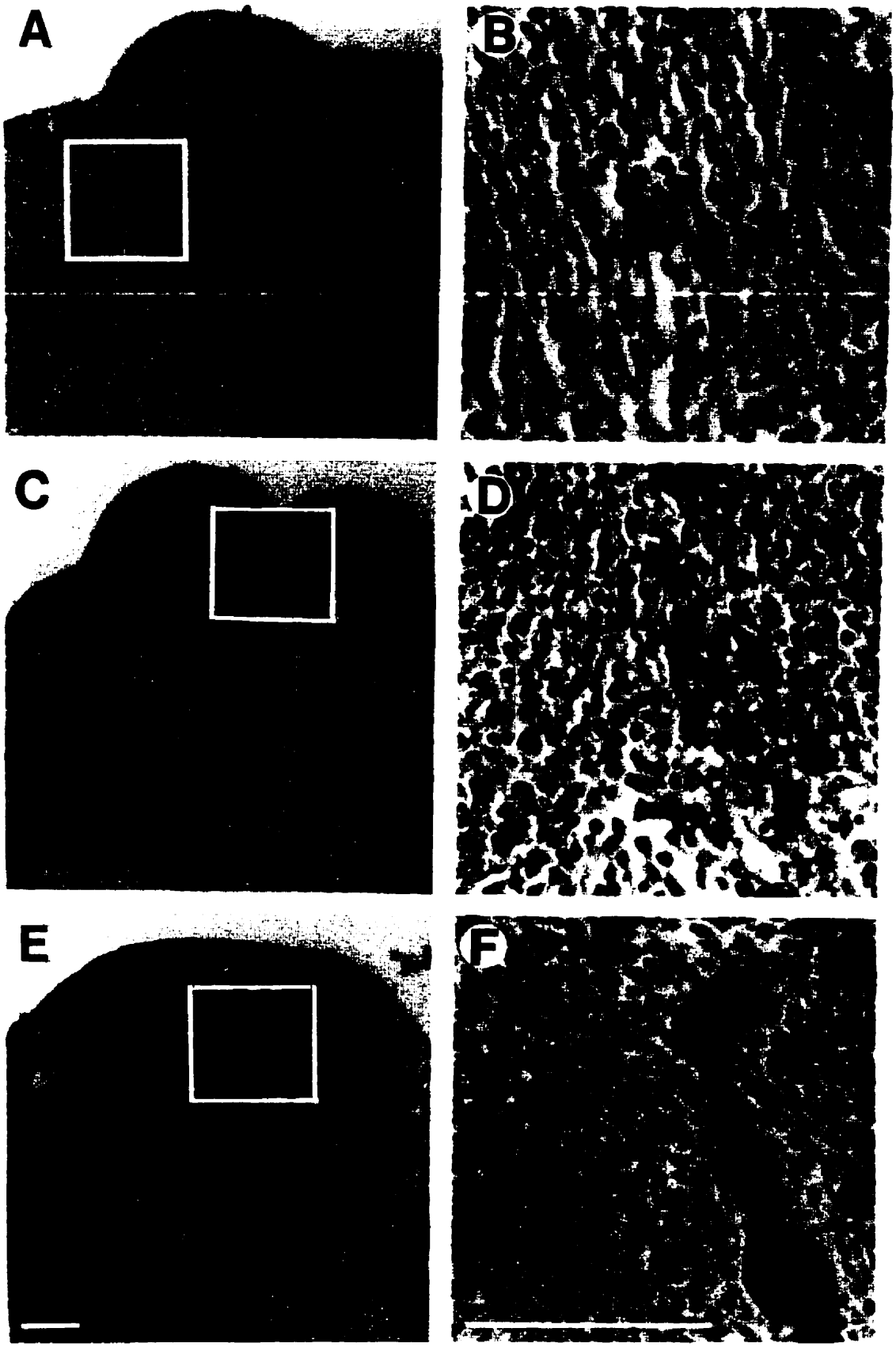
A
B
C
D
E
F

Figure 7: Immunohistochemical localization of cathepsin D in the forelimb. Limbs were cultured for 24 hours in control medium (A, B), or in the presence of 4-hydroperoxycyclophosphamide: 1 µg/ml (C, D) or 10 µg/ml 4-hydroperoxycyclophosphamide (E, F). The right panels represent a higher magnification of the corresponding boxes in the left panels. Bar= 25 µM.

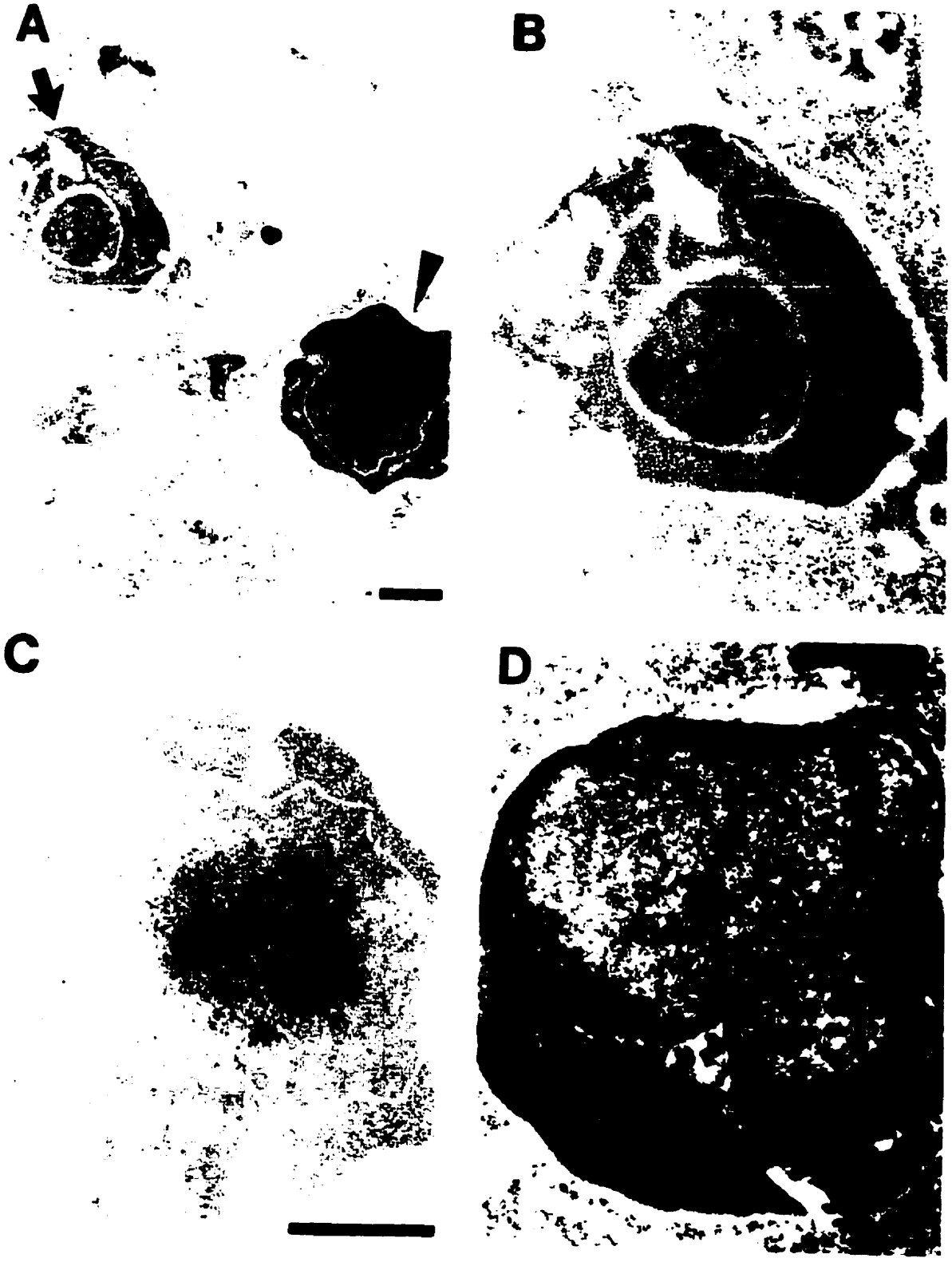
A
B
C
D

Figure 8: Ultrastructural localization of cathepsin D in cells of the interdigital areas of limbs exposed to 4-hydroperoxycyclophosphamide for 6 hours (1 µg/ml: A, B and C) or 24 hours (10 µg/ml: D). B and C represent a higher magnification of areas of the cell in A (pointed out by an arrow and arrowhead, respectively). Some of the immunogold particles are indicated by open arrows. Note that the free apoptotic body (B) shows much less labelling than the phagocytosed one (C). Bar= 1 µM.

Connecting Text

The results from the previous chapter characterized the response of limb buds to 4OOH-CPA treatment during development. A direct concentration- and time-dependent relationship with limb malformations was revealed. Apoptosis induction and acceleration were caused by drug treatment which was localized in specific areas of the limb. In this chapter we commenced the study of apoptosis regulation during limb development by examining the protein induction and localisation of cathepsin D, a gene reported to be involved in apoptosis. To further continue investigating apoptosis regulation, we investigated the pattern of induction of two other proteins, transglutaminase and clusterin, known to be involved in apoptosis. The result of these studies with more data on apoptosis localisation during limb development are presented in the next chapter.

CHAPTER THREE

Transglutaminase and Clusterin Induction during Normal and Abnormal Limb Development

S. Adel Moallem and Barbara F. Hales

Status: Published in *Biology of Reproduction* 55, 281-290 (1996)

ABSTRACT

Apoptotic cell death is important in pattern formation in the limb. The purpose of this study was to investigate the relationship between the occurrence of apoptosis in the mouse limb during normal and abnormal development and the expression of tissue transglutaminase and clusterin, two proteins associated with apoptotic cell death. Mouse limb buds were cultured *in vitro* in the absence or presence of a potent teratogen, an activated analog of cyclophosphamide, 4-hydroperoxycyclophosphamide (1 or 10 μg/ml). Using whole-mount *in situ* DNA labelling and confocal microscopy, apoptotic cells were localized in the interdigital areas of control limbs after culture for 24 h. The number of apoptotic cells in the interdigital areas of the limbs was increased in the presence of 4-hydroperoxycyclophosphamide (1 µg/ml). Exposure to a higher concentration (10 µg/ml) of 4-hydroperoxycyclophosphamide further increased the numbers of cells staining positively for apoptosis. The relative abundance of tissue transglutaminase increased 3-4 fold after 6 or 24 h of culture with either concentration of 4-hydroperoxycyclophosphamide; immunoreactive protein in drug-treated limbs decreased to control levels by 48 h. Transglutaminase immunoreactivity was localized in the interdigital areas of limbs 24 h after drug exposure. Clusterin immunoreactivity in the control limbs was weak. The abundance of clusterin was increased 3-4 fold in drug-treated limbs; this induction occurred only after 48 h of culture with 4-hydroperoxycyclophosphamide. Clusterin immunoreactivity in limbs after drug treatment for 48 h was localized to the interdigital areas; immunogold

electron microscopy of clusterin expression showed a specific labelling in phagocytosed apoptotic bodies. Thus, the number of cells staining positively for apoptosis in the limb was greatly increased in the interdigital areas during abnormal limb development. The expression of both transglutaminase and clusterin was altered in areas of the limb undergoing apoptosis during abnormal limb development.

INTRODUCTION

Apoptosis or programmed cell death is an important form of "physiological" cell death with distinct morphological and biochemical characteristics [1,2]. In the developing embryo, the spatio-temporal occurrence of apoptosis is precisely controlled and occurs in the neural tube, heart, palate, duodenal mucosa, and limb buds [3,4]. Mammalian limb development provides an excellent model in which to study the regulation of apoptosis; apoptosis occurs predominantly in the interdigital areas during normal limb development, as well as in an area designated the foyer proximale primare and in the apical ectodermal ridge [5,6].

Cyclophosphamide has been widely used as a model drug to study the role of alkylation of cellular macromolecules in antineoplastic activity, as well as in mutagenesis, carcinogenesis, and teratogenesis [7,8]. *In utero* exposure of the developing embryo to cyclophosphamide may lead to a variety of limb malformations including phocomelia, adactyly, syndactyly and polydactyly [9]. Murine limbs cultured during organogenesis *in vitro* with an activated analog of cyclophosphamide, 4-hydroperoxycyclophosphamide, exhibit a time and dose dependent growth retardation and dysmorphogenesis [10,11]; this treatment accelerated apoptosis in the interdigital areas of the limb [11].

There is growing interest in the role of apoptosis in normal and abnormal development. Little is known about either the tissue or cell specificity of the regulation of apoptosis during development, or about the mechanisms by which

exposure to a teratogen can perturb this process. Two molecules of interest during development for which there is evidence of altered regulation during apoptosis are tissue transglutaminase and clusterin.

Transglutaminases are a family of Ca^{2+}-dependent enzymes that catalyse acyl-transfer reactions between peptide-bond glutamine residues and the ε-amino group of lysine residues of other peptides [12]. The transglutaminase reaction leads to protein cross-links, which result in the formation of a cross-linked protein scaffold in apoptotic cells; the detergent-insoluble protein nets join cytoplasmic and membrane proteins, maintain cellular integrity during the formation of apoptotic bodies, and prevent the dead cells from breaking into fragments prior to their digestion [13-17]. There is strong evidence that tissue transglutaminase is a specific marker for apoptosis in many tissues [13-18]. Jiang and Kochhar [16] have correlated the induction of tissue transglutaminase activity in limb buds with apoptosis induced by exposure to retinoic acid. Cell transfection with a tissue transglutaminase cDNA resulted in a significant reduction in proliferative rate and a large increase in the number of apoptotic cells [19]; transfection with a cDNA segment in the antisense orientation led to dramatic decrease of both spontaneous and retinoic acid-induced apoptosis. Thus, the regulation of tissue transglutaminase is important in tissues undergoing apoptosis [19].

Clusterin, a glycosylated and sulfated glycoprotein, was first isolated from ram rete testis fluid as a protein that elicited clustering of Sertoli cells [20]. The rat homolog of clusterin, sulfated glycoprotein-2 or SGP-2, was subsequently isolated from Sertoli cells and detected in several sites of the rat reproductive system [21].

The expression of clusterin in the testis, epididymis, ovary, uterus, and a wide variety of tissue types during embryogenesis suggests an important role in reproduction and development [22]. Clusterin expression has been associated with programmed cell death or apoptosis in the prostate [23,24] and in the interdigital areas of mouse limb buds [23]. The role of clusterin during apoptosis is not clear. One of the proposed roles for clusterin is in lipid transport between cells; the induction of clusterin in apoptosis or tissue injury may provide a mechanism to eliminate excess lipid derived from dead or injured cells or to scavenge toxic lipid byproducts [25]. Alternatively, clusterin may act as an inhibitor of complement-mediated cytolysis by binding to the soluble C5b-7 to form a cytolytically inactive complex; this would protect both the apoptotic cell and its vital neighbours from complement attack [25,26].

The purpose of this study was to investigate the regulation of tissue transglutaminase and clusterin in the mouse limb bud undergoing apoptosis. The presence and localization of these two proteins during normal and teratogen-induced abnormal development were determined.

MATERIALS AND METHODS

Limb bud culture and drug treatments

Timed pregnant CD1 mice (20-25g), mated between 8 and 10 AM (day 0 of gestation), were purchased from Charles River Canada Inc. (St. Constant, QC) and

housed at the McIntyre Animal Centre (McGill University, Montréal, QC). Between 8 and 10 AM on day 12 of gestation, females were sacrificed by cervical dislocation, uteri were removed, and the embryos were dissected in sterile Tyrode's saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites, pooled and cultured in a chemically-defined medium as previously described [27]. 4-Hydroperoxycyclophosphamide (1 µg/ml or 10 µg/ml, a gift from Dr. N. Brock, Asta-Werke, Germany) or vehicle control (water) was added to the designated cultures after one hour. The cultures were terminated at the specified times. Three separate replicates were done for each experiment.

Whole Mount In situ DNA end labelling - Light and Confocal Microscopy

After 24 h of culture, limbs were fixed in 10% buffered formalin overnight at 4°C, washed in 70% ethanol, then rehydrated and treated for one hour in 5% H_2O_2 in methanol to quench endogenous peroxidase, followed by 3 washes in PBT (0.1% Tween-20 in PBS). Limbs were incubated with proteinase K (15 µg/ml) for five minutes at room temperature and then two times five minutes in glycine (2mg/ml). The limbs were fixed again in 4% paraformaldehyde for one hour, and then washed 3 times in PBT. The ApopTag kit (Oncor, Gaithersburg, MD, USA) was used to label and stain the 3'- OH ends of DNA, generated during the process of apoptosis. With this kit, terminal deoxynucleotidyl transferase (TdT) was used to incorporate digoxigenin-11-dUTP in the DNA for one hour at 37°C. Limbs were washed with 0.1% Tween-20-added stop/wash buffer for 30 min, then blocked in

10% fetal calf serum in PBT for one hour. The anti-digoxigenin-peroxidase and anti-digoxigenin-fluorescein conjugates were preabsorbed for one hour with 1% fetal calf serum in PBT. The anti-digoxigenin-peroxidase (light microscopy) or anti-digoxigenin-fluorescein (confocal microscopy) conjugates were applied to the limbs for one hour and then the limbs were washed 4 times in PBS. Peroxidase-incubated limbs were developed in peroxidase substrate until visible staining was detected, then they were washed in PBS and photographed with a light microscope. Fluorescein-incubated limbs were counterstained with propidium iodide, mounted under glass coverslips and examined using a confocal laser scanning microscope (Carl Zeiss LSM-410 invert). The fluorescein signal was imaged by exciting the sample with a 488-nm line from an argon/krypton laser and the resulting fluorescence was collected on a photomultiplier after passage through FT510, FT560, and BP515-540 filter sets. Likewise, the same field was excited with a helium/neon (543-nm line) laser and the propidium iodide signal was imaged on a second photomultiplier after passage through FT510, FT560 and LP590 filter sets. Multiple Z-sections were collected and 3 dimensional images were constructed using built-in LSM software. All images were archived on a Bernoulli multidisk. The fluorescein and propidium iodide images were overlaid and pseudo colored using the software. Finally, images were printed on a Kodak XLS8300 high resolution color printer.

Western blot analysis

At the designated times, three to four limbs per group were placed in liquid N_2 and stored at -80°C. Limbs were homogenized in the presence of liquid N_2,

dissolved in sample loading buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% ß-mercaptoethanol) and boiled for five minutes. Samples (50 µg protein per lane as determined by Bio-Rad protein spectrophotometric assay) were fractionated by SDS-PAGE using 10% acrylamide gels [28]. Biotinylated SDS-PAGE broad range standards (Bio-Rad Laboratories, Mississauga, ON) were used as molecular weight markers.

The fractionated proteins were transferred to a nitrocellulose sheet [29]. The blots were blocked with 5% nonfat dried milk in TBS-T (137 mM NaCl; 20 mM Tris, pH 7.4; 0.1% Tween 20) at room temperature for one hour and then incubated with tissue transglutaminase antibody (polyclonal rabbit anti-tissue transglutaminase IgG was purchased from Debrecen Medical University Apoptosis Research Foundation, Debrecen, Hungary) or clusterin antibody (polyclonal rabbit anti-clusterin IgG was kindly provided by Dr. S. R. Sylvester). Normal rabbit IgG was used as a control for non-specific binding. The blots were washed three times in TBS-T and incubated with biotinylated anti-rabbit antibody (1:500 in 5% milk/TBS) for 30 minutes. The antibody binding was visualized with streptavidin alkaline phosphatase conjugate (1:3000 in TBS) using 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium as substrates (Amersham Canada Ltd., Oakville, ON). Blots were photographed and bands were scanned using laser densitometry (Pharmacia, Montréal, QC). The relative absorbance of the relevant bands was calculated and normalized to the total absorbance. Three separate replicates were done for these experiments; values represent mean ± S.E.M. The data were analysed by two-way analysis of variance (ANOVA) using the SYSTAT for Windows version 5.05

software program (Systat Inc., Evanston, IL). The level of significance was $P \leq 0.05$.

Tissue transglutaminase and clusterin immunohistochemistry - Light microscopy

At the end of the culture period, limbs were fixed with Bouin's fixative at 4°C for 3 h, dehydrated with ascending ethanol concentrations, cleared with xylene and embedded in paraffin. Sections of 5 µm were cut, mounted on slides, and rehydrated with xylene and descending concentrations of ethanol and water. The sections were washed with PBS, treated with 0.3% H_2O_2 in PBS for 30 minutes, washed with PBS, blocked with 1.5% normal goat serum in PBS for 30 minutes and then incubated with the primary antibody (tissue transglutaminase 1:500 or clusterin 1:200) overnight. After washing with PBS, sections were stained using the avidin-biotin-peroxidase complex method with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Each section was reacted with DAB solution containing 0.05% 3,3-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO, USA) and 0.01% hydrogen peroxide in 0.05 M Tris buffer, pH 7.2. Tissue transglutaminase sections were counterstained briefly with haematoxylin and clusterin sections with methylene blue. Normal rabbit IgG replaced the primary antibody in control sections.

Clusterin immunohistochemistry - Electron microscopy

At the end of the culture period, limbs were fixed with 0.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h. After washing with buffer and dehydration with ethanol, tissue blocks were embedded in

LR White acrylic resin (MECA Ltd, Montréal, QC). Tissue blocks were polymerized in gelatin capsules for 24 h in a vacuum oven at 48°C. Ultrathin sections were cut and mounted on 200 mesh nickel grids. The grids were blocked with 5% normal goat serum for 15 minutes and incubated overnight with clusterin antibody (1:20). A higher concentration of clusterin antibody was utilized in this experiment to increase the sensitivity of the detection. After washing with buffer, the grids were incubated with colloidal gold-labelled goat anti-rabbit IgG (gold particles: 10 nm in diameter; 1:20 dilution; British BioCell Int., Cardiff, UK). As a control, the clusterin antibody was replaced by normal rabbit IgG. The grids were counterstained with uranyl acetate and lead citrate and examined in a Philips EM410 electron microscope. The same experiment was performed with tissue transglutaminase antibody; no immunolabelling was detected.

RESULTS

Apoptosis in control and drug-exposed cultured limbs

Intact limbs cultured for 24 h and then stained for apoptotic cells by *in situ* DNA labelling using a whole mount approach are shown in Figure 1 (light microscope) and Figure 2 (confocal microscope). Cells staining positively for apoptosis were found mainly in the interdigital areas of the control limbs, in an area underlying the apical ectodermal ridge (Fig 1A and B; Fig 2A). Exposure of the limbs for 24 h to the low concentration of 4-hydroperoxycyclophosphamide (1µg/ml)

increased the numbers of cells staining positively for apoptosis (Fig1C and D; Fig 2B); to some extent these cells remained localized in the interdigital areas, as in the control limbs, but now reached a greater distance into the limb. Exposure of the limbs to the higher concentration of 4-hydroperoxycyclophosphamide (10 µg/ml) resulted in a dramatic increase in the number of cells staining positively for apoptosis (Fig. 1E and 1F; Fig 2C). Under the light microscope, the thickness of the limbs prevented the resolution of specific cells or areas that were stained. Under the confocal microscope, it was possible to see that the green-stained apoptotic cells were still focussed in the interdigital areas. Therefore, while apoptosis occurred in control limbs, it was induced dramatically by drug-treatment. Apoptotic cells in drug-treated limbs remained localized chiefly in the interdigital areas.

Tissue transglutaminase induction in control and drug-treated limbs

A Western blot of tissue transglutaminase immunoreactivity in limbs cultured for 6, 24, and 48 h in the absence or presence of 4-hydroperoxycyclophosphamide is shown in Figure 3. Two bands were detected with immunoreactivity to anti-tissue transglutaminase antibody: a 80 kDa band and a 118 kDa band (Fig.3-A). An 80 kDa band has been reported previously for tissue transglutaminase [30], while the 118 kDa band is likely to represent the presence of a tissue transglutaminase complex with another protein; such complexes, with molecular weights in the range of 90 to >200 kDa, have been reported to be the result of a covalent linkage of tissue transglutaminase with other molecules [31]. Both bands were subjected to densitometric quantification. The 118 kDa band remained unchanged with length

of time in culture or upon treatment with 4-hydroperoxycyclophosphamide. The 80 kDa band also remained unchanged with length of time in culture. However, exposure to 4-hydroperoxycyclophosphamide at concentrations of 1 µg/ml or 10 µg/ml resulted in a significant increase over control (3-5 fold) in the absorbance or density of the 80 kDa band after 6 or 24 h of culture (Fig. 3-B). This induction of transglutaminase by drug exposure of the cultured limbs was transitory, disappearing by 48 h of culture. Thus, exposure of the limbs to 4-hydroperoxycyclophosphamide for 6 or 24 h dramatically increased the relative abundance of the tissue transglutaminase protein.

Immunohistochemical localization of tissue transglutaminase in control and 4-hydroperoxycyclophosphamide-exposed limbs is shown in Figure 4; these limbs were cultured either in the absence (Fig. 4, A and B) or the presence of 4-hydroperoxycyclophosphamide (Fig. 4, C, D, E, F) for 24 h, a time at which drug exposure increased the relative abundance of tissue transglutaminase in the limb. No immunostaining was observed in limbs cultured for 24 h when control IgG was substituted for anti-tissue transglutaminase antibody (data not shown). Control limbs cultured for 24 h showed very light tissue transglutaminase immunostaining, primarily localized over a few cells in the interdigital area (Fig. 4-A,B). In contrast, strong tissue transglutaminase immunoreactivity was observed in limbs exposed to 1 µg/ml (Fig. 4-C,D) or 10 µg/ml (Fig. 4-E,F) 4-hydroperoxycyclophosphamide. Intense immunostaining was localized specifically to the interdigital areas, particularly in cells with the condensed nuclei characteristic of apoptotic cells. After 6 h of culture the same pattern of immunostaining was found (data not shown). No

immunostaining was found in the limbs cultured for 48 h in the presence of either concentration of 4-hydroperoxycyclophosphamide (data not shown).

Clusterin induction in control and drug-treated limbs

Protein samples from rat epididymis and testis were electrophoresed for comparison with those from limb buds. Incubating the blots with control normal rabbit IgG revealed two non-specific bands, of 68 kDa and 46 kDa, in all samples (data not shown). Five major bands of clusterin immunoreactivity, at 61, 50, 42, 36 and 30 kDa were detected in rat caput epididymidis (Fig. 5-A, lane e). Two bands, of 60 and 36 kDa, were detected in rat testis (Fig. 5-A, lane t). Three specific bands, 61, 50, and 36 kDa, were detected in mouse limb buds (Fig. 5, lane 1-9), though at much lower concentrations than those in the epididymis and testis. The total intensity of these three bands in limbs cultured for 6 (lanes1-3), 24 (lanes4-6) or 48 (lanes7-9) h was determined by densitometry (Fig. 5-B). After 6 or 24 h of culture, the relative abundance (absorbance) of clusterin was not significantly different between control and drug-treated limbs. However, after 48 h of culture, the relative abundance of clusterin in the limbs treated with either 1μg/ml or 10μg/ml of 4-hydroperoxycyclophosphamide was increased 3-fold or 4-fold, respectively, above that found in the control limbs.

Immunohistochemical localization of clusterin in control and 4-hydroperoxycyclophosphamide-exposed limbs is shown in Figure 6; these limbs were cultured either in the absence (Fig. 6, A and B) or presence of 4-hydroperoxycyclophosphamide (Fig. 6, C, D, E, F) for 48 h, the time at which drug

exposure had an effect on the relative abundance of clusterin in the limb. Clusterin immunoreactivity was too low for detection in control limbs at this time and stage of development (Fig. 6, A and B). Weak immunoreactivity was observed in limbs exposed to the low concentration of 4-hydroperoxycyclophosphamide for 48 h; this immunoreactivity was detected in cells in the interdigital areas (Fig. 6, C and D). Clusterin immunostaining was more intense in limbs exposed to 10 µg/ml of 4-hydroperoxycyclophosphamide for 48 h; this immunoreactivity also was localized to the interdigital areas (Fig. 6, E and F).

Ultrastructural localization of clusterin in apoptotic bodies

The ultrastructural localization of clusterin in limb mesenchymal cells undergoing apoptosis, using immunogold electron microscopy, is shown in Figure 7. The ultrastructural morphology of cells in limbs exposed to 10 µg/ml of 4-hydroperoxycyclophosphamide for 48 h was poor, so immunogold electron microscopy was done on sections from limbs exposed in culture to the drug for only 24 h. Phagocytosed apoptotic bodies in the interdigital area of drug-treated limbs showed a specific labelling for clusterin (Fig. 7, A, B and C). Figure 7B and 7C are higher magnification views of the apoptotic bodies designated by the arrows in Fig. 7A. Immunogold particles are visible in the amorphous staining material located inside these bodies. As a control for the specificity of the reaction, the clusterin antibody was replaced with normal IgG; no significant labelling was found in these sections (data not shown). Thus, during apoptosis, clusterin can be immunolocalized to apoptotic bodies undergoing phagocytosis.

DISCUSSION

Treatment of mouse limbs in vitro with 4-hydroperoxycyclophosphamide, a potent teratogen, greatly increased the numbers of cells that stained positively for apoptosis. While tissue transglutaminase and clusterin were present in the untreated mouse limb during organogenesis *in vitro*, both were dramatically induced after exposure to 4-hydroperoxycyclophosphamide. Moreover, tissue transglutaminase and clusterin were localized primarily to areas in the limb undergoing apoptosis, the interdigital areas.

Disruption of normal development by teratogen exposure results in the induction of apoptosis first in areas of the limb programmed to undergo this type of cell death during normal development. The observation that exposure to a teratogen increased cell death in the embryo in areas of active cell death has been made in other studies [6,32,33]. The explanation for the increase in responsiveness of cells in the interdigital areas to a stressor is not known. These cells may be simply more sensitive to the stressor, i.e., have altered thresholds of response. Cells destined to form digits or other structures may not have the necessary protective "factors" to survive insult with a teratogen. Alternatively, cells in the interdigital areas may follow a unique pathway in differentiation that leads them to apoptosis. It will be necessary to elucidate the early events in apoptosis, and the mechanisms by which they are controlled, to distinguish among these possibilities.

It is clear that there are different regulatory mechanisms for the various genes required during apoptosis in the limb. The induction of tissue transglutaminase is apparent in the mouse limb by six h after exposure to 4-hydroperoxycyclophosphamide. Tissue transglutaminase expression may be required at a relatively early stage to help prevent the organelles of the dying cells from dispersing into the extracellular environment until the time at which the apoptotic bodies that are formed are disposed of by phagocytosis. Little is known about the regulation of tissue transglutaminase expression. The promoter region of the tissue transglutaminase gene has been cloned and characterized recently [34]. It is interesting that this promoter contains the consensus sequence for the activating protein-1 (AP-1) transcription factor. AP-1 proteins are expressed early during apoptosis [35], and persistent AP-1 activation may contribute to the induction of genes that are important in apoptosis such as tissue transglutaminase.

The time course of induction of cathepsin D in limbs exposed to 4-hydroperoxycyclophosphamide is intermediate: slower than that observed for tissue transglutaminase and more rapid than that for clusterin [11]. Cathepsin D expression was increased in treated limbs two to three-fold relative to controls after exposure to 4-hydroperoxycyclophosphamide for 24 h. Cathepsin D is required to digest proteins in the phagocytosed fragments.

Clusterin induction during apoptosis in the limb was a relatively late event. Analysis of the promoter region of the clusterin gene revealed the existence of several potential regulatory elements that may regulate the complex tissue-specific control of a gene which must be constitutively expressed in some tissues, but

repressed in others until induced during apoptosis [36]. An AP-1 site in this region of the clusterin gene may play a different role than the one in the transglutaminase gene. It has been suggested that this site alone, or in combination with other sites, contributes to the repression of clusterin expression in terminally differentiated cells [36].

The role of clusterin in apoptosis is still unclear. It may play a role in cell differentiation during development [22]. It is developmentally regulated in many tissues including the heart, kidney, lung and brain [37]. In the developing rat limb bud (day 14-16), intense immunostaining for clusterin was seen in the condensing mesenchyme of the developing digits. While some overlap between clusterin immunostaining and apoptosis was seen, most apoptotic cells in the interdigital mesenchyme and underlying surface ectoderm were not positive for clusterin immunostaining [38], consistent with our data for the untreated murine limb *in vitro*. Alternatively, clusterin may play a role in the protection of cells from injury or stress [38,39]. Clusterin is induced in injury states that are not always associated with apoptosis [23,40]. The treatment of rat embryos cultured *in vitro* with phosphoramide mustard, a metabolite of cyclophosphamide, was found to alter the expression and localization of clusterin in the embryo and its yolk sac; a DNA fragmentation pattern characteristic of apoptosis was found in the drug-exposed embryos, but not in their yolk sacs [41]. Moreover, *in situ* hybridization studies of two cell types, U937 and A431, have revealed that clusterin expression is confined to surviving cells after the induction of apoptosis, thus providing evidence that clusterin may be associated with cell survival within tissues that are regressing as

a consequence of apoptosis [42]. In an intriguing recent report it was suggested that clusterin depletion, rather than its induction, is associated with apoptosis [43]. It will be difficult to distinguish between these possibilities without more insight into the function of clusterin in apoptosis and tissue injury.

Thus, apoptosis plays an important role in the abnormal limb development induced by a teratogen such as 4-hydroperoxycyclophosphamide. The expressions of tissue transglutaminase and clusterin are altered in those limb regions that undergo apoptosis during drug-induced abnormal development. Further studies are needed to elucidate the signalling processes by which the cells that are destined to die respond to insult.

ACKNOWLEDGMENTS

This study was supported by the Medical Research Council of Canada. S. A. Moallem is the recipient of a studentship award from the Faculty of Medicine and Faculty of Graduate Studies and Research of McGill University. The authors would like to thank Dr. D. Laird for his assistance in confocal microscopy, Dr. S. R. Sylvester for providing the clusterin antibody and Ms. Marie Ballak for her assistance in the electron microscope studies.

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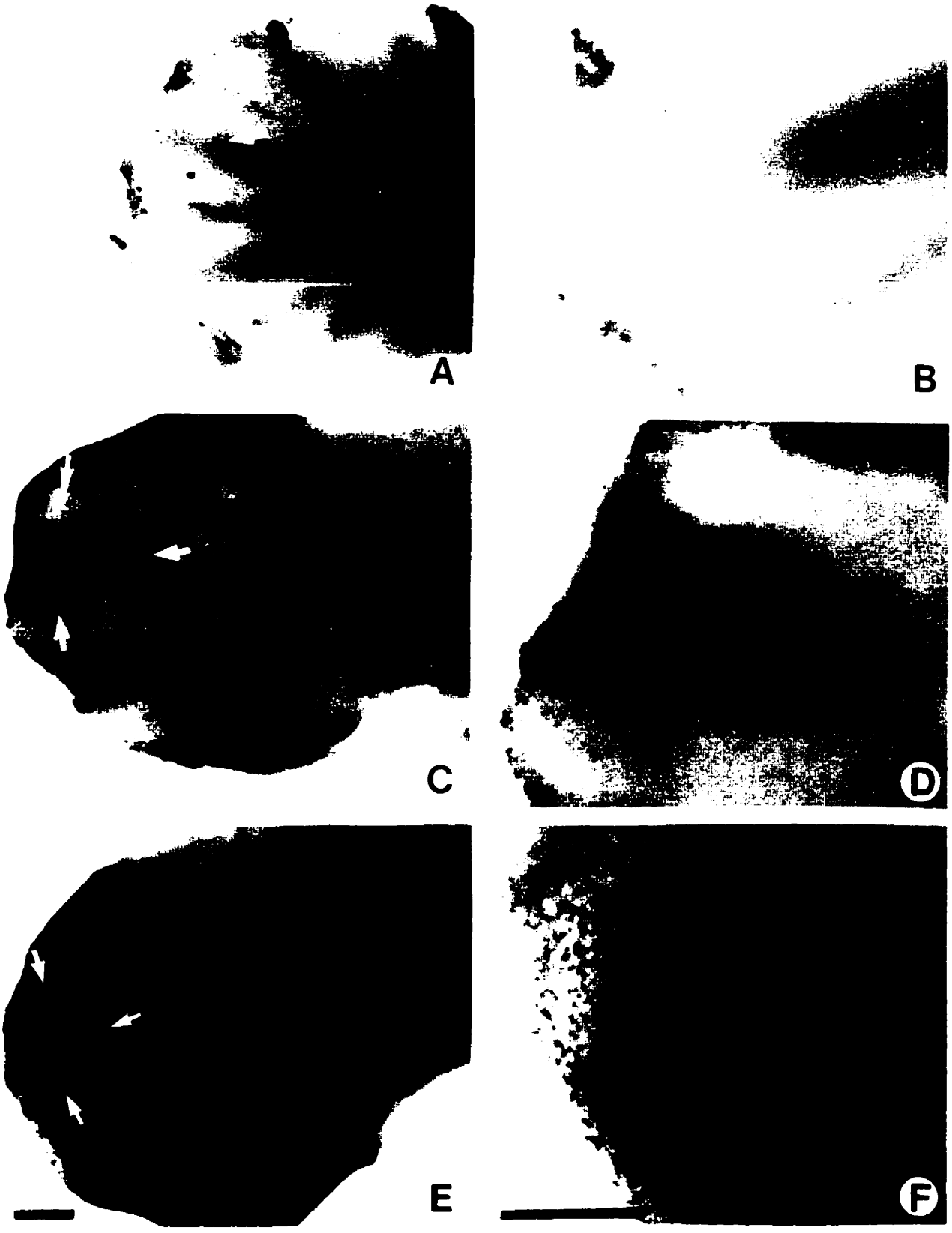
A
B
C
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E
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Figure 1: Whole mount in situ DNA labelling (light microscope). Limbs cultured for 24 h in control medium (A, B), in the presence of 1µg/ml (C, D) or 10 µg/ml (E, F) of 4-hydroperoxycyclophosphamide were stained for apoptosis as discussed in the Materials and Methods section and observed under the light microscope. Areas pointed by arrows in the right panel were shown at a higher magnification in the left panel. Bar=25 µM.

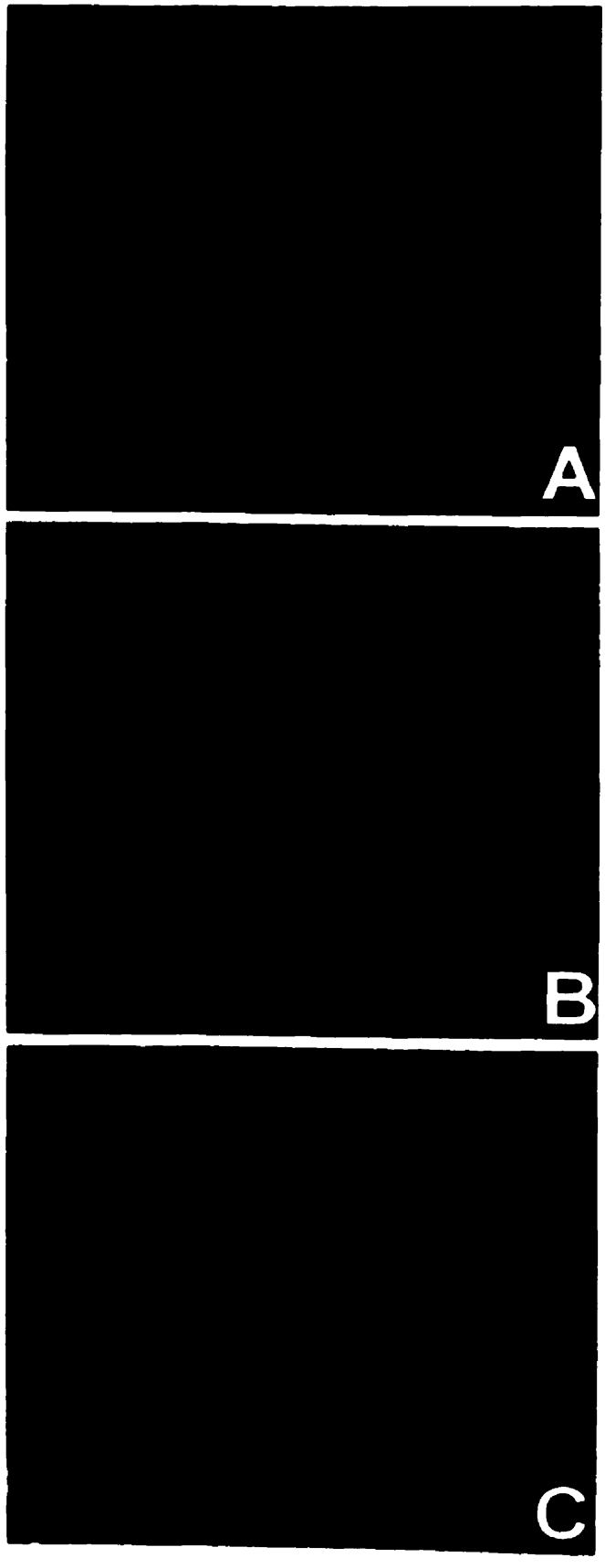
A
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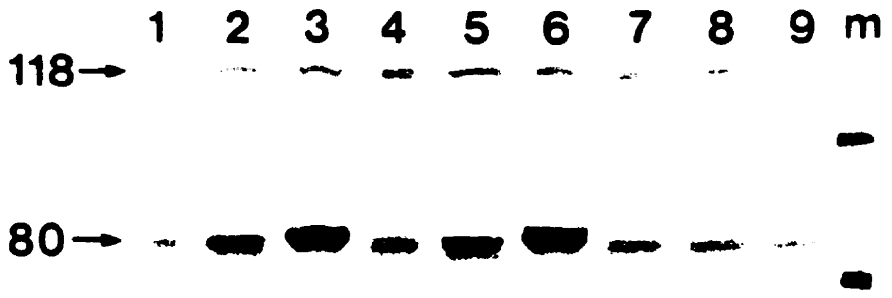
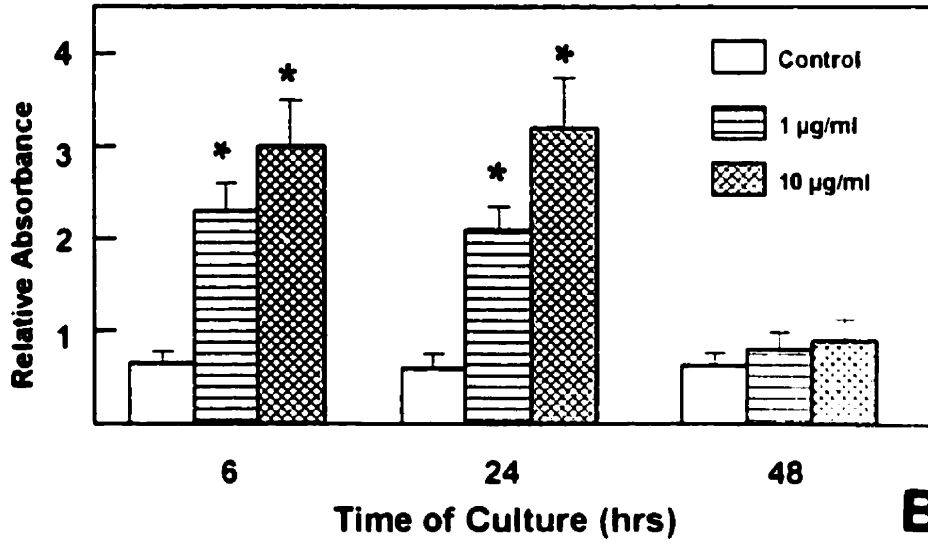
Figure 2: Whole mount in situ DNA labelling (confocal microscope). Limbs cultured for 24 h in control medium (A), in the presence of 1µg/ml (B) or 10 µg/ml (C) of 4-hydroperoxycyclophosphamide were stained for apoptosis as discussed in the Materials and Methods section and observed under the confocal microscope. X300


1 2 3 4 5 6 7 8 9 m
118
80

A


Relative Absorbance
4
3
2
1
Control
1 µg/ml
10 µg/ml
*
6
24
48
Time of Culture (hrs)

B

Figure 3: (A) Western blot analysis of immunoreactive tissue transglutaminase in the mouse forelimb. Protein samples from limbs cultured for 6 (lanes 1,2,3), 24 (lanes 4,5,6) or 48 (lanes 7,8,9) h in control medium (lanes 1,4,7) or in the presence of 4-hydroperoxycyclophosphamide, 1 µg/ml (lanes 2,5,8) or 10 µg/ml (lanes 3,6,9), were electrophoresed, blotted and immunostained with tissue transglutaminase antibody. Lane m depicts the molecular weight markers. (B) Plot of the densitometric analysis of the 80 kDa protein band. Data are expressed as means ± s. e. m. (n = 3).

* Significantly different from controls cultured for the same period of time ($P<0.05$).

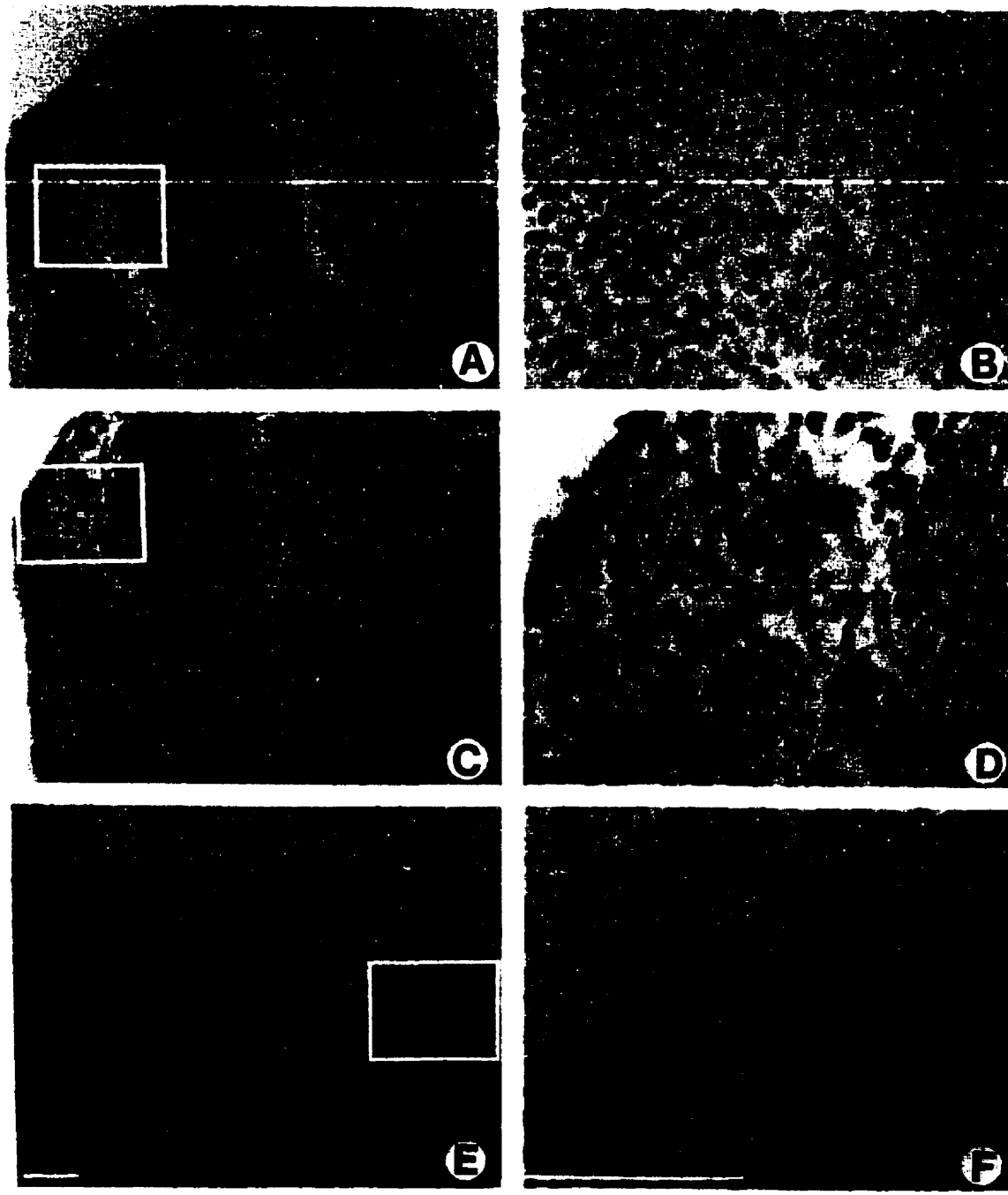

A
B
C
D
E
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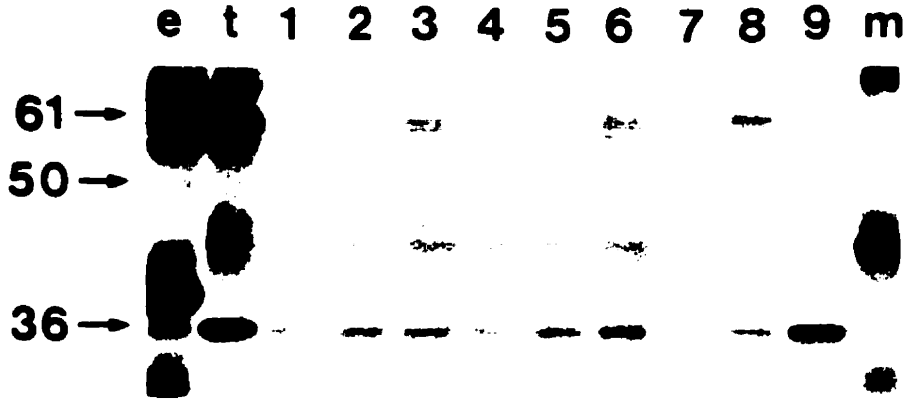
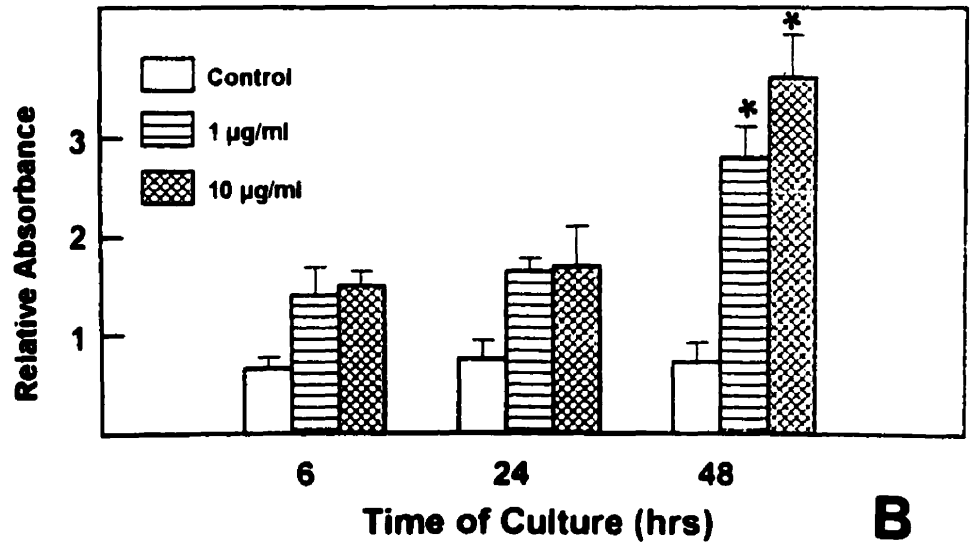
Figure 4: Immunohistochemical localization of tissue transglutaminase in the forelimb. Limbs were cultured for 24 h in control medium (A, B) or in the presence of 4-hydroperoxycyclophosphamide, 1 µg/ml (C, D) or 10 µg/ml (E, F). The right panels represent a higher magnification of the corresponding boxes in the left panels. Bars= 100 µM.

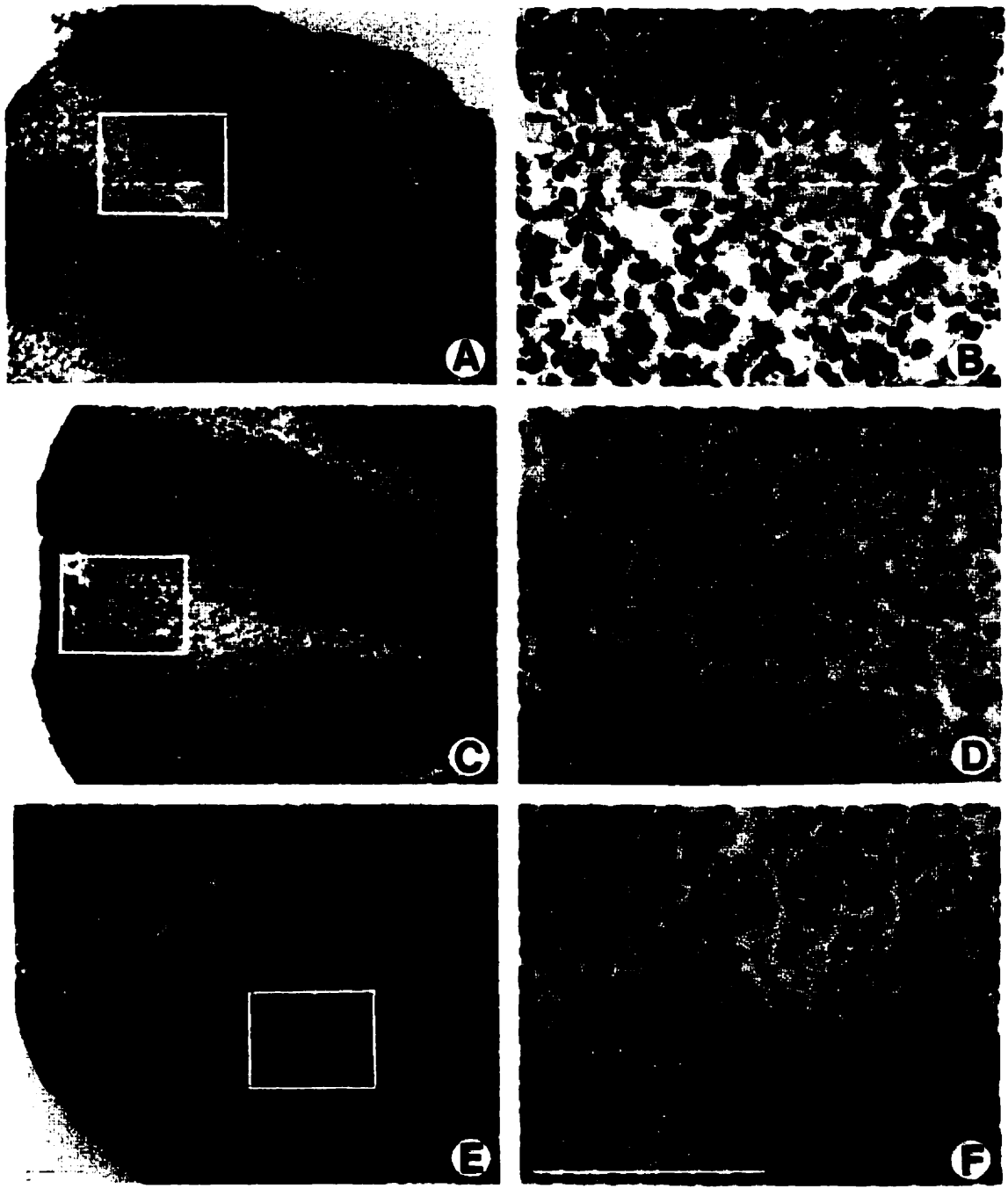

e t 1 2 3 4 5 6 7 8 9 m
61
50
36

A


Control
1 µg/ml
10 µg/ml
Relative Absorbance
1
2
3
*
*
6
24
48
Time of Culture (hrs)

B

Figure 5: (A) Western blot analysis of immunoreactive clusterin in the limb after various times in culture. Protein samples from limbs cultured for 6 (lanes 1,2,3), 24 (lanes 4,5,6) or 48 (lanes 7,8,9) h in control medium (lanes 1,4,7) or in the presence of 4-hydroperoxycyclophosphamide, 1 µg/ml (lanes 2,5,8) or 10 µg/ml (lanes 3,6,9), were electrophoresed, blotted and immunostained with clusterin antibody. Lane m depicts the molecular weight markers. Lane e is a protein sample from the adult rat caput epididymidis and lane t from the adult rat testis. (B) Plot of the densitometric analysis of the three limb clusterin protein bands at 61, 50 and 36 kDa. Data are expressed as means ± s. e. m. (n = 3). * Significantly different from controls cultured for the same period of time ($P<0.05$).


A
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C
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E
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Figure 6: Immunohistochemical localization of clusterin in the mouse forelimb. Limbs were cultured for 48 h in control medium (A, B) or in the presence of 4-hydroperoxycyclophosphamide, 1 µg/ml (C, D) or 10 µg/ml (E, F). The right panels represent a higher magnification of the corresponding boxes in the left panels. Bars= 100 µM.

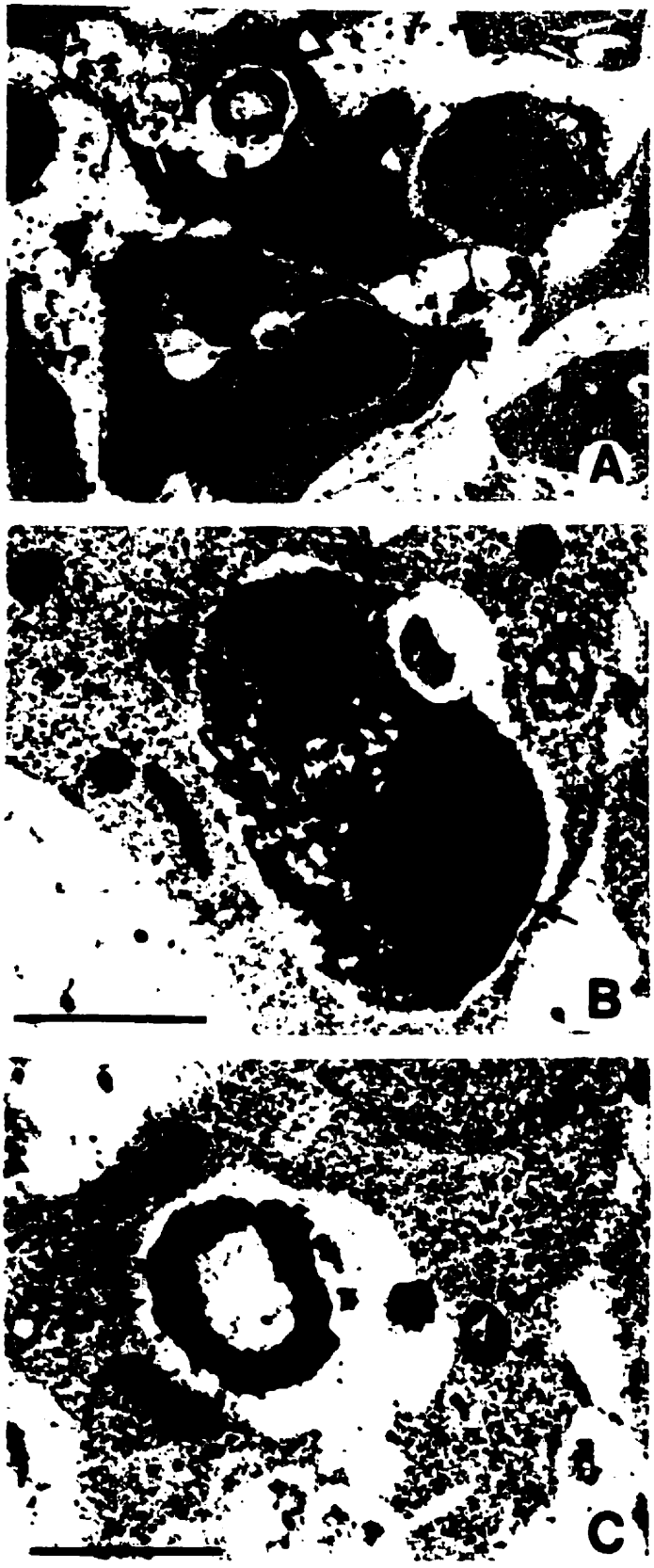
A
B
C

Figure 7: Ultrastructural localization of clusterin in cells of the interdigital areas of limbs exposed to 10 µg/ml 4-hydroperoxycyclophosphamide for 24 h. Panels B and C represent higher magnifications of the areas of the cell in A that are indicated by the filled and open arrowheads, respectively). Some of the immunogold particles are indicated by arrows in B and C. Bars= 1.5 µM.

Connecting Text

In the previous chapter we have continued characterizing and localizing apoptosis induction during normal and drug-induced apoptosis. Furthermore, the regulation of apoptosis was further studied by determining the induction and immunohistochemical localization of transglutaminase and clusterin. However, it was unclear what molecular target(s) might be affected by 4OOH-CPA treatment. Since the main molecular effect of 4OOH-CPA is causing DNA damage, we were interested to investigate possible genetic target(s) in this regard. As p53 had been identified as one of the most crucial genes in defending the genomic integrity against DNA damage, we chose this gene to study its role in drug teratogenesis and apoptosis. The results of these studies are presented in the next chapter.

CHAPTER FOUR

The role of p53 in 4-hydroperoxycyclophosphamide-induced limb malformations, apoptosis and necrosis

S. Adel Moallem and Barbara F. Hales

Status: published in *Development* 125: 3225-3234 (1998)

SUMMARY

The exposure of embryonic murine limbs *in vitro* to an activated analog of cyclophosphamide, 4-hydroperoxycyclophosphamide (4OOH-CPA), induced limb malformations and apoptosis. The purpose of this study was to investigate the role of the tumor suppressor/cell cycle checkpoint gene, p53, and of cell cycle arrest in the response of the limbs to cyclophosphamide. Limbs, excised on day 12 of gestation from wild type, heterozygous or homozygous p53-knockout transgenic murine embryos, were treated with vehicle (water) or 4OOH-CPA (0.3, 1.0 or 3.0 μg/ml) and cultured for six days. Exposure of wild type (+/+) limbs to 4OOH-CPA resulted in limb malformations, and reduced limb areas and developmental scores. The homozygous (-/-) limbs were dramatically more sensitive to the effects of 4OOH-CPA, as assessed by limb morphology, area and score. Heterozygous limbs exposed to the drug were intermediate for each parameter. Apoptosis, as assessed by the formation of a DNA ladder, was increased in drug-exposed wild-type limbs, but not in the drug-exposed homozygous limbs. Light and electron microscopy examination of the limbs revealed that drug-treatment of wild-type limbs induced the morphological changes typical of apoptosis, particularly in the interdigital regions. In contrast, there was no evidence of apoptosis in homozygous-limbs exposed to 4-OOH-CPA; morphological characteristics of necrosis such as cell membrane breakdown, mitochondrial swelling and cellular disintegration were evident throughout these limbs. Heterozygous limbs had cells dying with the characteristics of both apoptosis and necrosis. Fragments of poly(ADP-ribose) polymerase

characteristic of necrosis predominated in the drug-treated heterozygous and homozygous limbs. 4-OOH-CPA-treatment of limbs from wild type embryos led to arrest of the cell cycle at the G1/S phase. No cell cycle arrest was observed after drug treatment of homozygous limbs, where populations of cells in S and G2/M phases, as well as a population of sub G1 cells, were found. Thus, the presence of p53 and of p53 dependent apoptosis protect organogenesis-stage limbs from insult with a teratogen. The absence of p53 may decrease DNA repair capacity and contribute to the accumulation of DNA damage in these cells and in their daughter cells, and since they are unable to eliminate those cells by apoptosis, necrosis and/or major limb malformations occurred.

INTRODUCTION

Apoptosis is a form of active cell death which is distinct from necrosis or passive cell death (Kerr et al., 1972; Schwartzman and Cidlowski, 1993). It is characterized morphologically by chromatin condensation, cytoplasmic shrinkage and membrane blebbing. During apoptosis, an endogenous endonuclease is activated which catalyses internucleosomal DNA cleavage to multiple 180-200 bp fragments (Wyllie et al., 1980; English et al., 1989). Apoptotic cell death can be initiated by a number of external signals including cytotoxic anti-cancer drugs (Hickman, 1992), glucocorticoids, irradiation, heat shock and the withdrawal of some growth factors or hormones (Sen, 1992). When the regulation of apoptosis is disturbed, either by inhibition or inappropriate induction, disease can result (Bursch et al., 1992). Apoptotic cell death plays a crucial role in embryogenesis (Sanders and Wride, 1995). In the developing embryo, the spatio-temporal occurrence of apoptosis is precisely controlled and occurs in the neural tube, heart, palate, duodenal mucosa, and limb buds (Gerschenson, 1992; Wyllie, 1993).

p53, a positive regulator of apoptosis, was one of the first tumor suppressor genes identified (Oren, 1992). While it is not essential in most cells for normal cell cycle regulation, it acts as a cell cycle checkpoint gene that is induced in response to DNA damage. DNA damage causes a rapid increase in total p53 concentrations. This is achieved by increased gene transcription and the stabilization of normally rapidly degraded p53 protein; this increase in p53 leads to the suppression of cell growth or apoptosis (Smith and Fornace, 1996). It has been suggested that p53-

mediated cell cycle arrest provides more time for DNA repair to take place. However, recent findings suggest that p53 is involved actively in the DNA repair process (Smith et al., 1995; Li et al., 1996). It is not clear how a cell decides whether to arrest growth and repair DNA or to induce apoptosis in response to increased p53. Rowan et al. (1996) reported that in a p53 mutant cell line, p53 had a direct role in mediating cell-cycle arrest despite the loss of the ability to induce apoptosis. The process of apoptosis may be p53-dependent or independent. Thymocytes from p53-deficient mice are resistant to the induction of apoptosis by radiation, but not by anti-CD3 antibody or glucocorticoids; this observation suggests that the induction of apoptosis by radiation, but not other insults, is p53-dependent (Clarke et al., 1993; Lowe et al., 1993a). E1A, the adenovirus early region, is an oncogene that increases cellular susceptibility to apoptosis including its induction by anticancer agents (Lotem and Sachs, 1993). Parental embryonic fibroblasts are resistant to low doses of irradiation or several DNA-damaging chemotherapeutic agents (Lowe et al, 1993b). However, treatment of fibroblasts expressing E1A, with the same agents rapidly induces apoptosis, whereas there are no effects on the viability of p53-deficient E1A-expressing cells (Lowe et al, 1993b). Therefore, apoptosis caused by DNA damaging agents has a clearly established dependency on p53.

p53 is expressed extensively during mouse embryogenesis; however, the concentration of transcripts declines dramatically in cells undergoing terminal differentiation (Schmid et al. 1991). Although transgenic p53 "knockout" mice survived to birth, a high incidence of developmental abnormalities was found. Sah

et al. (1995) reported that a fraction of the p53-deficient embryos displayed defects in neural tube closure and, subsequently, exencephaly. Studies by Armstrong et al. (1995) revealed that these defects were found predominantly in the female embryos. More information is needed on the role of p53 during development. The role of p53 in mediating drug teratogenicity is not fully clear. With benzo[a]pyrene as the teratogen, p53 had a "teratological suppressor" function (Nicol et al., 1995). In this study, there was an increase in fetal resorption and other anomalies in p53 heterozygous and homozygous mice. However, two other anomalies, omphalocele and hemiangioendothelioma (red nevis of the skin) were conversely decreased in the heterozygous mice compared to the wild type ones; they suggested that these two anomalies might involve a cellular mechanism mediated by apoptosis. Norimura et al. (1996) found that radiation caused an increase in embryonic malformations in p53-homozygous mice; however, wild-type embryos had higher incidence of death. This reciprocal relationship of radiosensitivity to anomalies and to embryonic or fetal lethality supports the notion that embryonic or fetal tissues have a p53-dependent "guardian" of the tissue that aborts cell bearing radiation-induced teratogenic DNA damage. These results could be explained as that in wild-type embryos, p53-dependent apoptosis suppresses DNA damage-induced malformation by removing teratogen-injured cells. However, they did not have a clear explanation for the higher rate of death in these embryos. In another study, the presence of p53 was necessary for 2-chloro-2'deoxyadenosine exposure to induce eye defects (Wubah et al., 1996); p53-dependent apoptosis played a critical role in these eye defects as wild-type (+/+) embryos were more susceptible than the

homozygous (-/-) knockout embryos lacking p53. Taken together, this suggests that p53 has as its primary function the coordination of multicellular adaptive responses to diverse environmental stresses (reviewed in Hall and Lane, 1997).

Limb development has been used extensively to study growth, differentiation, patterning and cell death during development (Zakeri and Ahuja, 1994; Cohn and Tickle, 1996; Hurle et al. 1996; Serrano and O'Farrell, 1997). Apoptosis occurs predominantly in the interdigital areas and apical ectodermal ridge during normal limb development (Scott, 1979). Interference with this process by chemicals may be an important mechanism of action of teratogens that induce limb malformations (Alles and Sulik, 1989; Kurishita, 1989; Moallem and Hales, 1995; Moallem and Hales, 1996). Exposure to cyclophosphamide during organogenesis results in a spectrum of malformations that includes exencephaly or hydrocephaly, open eyes, cleft palate, kinky tails, and limb anomalies such as phocomelia, adactyly, oligodactyly, syndactyly, and polydactyly (Gibson and Becker, 1968; Hales, 1981). These effects are associated with induction of apoptosis by cyclophosphamide in the developing embryo. Although a causal relationship has not been proven yet, it is likely that disturbances in the physiological apoptosis by cyclophosphamide is a main mechanism in embryo and limb malformations. We have shown that 4-hydroperoxycyclophosphamide (4OOH-CPA), an active analog of the alkylating agent cyclophosphamide, induces concentration and time-dependent limb reduction malformations and apoptosis in the interdigital areas of cultured murine limbs (Hales and Jain, 1986; Moallem and Hales, 1995). Although it is well known that DNA, RNA and protein can be alkylated by cyclophosphamide, DNA is assumed to be the

primary target; DNA cross-linking leads to cytotoxicity and also may be important in the mediating the action of this drug as a teratogen (Mirkes, 1985).

The purpose of the present study was to investigate the role of p53 in mediating cyclophosphamide-induced limb malformations. Limbs from p53-transgenic knockout, heterozygous, and wild type mice were cultured in the absence or presence of 4OOH-CPA and examined for limb malformations, the presence of cell death by apoptosis or necrosis, and cell cycle stage.

MATERIALS AND METHODS

Limb bud culture and p53-genotype

p53-knockout heterozygous C57BL/6 mice (+/-) were purchased from Taconic Farms, Inc.(Germantown, NY, USA), and bred to form a colony at the McIntyre Animal Centre (McGill University, Montréal, QC). Mice (20-25 grams) were mated between 8 and 10 AM (day 0 of gestation). On day 12 of gestation, between 8 and 10 AM, females were sacrificed by cervical dislocation, uteri were removed, and the embryos were dissected in sterile Tyrode's saline solution; the embryos at this time were 5-6 mm in length with about 42 somite pairs. Forelimbs were excised just lateral to the somites, and cultured in a chemically-defined medium, as previously described (Kwasigrosh et al., 1984). 4-Hydroperoxycyclophosphamide (4OOH-CPA, 0.3, 1 or 3 µg/ml, a gift from Dr. N. Brock, Asta-Werke, Germany) or vehicle control (water) was added to the designated cultures after one hour.

The bodies of the embryos were used to determine the p53 genotype by polymerase chain reaction or PCR. "2A" primer (5' GGGACAGCCAAGTCTGTTATGTGC 3') , a mouse p53 exon 4 sense; "2B" primer (5' CTGTCTTCCAGATACTCGGGATAC 3'), a mouse p53 exon 6 antisense; and "57" primer (5' TTTACGGAGCCCTGGCGCTCGATGT 3"), a pol II from the neo cassette, antisense were used with 35 cycles as follows: 94 °C, 1 minute; 62 °C, 2 minutes; 72 °C, 6 minutes; autoextend 10 seconds per cycle. The resulting 1.2 Kb or 0.6 Kb band correspond to the + or - alleles, respectively.

Limb morphology

After 6 days the cultures were terminated and limbs (six to seven per bottle) were fixed and stained as previously described (Hales and Jain, 1986). The morphology of the limbs was examined with a dissecting microscope. Two-dimensional limb area, an indicator of limb growth was measured by image analysis with Micro-Plan II (Nikkon, Montréal, QC); limbs were scored for the extent of development in vitro according to the scoring system of Neubert and Barrach (1977). The data were analyzed by two-way ANOVA using the SYSTAT for Windows version 5.05 software program (Systat Inc., Evanston, IL). The level of significance was $p \leq 0.05$.

Other limbs were cultured for 24 hours, fixed and processed for light or electron microscope examination. For examination with the light microscope (Leica, Montréal, QC) limbs were fixed with buffered formalin; semi-thin sections were cut and stained with methylene blue. For the electron microscope studies, limbs were

fixed with 3.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours, then washed with buffer and postfixed with 1% osmium tetroxide in cacodylate buffer for 1 hour at 4°C. The samples were dehydrated with ethanol and embedded in Epon at 55°C for 24 hours. Ultrathin sections were cut and examined using a Philips EM410 electron microscope.

DNA electrophoresis

DNA was extracted and labeled with ^{32}P for gel electrophoresis (Tilly and Hsueh, 1993). Briefly, limbs were homogenized in liquid nitrogen and digested in a buffer containing proteinase-K. Samples were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and DNA was precipitated with ethanol. RNA was digested with RNase (Boehringer Mannheim Canada, Laval, QC), and the DNA re-extracted. DNA was quantified absorbance at 260 nm. The 3' end labeling was carried out by terminal transferase reaction (Pharmacia Biotech Inc., Baie d'Urfé, QC) in a medium containing DNA, [$\alpha^{32}P$]-ddATP and cobalt chloride. The labeled DNA was precipitated in 10M ammonium acetate and ethanol, washed, air-dried and resuspended in buffer. Samples equal to 1 µg DNA were loaded onto a 2% agarose gel and separated by electrophoresis for 3.5 hours at 50V. Gels were dried and autoradiographed.

Western blot analysis of poly(ADP-ribose) polymerase (PARP) fragmentation

At the designated times, three to four limbs per group were placed in liquid N_2 and stored at -80°C. Limbs were homogenized in the presence of liquid N_2, and dissolved in sample loading buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 6M urea; 10% glycerin; 0.003% bromophenol blue; 5% ß-mercaptoethanol). Samples (10 µg protein per lane as determined by Bio-Rad protein spectrophotometric assay) were fractionated by SDS-PAGE in 8% acrylamide gels (Studier, 1973). Kaleidoscope prestained standards (Bio-Rad Laboratories, Mississauga, ON) were used as molecular weight markers. The fractionated proteins were transferred to a nitrocellulose sheet (Towbin et al., 1979). The blots were blocked with 5% nonfat dried milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.4; 0.1% Tween 20) at room temperature for one hour and then incubated with 422 PARP antibody (1/1000, kindly provided by Dr. Guy Poirier) or the preimmune serum overnight. The blots were incubated with peroxidase-conjugated second antibody for one hour, developed with a ECL kit (Amersham Canada Ltd., Oakville, ON), and autoradiographed for <1 hour.

Flow cytometric analysis

Limbs were homogenized gently to minimize cellular breakdown and cell suspensions were made by filtering the homogenates through nylon meshes of 100 µm, 50 µm and 10 µm. Approximately 1 x 10^6 cells were collected by low speed centrifugation at 23°C and the cell pellet was resuspended in 0.1% sodium citrate and 0.1% Triton 20. Cells were washed and fixed in 2% paraformaldehyde in PBS

for two hours. After washing, they were treated with RNase (Boehringer Mannheim Canada, Laval, QC) and stained with propidium iodide (10 µg/ml). Samples were filtered through a 10 µm nylon mesh immediately before analysis by flow cytometry. Fluorescence > 570 nm emitted from the propidium iodide-DNA complex in each cell nucleus was measured with a FACScan flow cytometer. (Becton Dickinson Canada Inc., Mississauga, ON). Approximately 10,000 cells were examined for each sample. The percentages of cells within the G_1, S, and G_2/M phases of the cell cycle were determined by analysis with the LYSIS II system. Three replicates were done for each experiment. Data were analyzed by ANOVA and Bonferroni T-test (Glantz, 1992).

RESULTS

Limb morphology after teratogen exposure is dependent on p53-genotype

Limbs from wild-type p53 (+/+) and heterozygous (+/-) embryos developed to a similar extent in the control medium (Fig.1); scapula, long bones and paw skeleton were observed. The majority (80%) of the limbs from p53 deficient homozygous (-/-) embryos also developed normally in the control medium, but approximately 20% were malformed. A malformed sample is shown in Figure.1. Treatment with 4OOH-CPA caused concentration-dependent limb reduction malformations in wild-type

limbs (Fig.1). The effects of 4OOH-CPA on the development of both the long bones and paw skeleton in the (+/+) limbs was concentration dependent with a dramatic reduction in the formation of these cartilaginous anlagen at the highest drug concentration (3 µg/ml). In the limbs from p53-deficient (-/-) embryos there was a significant response to 4OOH-CPA even at the lowest concentration while higher concentrations of 4OOH-CPA (≥ 1 µg/ml) caused dramatic limb reduction malformations (Fig.1). Interestingly, the heterozygous limbs (+/-) had an intermediate response to 4OOH-CPA treatment (Fig.1).

The areas and developmental scores of limbs from embryos of each genotype from each treatment group are presented in Figure 2. The limb areas (Fig. 2A) represent the extent of limb growth; the limb scores (Fig. 2B) describe the degree of limb differentiation (Neubert and Barrach, 1977). The p53 genotype did not affect the limb area in control cultures; there was a significant ($P \leq 0.05$; ANOVA and Bonferroni T-test) 4OOH-CPA concentration-dependent decrease in the areas of limbs from (+/+) embryos. While the areas of limbs from heterozygous embryos were not significantly different from controls, exposure of the limbs from homozygous (-/-) embryos to each concentration of 4OOH-CPA resulted in limb areas that were approximately half of those from the (+/+) embryos.

As observed for limb area, p53 genotype did not affect the limb scores in control cultures. A 4OOH-CPA concentration-dependent decrease was observed in the scores of limbs from (+/+) embryos; for each drug concentration, the scores of (-/-) limbs exposed to 4OOH-CPA were significantly lower than those from (+/+) embryos. The response of the (+/-) limbs was intermediate, indicating that the

presence of a functional wild-type allele of the p53 gene in these limbs has an impact.

DNA fragmentation pattern

While previous studies have shown that 4OOH-CPA induces apoptosis in organogenesis-stage murine limbs (Moallem and Hales, 1995), it is not clear whether this apoptosis is p53 dependent. If p53 dependent apoptosis plays a critical role in limb malformations, the p53-deficient limbs would have had a decreased, rather than an increased susceptibility to limb malformations. To determine the p53 dependence of apoptosis in the limb, DNA was extracted from the limbs and quantified by spectrophotometric measurements. Then, 1 µg/ml DNA was labeled with a ^{32}P-labeled nucleotide and electrophoresed (Fig. 3). A smear of radioactivity, probably indicative of a low amount of DNA fragmentation, was observed in control limbs from embryos of all genotypes. An increase in "DNA ladder" formation, typical of apoptotic cell death, was observed in 4OOH-CPA-exposed (+/+) limbs and in (+/-) limbs, but not in drug-exposed (-/-) limbs. Thus, the apoptosis induced by the exposure of limbs during organogenesis to 4OOH-CPA is p53-dependent; 4OOH-CPA failed to induce apoptosis in the limbs from the (-/-) p53-deficient embryos.

Morphological analysis of the type of cell death in the limb

To investigate the type of cell death in drug-treated limbs of each genotype, limb

sections were examined with the light and electron microscope. Light microscope examination of control limbs showed very few, if any, dying cells (Fig. 4A,C,E); the p53 genotype did not affect the occurrence of cell death in these limbs. 4OOH-CPA treatment (3 µg/ml) of the (+/+) limbs resulted in an increase in the number of cells with shrunken cytoplasm and condensed nuclei compared to the control limbs, especially in the interdigital regions (Fig. 4B). More dying cells were found in the limb after drug treatment of the heterozygous limbs (Fig 4D). Many pyknotic and fragmented cells appeared in 4OOH-CPA-treated (-/-) limbs (Fig. 4F), not only in the interdigital areas, but throughout the limbs.

The morphology of cells in the limbs from each genotype and treatment group was examined in more detail with the electron microscope (Fig. 5). Only a few cells had the condensed chromatin and shrunken cytoplasm characteristic of dying cells in control limbs (Fig.5A,C,E). While p53 genotype did not appear to affect the incidence of dying cells, cells in the limbs from heterozygous (+/-) (Fig.5C) and homozygous (-/-) (Fig.5E) embryos appeared to have less cell-to-cell contact compared to cells in the limbs from wild-type embryos (+/+) (Fig.5A). There were fewer cells in each microscopic field in the limbs from (+/-) (25% reduction) and (-/-) (45% reduction) embryos, compared to the (+/+) limbs. Most of the cells in the sections of limbs from (+/+) embryos cultured in control medium appeared lightly stained with a cuboidal shape and a large nucleus (Fig.5A); few cells had the condensed chromatin and withdrawn cytoplasm typical of apoptosis. Exposure to 3 µg/ml 4OOH-CPA (Fig. 5B) resulted in the appearance of numerous cells with the morphological features of apoptosis; some of these cells were still intact while

others were in the process of undergoing phagocytosis. Drug treatment of the heterozygous (+/-) limbs induced a further increase in the numbers of dying cells (Fig.5D), compared to their control counterparts (Fig. 5C). Some cells had the condensed chromatin and intact plasma membrane typical of apoptosis while others had disintegrated cellular structures, suggesting that they were dying by necrosis as well as by apoptosis. Examination of sections from the 4OOH-CPA treated p53-deficient (-/-) limbs revealed many cells with ruptured cell membranes, and disintegrated organelles, confirming the occurrence of a high level of necrosis (Fig. 5F). Apoptosis was not observed. Thus, the type of cell death in the developing limb in response to 4OOH-CPA is p53-genotype dependent. Moreover, the cell death type is affected by the p53 gene dosage. Finally, in the absence of the p53 gene dying cells are no longer localized to specific interdigital areas.

Poly(ADP-ribose) polymerase (PARP) protein fragmentation

To further differentiate between cell death by apoptosis versus necrosis in the limb, the poly(ADP-ribose) polymerase (PARP) cleavage pattern was investigated by Western blot analysis. Shah et al. (1996) reported that PARP is cleaved to different fragments in response to apoptosis (89 kDa fragment) or necrosis (50, 40 and 35 kDa fragments). No fragmented PARP was detected in wild type limbs cultured in control medium (Fig. 6). The 89 kDa fragment of immunoreactive PARP, characteristic of apoptosis, was detected in (+/+) limbs after 4OOH-CPA treatment;

the small molecular weight fragments of PARP, characteristic of necrosis, were not observed in these limbs. While the predominant PARP fragment observed in the control heterozygous (+/-) and homozygous (-/-) limbs in the absence of drug treatment was the 89 kDa fragment, lower amounts of the smaller molecular weight fragments (50, 40 and 35 kDa) characteristic of necrosis appeared also to be present, though higher in the homozygous limbs than the heterozygous limbs. The relative proportion of these small molecular weight fragments to total immunoreactive PARP increased dramatically in both the heterozygous (+/-) and homozygous (-/-) limbs exposed to 4OOH-CPA. However, the apoptotic fragment could still be detected in the treated homozygous limbs, suggesting a p53-independent occurrence of apoptosis. Thus, using both morphological and biochemical criteria (PARP and DNA fragmentation patterns), we can conclude that cell death occurs predominantly by necrosis in the 4OOH-CPA-exposed limbs from p53 deficient homozygous (-/-) and heterozygous (+/-) embryos, while some indication of low rate of apoptosis was still present in the limbs. It is conceivable that increasing the dosage of drug treatment might completely switch cell death from apoptosis to necrosis.

Cell cycle analysis

Flow cytometry was done to analyze the effects of exposure to 4OOH-CPA on cell cycle regulation in limbs derived from embryos with each p53 genotype. A normal

distribution of cell phase populations was found in suspensions from control wild type (+/+) limbs. Interestingly, there was a significant difference ($P \leq 0.05$) in the percentage of S-phase cells between limbs of the three genotypes cultured in control medium. This has been supported by other studies (Harvey et al., 1993), but it is unclear why this phenomena occurs and how significant it is. Further studies are needed to elucidate the biological importance of this observation.

Exposure of limbs from wild type embryos to 4OOH-CPA caused a cell cycle block at the G1/S phase; this block was evident from the absence of cell populations at the S and G2/M phases (Fig. 7). Similarly, the cell cycle was blocked at the G1/S phase in response to 4OOH-CPA treatment of limbs from heterozygous (+/-) embryos. While there were fewer cells from drug-treated homozygous (-/-) limbs in the S and G2/M phases of the cell cycle compared to the vehicle-treated samples ($p<0.05$); exposure to 4OOH-CPA did not lead to a cell cycle block in limbs from p53-deficient (-/-) embryos. Indeed, more cells were in the S and G2/M phases of the cell cycle in the (-/-) limbs compared to the wild-type (+/+) drug treated limbs (increased 20 fold and 3 fold, respectively; $p<0.001$). Thus, in the absence of p53 many cells with DNA damage continued to cycle and failed to show a complete block at the G1/S phase.

Interestingly, there was an increase in the number of hypodiploid cells represented by the sub G1 fraction in response to 4OOH-CPA treatment; this was most pronounced in the homozygous (-/-) limbs. These observations are consistent with previous findings (Smith et al., 1995; Li et al., 1996; Smith and Fornace, 1996) in which p53 acts as a cell cycle checkpoint gene to ensure efficient DNA repair in

response to DNA damage; the absence of p53 leads to continuous cell cycling with insufficient time for DNA repair.

DISCUSSION

The absence of p53 rendered embryonic limbs more susceptible to alkylating agent-induced malformations, demonstrating that p53 plays a protective role against teratogenic insults during embryonic development. This is consistent with previous studies with benzo[a]pyrene as the teratogen, in which Nicol et al. (995) demonstrated a "teratological suppressor" function for p53. However, investigations of other insults during development have lead to opposite conclusions. Norimura et al. (1996) showed that irradiation of p53 (+/+) mice on day 9.5 of gestation caused more embryonic deaths than anomalies; however, in p53 (-/-) mice, there were more embryonic anomalies than deaths. Wubah et al. (1996) found that the eye malformations induced by a genotoxic agent, 2-chloro-2'-deoxyadenosine, were due to p53-dependent apoptosis. The absence of p53 in the (-/-) mice resulted in a sharp decrease in the incidence of apoptosis and hence prevented the occurrence of the malformations. Thus, p53 served to "enhance" the susceptibility of embryos to eye malformations. However, it is not clear if cells with DNA damage caused by 2-chloro-2'-deoxyadenosine in the p53 (-/-) embryos can transmit this damage to their daughter cells, leading to subsequent developmental abnormalities. The ability

of p53 to either "suppress" or "enhance" teratogenicity may depend on the role of p53-dependent apoptosis in mediating the defect. More studies are needed to clarify the role of p53 and p53-dependent apoptosis in mediating teratogen-induced malformations during organogenesis.

In exploring the mechanism by which p53 "suppressed" the response of the organogenesis-stage limb to insult, we found that necrosis was the major type of cell death in the limbs exposed to 4OOH-CPA in the absence of p53. Initially apoptosis and necrosis were considered as morphologically and biochemically different modes of cell death. However, there is increasing evidence that the two type of demise can occur simultaneously in tissues exposed to the same stimulus, and often the intensity of the stimulus decides the prevalence of either apoptosis or necrosis. Thus, both types of cell death may be triggered by the same signals and share early events. Many signals as diverse as heat shock (Schrek et al., 1980), viruses (Subramanian et al., 1995), protein synthesis inhibitors (Lennon et al., 1991), oxidative stress (Dypbukt et al., 1994), hypoxia (Shimizu et al., 1996b), Ca^{2+} ionophores, radiation (Fukuda et al., 1993), glutamate (Ankarcrona et al., 1995), and nitric oxide (Bonfoco et al., 1995) can induce both apoptosis and necrosis (Leist and Nicotera, 1997). Second messengers and downstream steps such as ATP (Leist et al., 1997) and bcl-2 (Shimizu et al., 1996a) have been shown to be involved in both apoptosis and necrosis. Although it is unlikely, this study did not exclude completely the possibility that due to increased sensitivity to apoptosis in the treated homozygous limbs, early massive apoptosis was followed by failure of complete phagocytosis, leading to "secondary necrosis" (Vermes et al., 1997; reviewed in

Slater et al., 1995).

Previous studies from our lab and others have implicated cell death by apoptosis in both normal and teratogen-induced abnormal limb development (Alles and Sulik, 1989; Kurishita, 1989; Moallem and Hales, 1995). Nevertheless, p53 deficient (-/-) limbs were resistant to drug-induced apoptosis; examinations with the light and electron microscope clearly disclosed the presence of necrotic cells in (-/-) limbs. It may be significant that the specific localization of dying cells to the interdigital regions was lost in the (-/-) limbs; necrotic cells appeared throughout the limb. The basis for differences in the response of cells in the interdigital areas versus those in the cartilaginous anlagen to stimuli is not well understood. In response to insult, p53 may direct interdigital cells to undergo death by apoptosis, while cells in the digital areas may undergo growth arrest and DNA repair. The loss of p53 may block this regulatory function and lead to sustained cell division or necrosis, depending on the extent of DNA damage. Further investigation of the p53-dependent change of cell death from apoptosis to necrosis in the limb after insult may contribute to our understanding of the role of p53 and of cell death in limb pattern formation.

The amount of stress or insult (i. e. dose and time of exposure) can affect the type of cell death (Lennon et al., 1991; Dypbukt et al., 1994; Sato et al., 1995). Boyle and Hickman (1997) recently suggested that the threshold for drug-induced apoptosis is determined not only by the extent of cytotoxic damage but also by the trophic environment and the ability of a cell to modulate survival signals that attenuate toxicity. The p53 protein may serve to "modulate" the response of the

limbs to insult. The absence of p53 may increase the impact of an insult on cell function in the limbs of the p53 deficient animals. The presence of lower amounts of p53 in the heterozygous mice (gene dosage) resulted in an intermediate response, suggesting that an insult may "overwhelm" the p53 defensive mechanisms to arrest the cell cycle and/or induce apoptosis.

p53 has an important function as a cell cycle "checkpoint" gene (Lane, 1992). The cause and effect relationship between expression of wild type p53 and G1 arrest after irradiation was established by Kastan et al. (1992) and Kuerbitz et al. (1992). According to the model that has been proposed, p53 helps to maintain genetic stability by initiating a transient G1 cell cycle arrest; this arrest allows time for the repair of damaged DNA before entry into S phase. Recently, an additional role for p53 as a G2 cell cycle checkpoint was discovered (Cross et al., 1995; Paules et al., 1995). Our results are in accordance with the above studies and indicate that even in embryonic tissues, p53 functions to block the cell cycle at the G1 phase in response to insult with a DNA-damaging agent. Although it is not clear which molecule(s) p53 targets to induce cell cycle arrest in the limb, it is attractive to speculate that one target may be insulin-like growth factor-1 (IGF-1). p53 inhibited the activity of insulin-like growth factor-1 (IGF-1) by direct transcriptional induction of insulin-like growth factor binding protein 3 (IGF-BP3) (Buckbinder et al., 1995); it was demonstrated that the cellular p53 induced IGF-BP3 gene expression in fibroblasts in response to DNA damage (Buckbinder et al., 1995). Consequently, the induced IGF-BP3 inhibited the IGF-1 mitogenic signal. IGF-1 is a strong candidate as a major mitogen involved in the morphogenesis of the developing

limbs (Geduspan et al., 1992; Dealy and Kosher, 1995); in addition, IGF-BP3 has been found in the developing limb (Bhaumick, 1993).

It is intriguing that a "subset" of p53 (-/-) homozygous limbs cultured in the control medium did not develop normally. Other investigators have reported that proportion of p53 (-/-) homozygous embryos have developmental abnormalities, namely neural tube defects (Armstrong et al., 1995; Sah et al., 1995). These studies did not report any abnormal limb development. The added "stress" of culture in 50% O_2 may disturb limb organogenesis in the p53 deficient embryos. Oxidative stress occurs during the in vitro culture of whole embryos (Ozolins and Hales, 1997); high oxidative stress can cause abnormal development (Ishibashi et al., 1997; reviewed in Wells et al., 1997). Another interesting features of this group of limbs are the appearance of the low molecular fragments of PARP (Fig. 6), and also the difference in the distribution of cell populations compared to the control wild type limbs (Fig. 7). Both of which can contribute to other unknown effects that eventually can lead to the abnormal development of a percentage of limbs in this group.

Alternatively, cell-to cell-contacts may be abnormal in the limbs from p53 deficient embryos. We found a decrease in cell density and less contact with neighboring cells in sections from p53-deficient, vehicle-treated limbs, compared to sections from the limbs of wild type embryos. Cano et al. (1996) reported that there was a 70% reduction in the expression of E-cadherin, a calcium dependent cell-cell adhesion molecule, including focal loss at cell-cell contacts in p53-null cells. Also, Kaneko et al. (1996) reported that there was a high frequency of association

between p53 mutations and negative expression of CD54, an intercellular adhesion molecule, in Burkitt's lymphoma. Cells in the p53 deficient homozygous limbs may be more susceptible to environmental stress, including in vitro culture, as a consequence of the loss of cell-cell adhesion. The relationship between cell adhesion and the induction of apoptosis has been investigated and some evidence are available showing loss of cell-cell contact might trigger apoptosis, suggesting the existence of an antiapoptotic signal(s) in the extracellular matrix (Peluso, 1997; Levkau et al., 1998). However, another aspect of this relationship is the loss of cell contact as a morphologic feature of apoptosis (reviewed in Schwartzman and Cidlowski, 1993). Thus, the cause-effect relationship of loss of cell contact and apoptosis is not fully understood.

We propose that in wild type (+/+) limbs, 4OOH-CPA induces DNA damage which may lead to arrest of the cell cycle in the digital areas at the G1 phase, allowing the utilization of DNA repair systems to restore normal DNA structure/function. Some cells in the interdigital areas may have more extensive DNA damage, due to a higher susceptibility of cells to DNA damage and/or to a less efficient DNA repair system. Unrepaired interdigital cells may be removed by the activation of controlled cell death, namely apoptosis. Thus, insult by a teratogen such as 4OOH-CPA leads to an increase in apoptosis, this increased apoptosis removes cells with damaged DNA from the organism. This increased apoptosis causes moderate limb malformation, but it serves to protect the limb against the greater insult and malformations represented by the survival of these cells with damaged DNA and their subsequent growth and cycling (Fig.8). The rate of limb

malformations after insult is higher in p53 deficient (-/-) embryos exposed to a DNA-damaging teratogen due to the continuity of the cell cycle in surviving cells which leads to the accumulation of damaged DNA and also the occurrence of massive necrosis. Accumulation of DNA damage and/or necrosis lead to severe limb malformation in the -/- p53 mice.

Acknowledgments

The authors should like to thank Dr. Bernard Robaire for his thoughtful and helpful discussion of the manuscript, Dr. Guy G. Poirier for providing the PARP antibody, Dr. U. Saragovi for his assistance in flow cytometry and Ms. Marie Balak for her assistance in the electron microscope studies.

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0

0.3 µg/ml

1 µg/ml

3 µg/ml

Fig. 1: p53 wild-type (+/+), heterozygous (+/-), and homozygous (-/-) limbs cultured for 6 days with vehicle (water) or 4-hydroperoxycyclophosphamide (0.1, 1 or 3 µg/ml). Limbs were fixed, stained, and photographed. Bar=0.3 mm

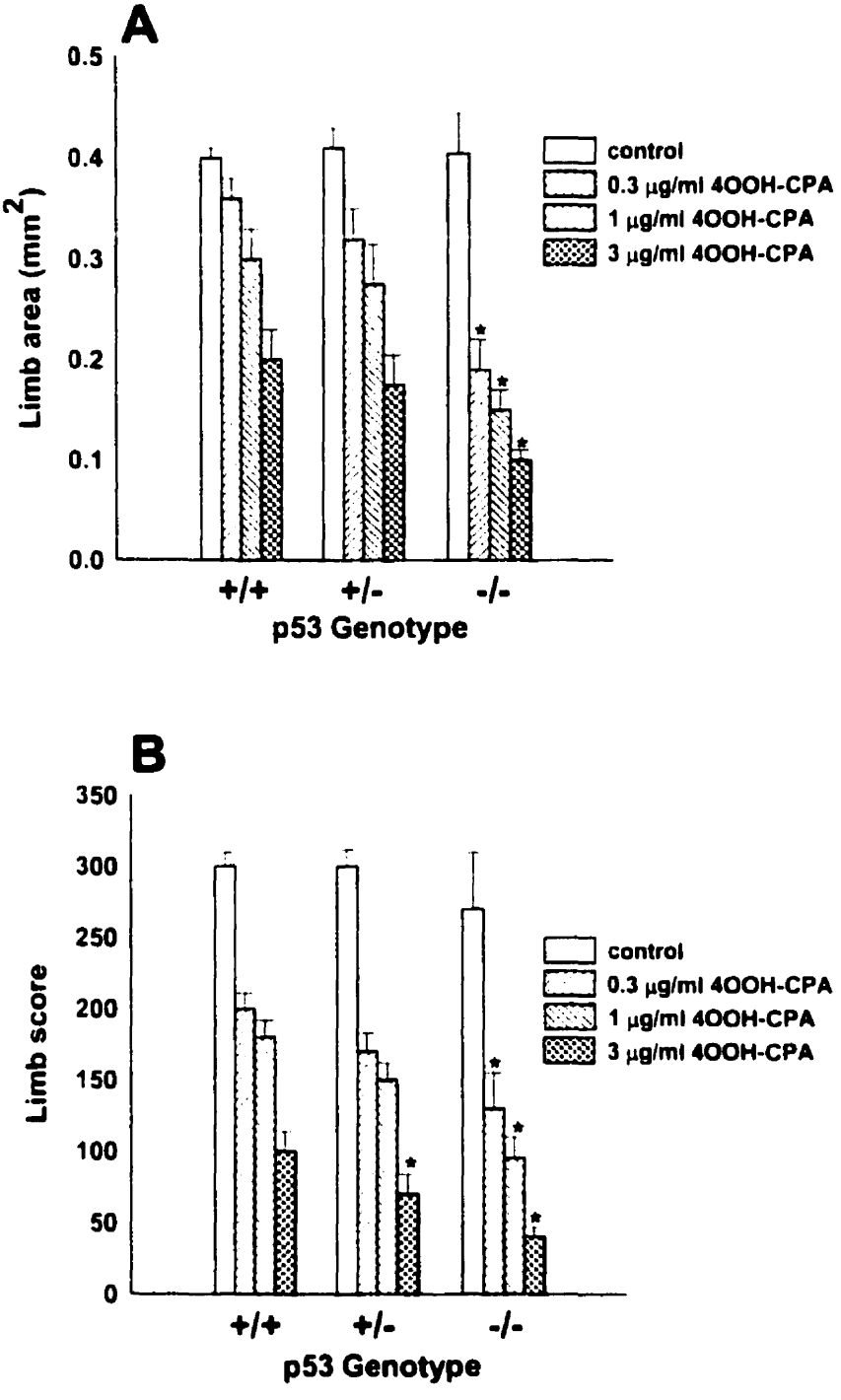
A
0.5
0.4
0.3
0.2
0.1
0.0
Limb area (mm2)
control
0.3 µg/ml 4OOH-CPA
1 µg/ml 4OOH-CPA
3 µg/ml 4OOH-CPA
+/+
+/-
-/-
p53 Genotype
B
350
300
250
200
150
100
50
0
Limb score
control
0.3 µg/ml 4OOH-CPA
1 µg/ml 4OOH-CPA
3 µg/ml 4OOH-CPA
+/+
+/-
-/-
p53 Genotype

Fig. 2: Image analysis and developmental scores of control or 4OOH-CPA-treated limbs of different p53 genotypes. (A) The effect of 4OOH-CPA-treatment on limb area in mm^2 of the limbs from embryos with each p53 genotype. Bars represent means ± standard errors of the mean (n=3). * indicates a significant difference, $P<0.05$, between -/- limbs exposed to each drug treatment and the wild type (+/+), as evaluated by ANOVA and Bonferroni T-test. (B) The effect of 4OOH-CPA-treatment on the scores of the limbs from embryos with each p53 genotype. Bars represent means ± standard errors of the mean (n=3). * indicates a significant difference, $P<0.05$, between -/- and +/- limbs exposed to each drug treatment and the wild type (+/+), as evaluated by ANOVA and the Bonferroni T-test.

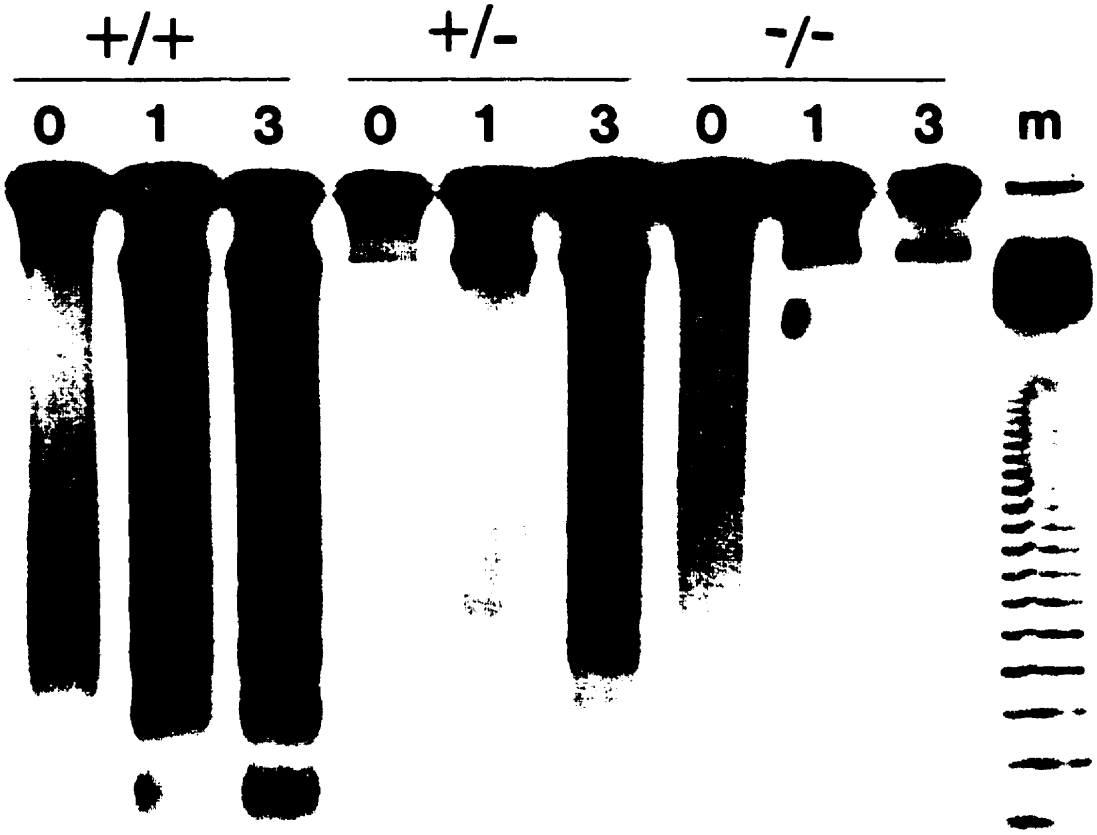
+/+
+/-
-/-
0 1 3 0 1 3 0 1 3 m

Fig. 3: The DNA fragmentation pattern of p53 wild-type (+/+), heterozygous (+/-), and homozygous (-/-) limbs cultured for 24 hours with vehicle (water) or 4-hydroperoxycyclophosphamide (1 or 3 µg/ml); DNA was extracted, labeled with ^{32}P, electrophoresed and autoradiographed. m= molecular size markers.

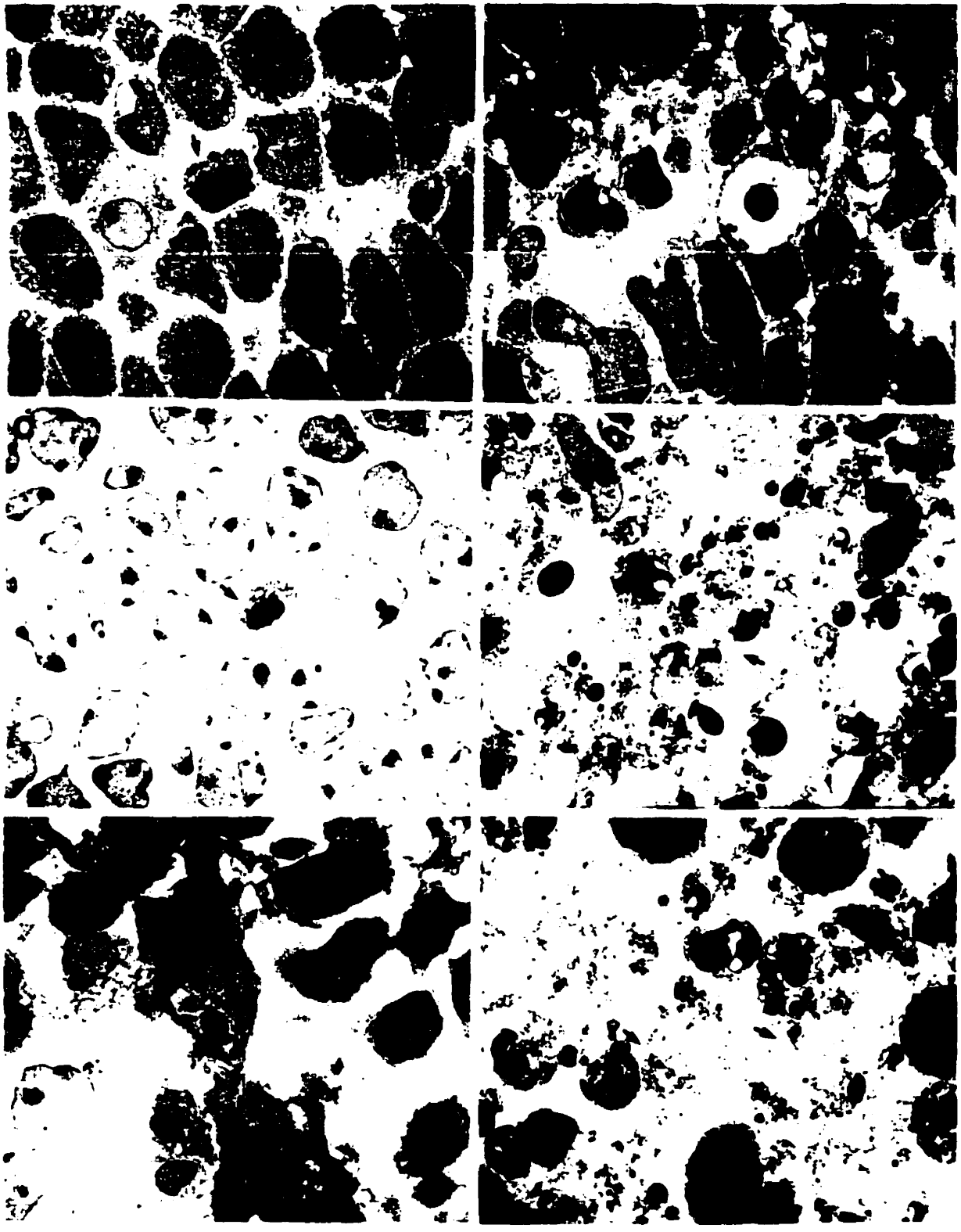

Fig. 4: Light micrographs of limbs from each p53 genotype (A, B wild-type; C, D heterozygote; E, F homozygote) after culture for 24 hours in the control medium (A, C, E) or in the presence of 3 µg/ml 4OOH-CPA (B, D, F). Arrows point to dying cells. Bar = 12 µM.

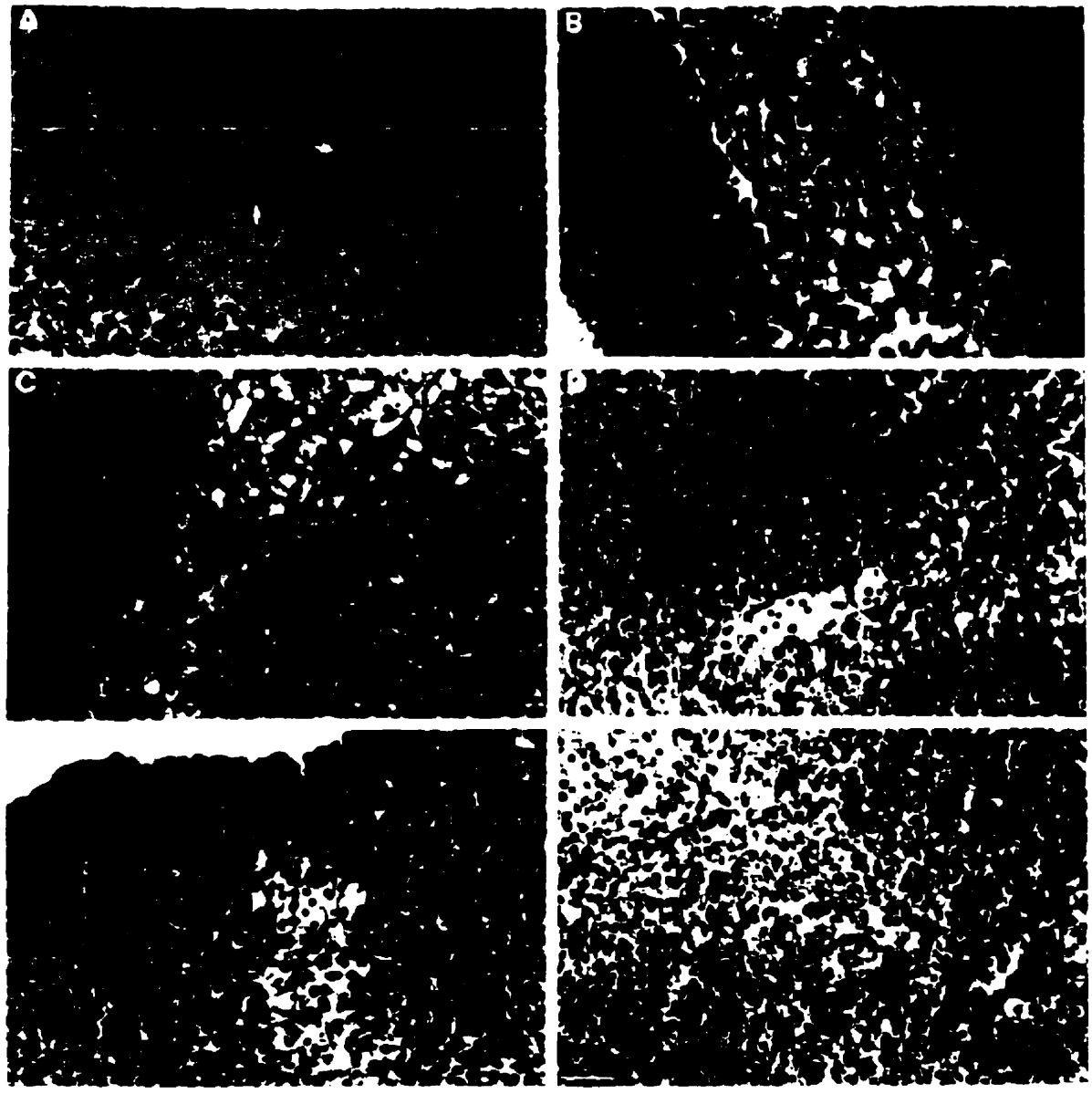
A
B
C

Fig. 5: Electron micrographs of limbs from each p53 genotype (A, B wild-type; C, D heterozygote; E, F homozygote) after culture for 24 hours in the control medium (A, C, E) or in the presence of 3 µg/ml 4OOH-CPA (B, D, F). Arrows point to some of the dying cells. Bar = 13 µM.

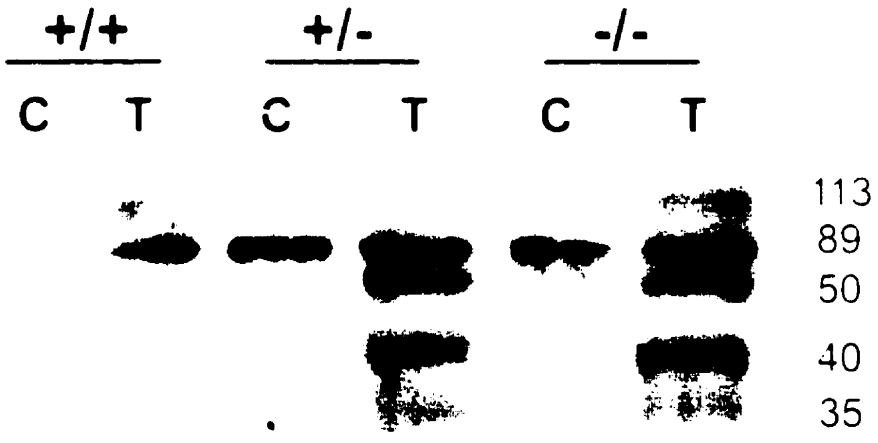
+/+
+/-
-/-
C
T
C
T
C
T
113
89
50
40
35

Fig. 6: Western blot analysis of immunoreactive PARP in the limbs of wild type, heterozygous, and p53 deficient embryos. Protein samples from limbs cultured for 24 hours were electrophoresed, blotted and incubated with PARP antibody. (C) Limbs cultured in control medium. (T) Limbs cultured in the presence of 3 µg/ml 4OOH-CPA. The size of the molecular weight markers in kD is indicated on the right of the figure.

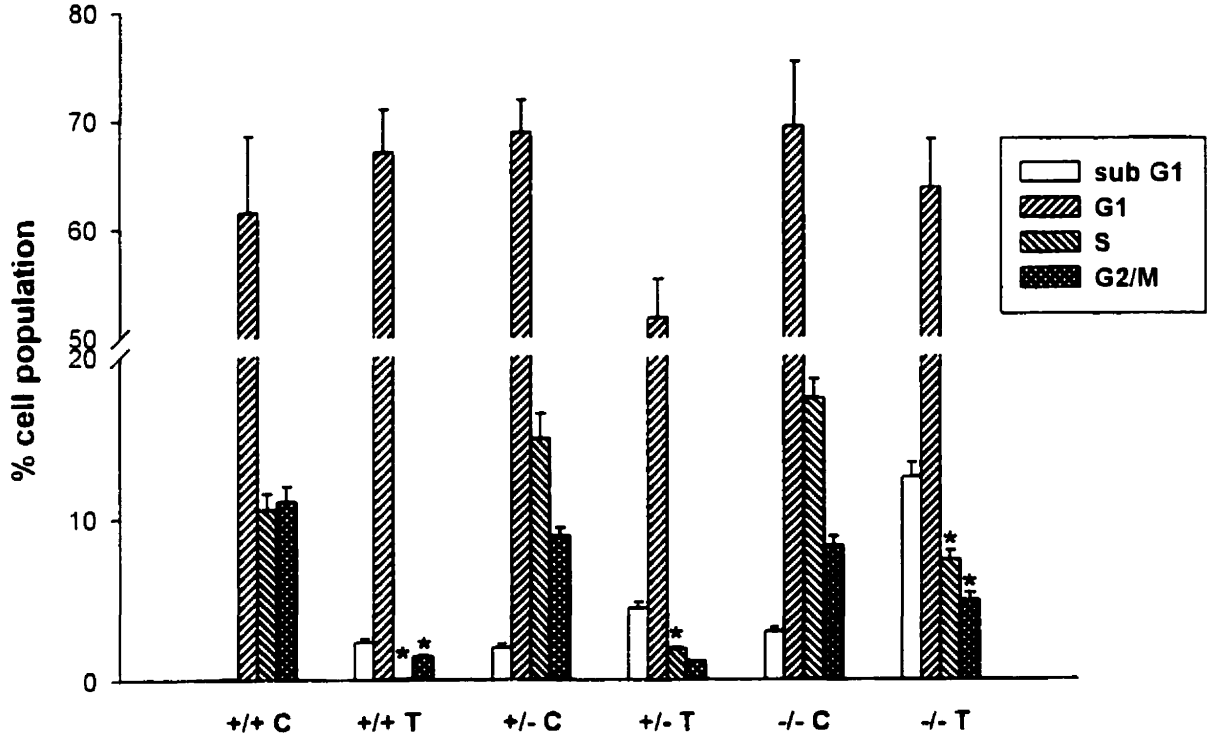
80
70
60
50
30
20
10
0
% cell population
sub G1
G1
S
G2/M
+/+ C
+/+ T
+/- C
+/- T
-/- C
-/- T

Fig. 7: Flow cytometric analysis of the effect of p53 genotype on cell cycle phase in limbs cultured for 24 hours in the presence of vehicle (C) or 3 µg/ml 4OOH-CPA (T). Bars are means ± standard errors of the means (n = 3). Compare bars depicted with * with their wild-type counterpart ($p<0.01$).

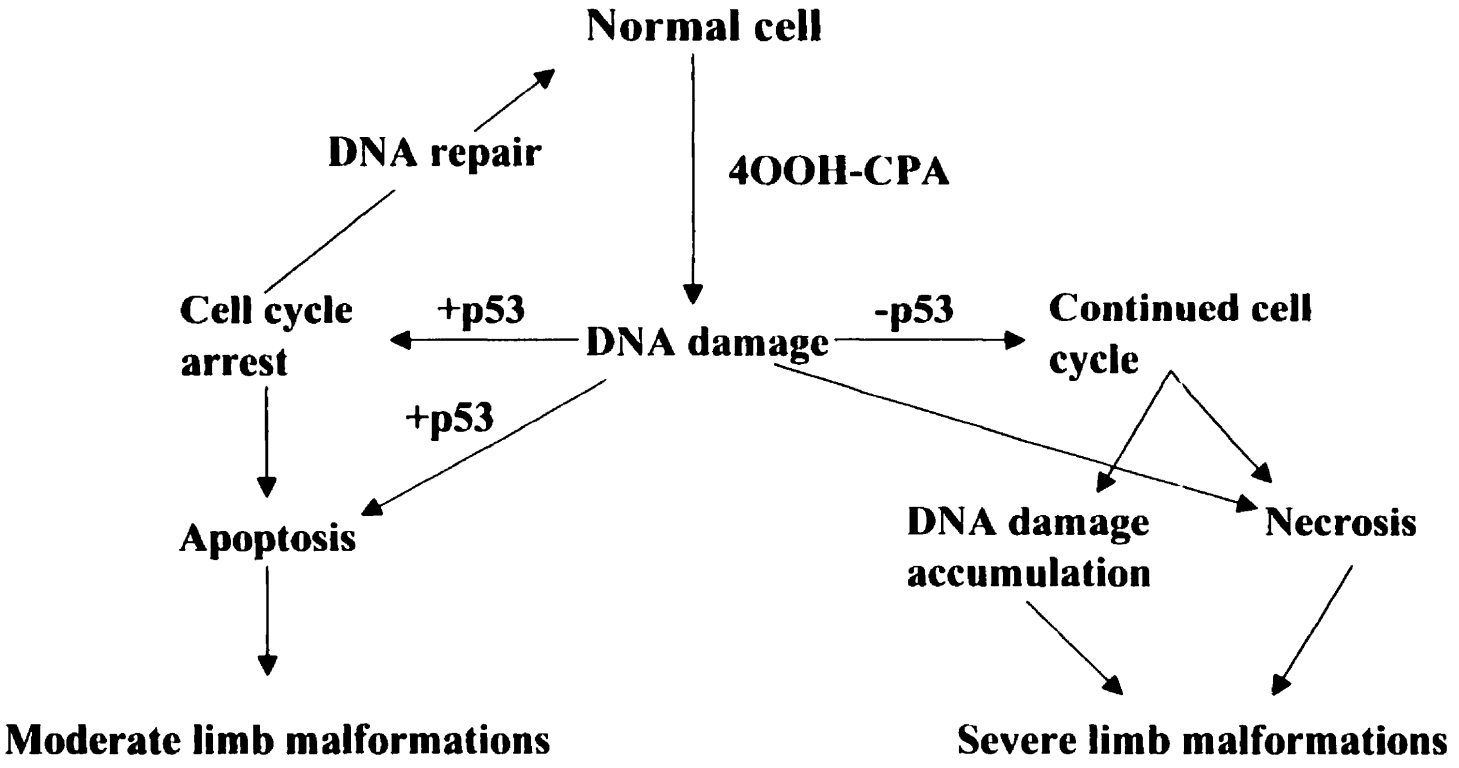
Normal cell
DNA repair
4OOH-CPA
Cell cycle arrest
+p53
DNA damage
-p53
Continued cell cycle
+p53
Apoptosis
DNA damage accumulation
Necrosis
Moderate limb malformations
Severe limb malformations

Fig. 8: Schematic diagram of the proposed pathways for limb development after teratogen treatment in the absence and presence of p53.

CHAPTER FIVE

DISCUSSION

In this dissertation I have studied apoptotic cell death during limb development, the effect of 4-hydroperoxycyclophosphamide (4OOH-CPA) on limb development and apoptosis, the expression patterns of three apoptosis marker molecules: cathepsin D, transglutaminase and clusterin, and the role of p53 gene in these processes. Although the results of all of the studies were discussed in their relevant chapters (Two, Three, and Four), in this chapter I will discuss briefly these results and present a model for p53 function in mediating apoptosis and the cellular events occurring in the absence of p53.

5.1 The induction of apoptosis and developmental malformations

In Chapter two and three of this thesis, I have demonstrated that 4OOH-CPA induces a concentration and time-dependent limb reduction malformations. Furthermore, 4OOH-CPA accelerates the induction of apoptosis, also in a concentration and time-dependent manner, specifically in regions where physiological apoptosis takes place. This close association between apoptosis and limb malformations suggests that the disruption in physiological apoptosis plays an important role in causing limb malformations. Indeed, this has been suspected by other investigators (Jiang and Kochhar, 1977; Alles and Sulik, 1989; Kurishita, 1989; Kochhar et al., 1993). It has been demonstrated that exposure of an embryo during organogenesis to a teratogen, such as retinoic acid or 5-azacytidine,

increases cell death in regions where programmed cell death normally occurs. Establishing such a cause-effect relationship between the induction of apoptosis and malformations has been a difficult task to achieve. Evidence to support this hypothesis can be found in some studies, including data from this dissertation (Chapter Two and Three), which have shown that any disruption in the limb apical ectodermal ridge (AER) area leads to severe limb malformations (Scott et al, 1977; Kochhar et al., 1993). This effect is thought to result as a consequence of the importance of AER-mesodermal interactions in normal limb development (Fallon and Kelly, 1977; Todt and Fallon, 1984). One reason for the difficulty in proving a cause-effect connection has been the unspecific action of most teratogens in inducing and/or accelerating apoptosis. This also applies to our study; although there is a close concentration and time-dependent association between the induction of apoptosis and limb malformations, this does not prove a cause-effect connection. Other studies have established similar associations. For example, Torchinsky et al. (1995) found a strong correlation between the rate of cyclophosphamide-induced apoptosis in different embryonic organs and the severity of anomalies in those organs. Additionally, probably apoptosis is more easily detectable than other teratological processes, which may create a bias in the interpretation of experimental results. If a well-established and specific induction of apoptosis takes place with a clear association with developmental malformations, and also if this apoptosis can be inhibited selectively, resulting in protection against malformations, the evidence for the existence of a cause-effect relationship would be strengthened significantly.

In this study (Chapter Two), DNA fragmentation was detected as a measure of the induction of physiological and drug-induced apoptosis. However, even though based on morphological criteria the type of cell death during normal limb development was found to be apoptotic, some investigator were not able to detect a DNA "ladder", the biochemical hallmark of apoptosis (Zakeri et al., 1990; Lockshin et al., 1991); this led them to suggest that programmed cell death during normal limb development is not classical apoptosis. More recently, results from a study by Mori et al. (1995) were in accordance with our data. They were able to detect the typical DNA fragmentation in the interdigital tissue of the fetal mouse limb. This discrepancy with Zakeri et al. (1990) and Lockshin et al.'s (1991) results may be explained based on differences in the tissues that were examined. Mori et al. (1995) isolated the interdigital tissue, then DNA was extracted and electrophoresed. This permitted an "enrichment" of the number of apoptotic cells in the sample, which may have enhanced the detection of any DNA ladder in the sample.

5.2 Three molecular markers of apoptosis and their role

In this thesis, we have demonstrated that cathepsin D, transglutaminase and clusterin proteins are induced in response to 4OOH-CPA treatment (Chapters Two and Three). These inductions showed a pattern of spatio-temporal specifity. All three proteins showed a relatively high specific localization in the limb with more in the interdigital area and less in the ectoderm. However, the time course of their induction differed. While the transglutaminase protein was induced after only 6

hours of 4OOH-CPA treatment, cathepsin D and clusterin were induced after 24 and 48 hours of 4OOH-CPA treatment, respectively. The durations of their inductions were different also. Transglutaminase induction lasted for only 24 hours, while cathepsin D and clusterin were still induced after 48 hours in culture. This might be significant in relation to their function during apoptosis. Transglutaminase is thought to play an important role in stabilizing apoptotic cells and helping them keep their cellular integrity (Fesus and Thomazy, 1988). Thus, this function might be needed starting at the early stage of apoptosis. However, the function of cathepsin D in lysosomes might be required at later stages to digest apoptotic bodies and help clear cellular debris (reviewed in Nixon and Cataldo, 1993). The role of clusterin is less certain. It may play a late role in the protection of cells from injury or stress (Little and Mirkes, 1995; Silkensen et al., 1994).

Also interesting were the findings of the subcellular localizations of cathepsin D and clusterin. For both markers, apoptotic bodies during the process of phagocytosis showed the highest immunoreactivity. This supports the role of cathepsin D as an enzyme involved in digesting apoptotic bodies. However, the basis of localization of clusterin is not well-understood, but it suggests for a role in later phases of apoptosis.

5.3 Role of the p53 gene in limb development and teratogenesis

Schmid et al. (1991) have shown that p53 was differentially regulated during embryogenesis, this observation suggests that p53 may have an important role in

the regulation of cellular proliferation and differentiation, and raises the possibility that p53 might be involved in controlling specific steps during embryogenesis. This finding that p53 is involved in embryogenesis is in a way contradictory to the finding that p53-knockout mice develop normally (Donehower et al., 1992), and that the incidence of developmental aberrations in such mice is rather low (Sah et al., 1995). Either p53 plays no direct role in normal development, and the low frequency of abnormality represents secondary events, or alternative pathways for p53 function exist during embryogenesis. However, Komarova et al. (1997) have shown that p53-responsive *lacZ* expression is poor under normal conditions in most embryonal and adult tissues, with the exception of the developing nervous system. Adriamycin or γ-radiation treatment stimulated induction of the transgene in several mouse organs (spleen, thymus, intestine) and in most cells of early, but not late embryos, correlating with the p53 mRNA expression, the degree of induction of p53 in these tissues, and the induction of apoptosis. Although it was reported that p53 is dispensable for growth, differentiation, and embryonic development (Donehower et al., 1992), subsequent studies have shown that ~16% of 13.5-day p53 -/- embryos, which were all females, displayed marked exencephaly, with an overgrowth of brain tissue (Sah et al., 1995). No reports of limb anomalies in the absence of p53 were presented. However, in our *in vitro* studies in this dissertation, 20% of p53 -/- limbs showed limb reduction malformations (Chapter Four). It is not known whether these limbs represent only female embryos in parallel to the *in vivo* defects in the CNS.

Treatment with 4OOH-CPA resulted in different phenotypic outcomes

depending on the genotype of the mice. The observation that there was an inverse relationship between the number of p53 gene copies and the severity of the 4OOH-CPA-induced malformations revealed a fundamental embryoprotective role for the p53 gene in drug-induced teratogenesis. Nicol et al. (1995) suggested a "teratological suppressor" function for p53, with benzo[a]pyrene as the teratogen. This function may be explained by the role of p53 in inducing cell cycle arrest in response to DNA damaging agents (Kastan et al., 1991; Nelson and Kastan, 1994). Based on this function of p53 as a checkpoint factor, it was proposed that DNA damage would induce transient G1 arrest in cells with wild-type, but not mutant p53, allowing time for damaged DNA to be repaired. Therefore, in response to DNA damage caused by 4OOH-CPA metabolites, cells in the (-/-)-limbs may not be capable of arresting their cell cycle (to start DNA repair) and/or inducing apoptosis (to remove cells with damaged DNA). Thus, DNA damage would accumulate in these cells and their daughter cells, leading to major malformations.

Comparing these results with other studies revealed a complicated picture relative to the role of p53 in the teratogenicity of benzo[a]pyrene (Nicole et al., 1995), 2-chloro-2'deoxyadenosine (Wubah et al., 1996) and irradiation (Norimura et al., 1996). Nicol et al. (1995) found that benzo[a]pyrene caused an increase in fetal resorption and other anomalies in p53 heterozygous and homozygous mice. However, two other anomalies, omphalocele and hemiangioendothelioma (red nevus of the skin) were conversely decreased in the heterozygous mice compared to the wild type ones; they suggested that these two anomalies might involve a cellular mechanism mediated by apoptosis. Norimura et al. (1996) found that

radiation caused an increase in embryonic malformations in p53-homozygous mice; however, wild-type embryos had higher incidence of death. This reciprocal relationship of radiosensitivity to anomalies and to embryonic or fetal lethality supports the notion that embryonic or fetal tissues have a p53-dependent "guardian" of the tissue that aborts cell bearing radiation-induced teratogenic DNA damage. The explanation for the results of the studies of Nicol et al. (1995) and Norimura (1996) is that in wild-type embryos, p53-dependent apoptosis suppresses DNA damage-induced malformation by removing teratogen-injured cells. However, they did not have a clear explanation for the higher rate of death in these embryos. In our study, we suggest that p53-dependent apoptosis in response to 4OOH-CPA-treatment removes injured cells that might have caused higher incidence of limb malformations. However, these "deleted" cells should be replenished probably by a "cell-replacement repair". If this repair process can not fully compensate for dead cells, limb malformations are expected. On the other hand, in p53-null limbs the above process does not take place. However, more destructive pathways were detected, i.e. failure of cell cycle arrest and/or induction of apoptosis. Both can lead to an increase in the severity of limb malformations.

I am proposing that in wild-type limbs p53 either arrests the cell cycle to allow for DNA repair, or in cases of severe DNA damage that is irreparable, engages the apoptosis program. In a way this is beneficial to the organism by eliminating cells with damaged DNA, which otherwise would lead to propagation of potentially deleterious mutations and damaged genome. However, since this elimination process occurs in cells that under normal conditions do not die, this cell death

lead to some abnormal limb development (as it was evident in Chapter Four). On other hand, in the absence of the protection role of p53 in p53-knockout limbs, neither cell cycle arrest nor apoptosis occurs in response to DNA damage caused by the drug treatment. It is well known that p53-null cells are prone to mutations and chromosomal abnormalities in normal conditions or after treatment with DNA damaging agents (Harvey et al., 1993; Lee et al., 1994). Severe limb malformation in the p53-knockout group can be attributed to the above two factors, i.e. failure of cell cycle arrest and loss of the ability to induce apoptosis; as both of these can lead to a major harm in the cellular genome which is spreading the DNA damage across next cellular generations. Furthermore, another contributing factor in this severe limb malformation is cell death by necrosis. An aspect that guarantees no repair or restoration would be possible at the cellular level to compensate for such genomic damage.

In summary, it can be postulated that loss of p53 may either impair normal developmental processes directly or act indirectly by preventing the exclusion of genetically abnormal cells from the developing embryo. This is supported by our observation that p53 is necessary for suppressing teratogenesis as a consequence of 4OOH-CPA-induced DNA damage. This postulate is also favoured by the study that showed that induction of apoptosis in teratocarcinoma cells (resembling cells of the early embryo) in response to DNA damage entailed the activation of latent p53 (Lutzker and Levine, 1996).

5.4 Role of p53 gene in cell cycle arrest and apoptosis

We have shown that the presence of the p53 gene is necessary for the induction of both cell cycle arrest and apoptosis in response to 4OOH-CPA treatment (Chapter Four). p53 is most likely important in determining the fine balance between growth, differentiation and cell death that are fundamental for the normal life of each organism. It is not clear, however, how the cell interprets the signalling of p53 towards a specific pathway. Perhaps there are different signalling pathways through which p53 exerts its 'orders' to the cell. Numerous studies have clearly established that growth arrest and apoptosis are independent functions of p53 (Rowan et al., 1996). Whereas certain oncogenic disruptions, availability of survival or growth factors, and cell type are known to determine whether p53 causes growth arrest or apoptosis, the amount of DNA damage and levels of p53 induced now also appear to contribute in determining the cellular effect (Gottlieb et al., 1996; Lassus, et al., 1996; Ronen, et al., 1996). p53 has been proposed to be involved in maintaining the stability of the genome (Livingstone et al., 1992; Yin et al., 1992), and both cell cycle arrest and apoptosis can be considered mechanisms by which this may be accomplished. In the presence of DNA damage, cells will either arrest, presumably to allow DNA repair, or undergo cell death, in a p53-dependent manner. Thus, in either case, the propagation of potentially deleterious mutations can be averted.

Our flow cytometric analysis revealed that the percentage of cells in the S phase was higher in the non-treated (-/-) limbs than in the non-treated wild-type limbs. This has been supported by other studies (Harvey et al., 1993), but it is

unclear why this phenomena occurs and how significant it is. The observed cell cycle arrest in wild-type limbs in response to 4OOH-CPA is induced by DNA damage and is mainly mediated by transactivation-dependent mechanisms. The induction of wild-type p53 protein levels in response to DNA damage is thought to be regulated by a posttranscriptional mechanism, with metabolic stabilization of the p53 protein being, in part, an important feature of this process (Kastan et al., 1991; Tishler et al., 1993). DNA strand breaks, known to be caused by cyclophosphamide (Hengstler et al., 1997), may be the primary signal leading to the induction of p53 protein levels following DNA damage (Nelson and Kastan, 1994; Zhan et al., 1994). These studies suggested that double-strand breaks and single-strand gaps of greater than 30 nucleotides are sufficient to induce a p53-dependent G1 arrest and, furthermore, that as few as one double-strand break may be adequate for p53 induction.

Elucidation of the mechanism by which p53 mediates a G1 cell cycle arrest was facilitated by the identification of several growth arrest-related gene products that are transcriptionally activated by p53. The induction of p53 in response to DNA damage activates the expression of several genes, including *gadd45* (*g*rowth *a*rrest and *D*NA *d*amage-inducible), p21 (WAF-1, cip1) and mdm2 (Kastan et al. 1992; Barak et al., 1993; El-Diery et al., 1993). It seems that the G1 cdk (cyclin-dependent kinase) inhibitor p21 is the main mediator of p53 cell cycle arrest. Thus far, p21 is the only cdk inhibitor known to be regulated by p53 in response to DNA damage. p21-mediated inhibition of cyclin E- and cyclin D-associated cdk2 complexes prevents the phosphorylation of a number of protein targets that are

required for the timely progression of cells into the next phase of the cell cycle (Matsushime et al., 1992; Nevins, 1992).

We have found an increase in p53-dependent apoptosis in response to DNA damage in treated limbs (Chapter Four). To date, the cell death-promoting gene, Bax, appears to be the only member of a family of apoptotic genes that is upregulated by p53 in response to DNA damage (Miyashita et al., 1994; Selvakumaran et al., 1994). Bax contains regions of homology to the antiapoptotic gene, bcl-2; bcl-2 enhances cell survival, whereas Bax accelerates apoptosis. The two proteins can form heterodimers with each other in which the ratio of bcl-2 to Bax determines cell survival or death following an apoptotic stimulus, suggesting that this family of proteins play a crucial role in the regulation of the apoptotic process (Oltvia et al., 1993). It is unknown, however, whether Bax specifically plays a role in p53-mediated apoptosis. The regulation of p53-mediated apoptosis is undoubtedly complex, and the relative contributions of apoptosis-related gene products may be tissue or cell type specific.

5.5 Apoptosis vs necrosis

In exploring the mechanism by which p53 "suppressed" the response of the organogenesis-stage limb to insult, we found that the type of cell death observed in the limbs exposed to 4OOH-CPA switched from apoptosis to necrosis in the absence of p53. Initially apoptosis and necrosis were considered as morphologically and biochemically different modes of cell death. However, there

is increasing evidence that the two types of cell demise can occur simultaneously in tissues exposed to the same stimulus, and often the intensity of the stimulus decides the prevalence of either apoptosis or necrosis. Thus, both types of cell death may be triggered by the same signals and share early events. Many signals as diverse as heat shock (Schrek et al., 1980), viruses (Subramanian et al., 1995), protein synthesis inhibitors (Lennon et al., 1991), oxidative stress (Dypbukt et al., 1994), hypoxia (Shimizu et al., 1996a), Ca^{2+} ionophores, radiation (Fukuda et al., 1993), glutamate (Ankarcrona et al., 1995), and nitric oxide (Bonfoco et al., 1995) can induce both apoptosis and necrosis (Leist and Nicotera, 1997). Second messengers and downstream steps such as ATP (Leist et al., 1997) and bcl-2 (Shimizu et al., 1996b) have been shown to be involved in both apoptosis and necrosis.

If indeed common events are shared by apoptosis and necrosis, what is the significance of these different modes of cell death? In their most typical appearance apoptosis and necrosis have little similarity and are clearly distinguishable mechanistically and morphologically (Searle et al., 1982). However, it can be argued that the death program may not be uniformly progressing in all conditions. If left undisturbed, the death program would predominantly yield an apoptotic-like morphology, whereas when elements of the program are disturbed or inhibited (such as in the p53 knockout mice as outlined in Chapter Four), or when the insult is so high that some of the subroutines cannot be terminated, then the shape of death can change. According to this view, apoptosis and necrosis may be seen as extremes of a continuum of possible shapes of cell death, and necrosis may be the

result of an aborted apoptosis (Leist et al., 1997). In agreement with this suggestion, it has been shown that with increasing intensity (exposure time or concentration) of the insult, cell death changes its shape from apoptotic to necrotic. In the case of glutamate, nitric oxide, reactive oxygen species, and many other toxins, this overstimulation leads to recruitment of additional lethal reactions, which cause cell lysis prior to the completion of the "default" apoptotic program (Dypbukt et al., 1994; Ankarcrona et al., 1995; Bonfoco et al., 1995). Notably, it has been observed that intracellular energy levels are rapidly dissipated in necrosis, but not in apoptosis (Dypbukt et al., 1994; Ankarcrona et al., 1995). These results suggest that while initial events may be common to both types of cell death, certain metabolic conditions would be required to activate downstream controllers, which direct cells towards the organized execution of apoptosis.

Since most of the above studies have been performed in cell culture, the question arises as to whether the viewpoint of apoptosis and necrosis as extremes of a continuum of multiple forms of death is supported by *in vivo* observations. In fact, it seems that in many pathological situations apoptosis and necrosis coexist. An example for this is ischemic brain damage (Arenas and Persson, 1994; Li et al., 1995; Charriaut-Marlangue et al., 1996). In the core of ischemic regions, necrotic cell death is prevalent. Towards the border regions, where energy-depletion and excitotoxic stimulation are less severe and prolonged, apoptotic neuronal death is commonly observed. The question remains open as to whether the sequential activation of apoptosis and necrosis is based on similar or different mechanisms of cell death. *In vivo* evidence suggests that cell death, either by necrosis or by

apoptosis, may be the manifestation of a completely different execution of an initially similar program (Lacronique et al., 1996; Rouquet et al., 1996; Kunstle et al., 1997).

In summary, as has been stated that the amount of stress or insult (i. e. dose and time of exposure) can affect the type of cell death (Lennon et al., 1991; Dypbukt et al., 1994; Sato et al., 1995). Boyle and Hickman (1997) recently suggested that the threshold for drug-induced apoptosis is determined not only by the extent of cytotoxic damage but also by the trophic environment and the ability of a cell to modulate survival signals that attenuate toxicity. The p53 protein may serve to "modulate" the response of the limbs to insult. The absence of p53 may increase the impact of an insult on cell function in the limbs of the p53 deficient animals. The presence of lower amounts of p53 in the heterozygous mice (gene dosage) resulted in an intermediate response, suggesting that an insult may "overwhelm" the p53 defensive mechanisms to arrest the cell cycle and/or induce apoptosis.

5.6 Conclusions and Overview

In this study many mechanistic aspects of the effect of 4OOH-CPA on limb development and apoptosis have been revealed. I have started this thesis by characterizing the effect and time course of two concentrations of a DNA damaging agent, 4OOH-CPA on limb development. The next question was what role apoptosis plays in this process, if any. I found that apoptosis was induced by drug treatment and specific patterns of timing, localization and regulation (by means of three proteins) were revealed. The next question was whether this induction of

apoptosis was the cause of limb malformation inflicted by 4OOH-CPA, or it was just an associated event. One approach to address this question was to do intervention studies by inhibiting the induction of apoptosis and examining whether this prevents limb malformations. We picked the p53 gene as our next target for two reasons. First, it was known at the time that apoptosis induced by DNA damaging agents is p53-dependent (Clarke et al., 1993; Lowe et al., 1993). Thus, I hoped, by using a p53-knockout transgenic mice, that it would be possible to prevent the occurrence of limb malformations. Second, the role of p53 in protecting the genome against DNA damage was intriguing. Since the molecular target of our model drug is DNA and it was known to cause DNA damage as its main mechanism of action. I wanted to investigate the p53 role in 4OOH-CPA-induced limb malformations and apoptosis. To my surprise, p53-homozygous mice were more sensitive to drug-induced limb malformations, and not more resistant. More investigation led us to unveil a "protector" role for p53 in limb development against teratogen insults.

Thus, after all, it is still not clear whether induction of cell death is the obligatory cause of limb malformations, since our approach of p53 knockout generated more complex outcome than we anticipated and could not answer that question. Another approach, is to use other inhibitors of apoptosis. The ideal inhibitor should be relatively non-toxic during development and inhibit apoptosis as "cleanly" and specifically as possible, i.e. inhibit the occurrence of drug-induced apoptosis with no other side effects such as interfering with other cellular processes and/or causing developmental malformations. The best candidates are probably inhibitors of caspases (Hara et al., 1996). Caspases are protease proteins that

have been shown to play a relatively universal role in the execution phase of apoptosis (reviewed in Cohen, 1997 and Nicholson and Thornberry, 1997). Indeed, preliminary data showed that inhibitors of caspase-1 prevented physiological apoptosis during limb development (Milligan et al., 1995; Jacobson et al., 1996). This avenue can be followed for a more thorough investigation, particularly assessing their role in drug-induced apoptosis.

Together, this study could be considered a forward step in understanding an important mechanism in drug teratogenesis and can be utilized for further studies in this field.

ORIGINAL CONTRIBUTIONS

My great respect and courtesy to the third millennium author(s) of

"Prevention, Treatment and Cure of Birth Defects"

I hope data from this thesis will

be cited in that publication

1. The time-course and concentration-response of the effects of 4-hydroperoxy-cyclophosphamide on the limbs of murine embryos were characterized. Increasing either the time of exposure or the drug concentration leads to an increase in the severity of limb reduction malformations.

2. The time-course and concentration-response of the effects of 4-hydroperoxy-cyclophosphamide on the induction of apoptosis during limb development were determined. Only 12 hours of exposure to 1 μg/ml of 4OOH-CPA was sufficient to trigger apoptosis as detected by DNA gel analysis.

3. Apoptotic cells were localized in specific areas of limb during normal development or after drug treatment. Drug treatment at low concentration (1 μg/ml) accelerated the death of cells destined to die in the interdigital area.

4. The inductions of cathepsin D, transglutaminase and clusterin proteins were investigated and their time-course, quantification and cellular and subcellular localization determined in normal or drug-induced apoptosis. A specific pattern of induction was found for these proteins in the developing limb. This suggested that these proteins play an important role during normal and drug-induced apoptosis.

5. The role of p53 in protecting developing limbs against 4-hydroperoxy-cyclophosphamide was revealed. A "gene-dose" effect was also detected,

i.e. homozygous limbs were more sensitive to drug treatment than the heterozygous limbs.

6. The dependency of the drug-induced apoptosis on the presence of the p53 gene in limb development discovered. Homozygous limbs were resistant to the induction of apoptosis by 4-hydroperoxycyclophosphamide.

7. The type of cell death switched from apoptosis to necrosis in p53-homozygous limbs treated with 4-hydroperoxycyclophosphamide. This was evident by morphological examination and also the pattern of fragmentation of the poly(ADP-ribose) polymerase protein. This might have contributed to the increased intensity of limb malformations in these limbs.

8. Loss of p53 rendered limb cells unable to arrest their cell cycle in response to the DNA damaging agent 4-hydroperoxycyclophosphamide. Again, this potentially leads to a higher rate of limb malformations.

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