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Mechanisms of Cell Death in the Developing Mouse Limb

by
Harleen Singh Ahuja

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

Mechanisms of Cell Death in the Developing Mouse Limb

by

Harleen Singh Ahuja

Adviser: Dr. Zahra Zakeri

Cell death is an important biological process that selectively eliminates cells in embryonic and adult tissues. The mechanisms underlying this selective elimination are unknown. The aim of this work has been to use several approaches to study cell death in the developing mouse limb. We have both examined the expression of cell death implicated genes and established the nature of cell death during limb development. Using a line of mutant mice which displays a limb deformity, we show that this defect is the result of a deformity in the pattern of cell death. To address the regulation of cell death, we performed a comparative study of normal and mutant limbs by analyzing the expression profile of genes associated with cell death, limb patterning, and cell cycle. These genes have been directly or indirectly implicated in cell death. We show that clusterin is not universally associated with cell death. We did not find any alteration in the pattern of expression of bcl-2 (b cell lymphoma) or Msx (muscle segment) genes in normal versus mutant limbs. Tissue transglutaminase and cyclin dependent kinase 5 (Cdk5) were associated specifically with dying cells of

the developing limb. Finally, we have shown that we can selectively increase cell death in the limb by exposure of embryos to retinoic acid (RA). We illustrate that the nature of RA cell killing is similar to normal cell death and that the genetically altered pattern of cell death in the Hm limb can be overcome by treatment of the embryo with RA at day 14 of gestation. To determine the mechanism of this selective cell killing by RA, we examined the expression of the retinoic acid receptor ß and a transgene containing a retinoic acid response element. The regulation of RA action seems to be independent of receptor expression and endogenous RA availability. We conclude that cell death is an important aspect of limb development and that it is dependent on a variety of signaling molecules that initiate the process, such as RA, and a number of factors such as Cdk5 and tissue transglutaminase that mediate it.

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Chapter 1. The Concept of Cell Death

A. Normal incidence of cell death

B. Function of cell death

C. Cell death and disease

D. Types and morphology of cell death

E. Genetic regulation

The Concept of Cell Death

Cell death is a fundamental aspect of embryonic differentiation, morphogenesis, teratogenesis, and aging of the central nervous system. As an integral part of normal embryonic growth and differentiation, it determines the sculpting of structures in both vertebrates and invertebrates. For example, in the mammalian embryonic central nervous system, over one third of the newly formed neurons die. Alteration of the pattern of cell death by genetic anomalies or environmental factors results in developmental abnormalities. Significant cell loss during aging and pathology derives from a controllable form of physiological cell death. Thus an understanding of the mechanism of cell death has considerable value.

A. Normal incidence of cell death

Under physiological conditions cell death is involved in regulating the size of tissues in adult life and in the programmed elimination of cells that accompanies embryonic and fetal development (Walker et al., '88; Raff, '92; Cohen, '93; Coucouvanis et al., '95).

In normal adult tissues, cell turnover is a consistent feature of rapidly proliferating mammalian cell populations, exemplified by gut crypts as well as spermatogonia in the basal compartment of seminiferous tubules (Potten, '77; Allan, '87; Coucouvanis et al., 93), and slowly proliferating mammalian cell populations where cell death must quantitatively balance mitosis over a period of time and a

steady state balance is achieved (Wyllie, '80; Berges and Isaacs, '93). Cell death plays an active role in the removal of epidermal cells from the skin (Young, '87; Danno and Horio, '82; Vaux, '93). Normal involution also occurs as a result of physiological fall in trophic hormonal stimulation in human premenstrual endometrium (Hopwood, '76; Otsuki et al., '94). Cellular immune reactions selectively eliminate cells infected by certain viruses (Zingernagel and Doherty, '75; Debbas and White, '93; Vaux et al., '94; Shen and Shenk, '95), thus helping to eradicate the infection as well as selectively deleting transformed cells.

Cell death is as much a part of normal development as proliferation or differentiation (Glücksmann, '51; Saunders, '66; Hinchliffe, '81; Coucouvanis et al, '95). For example, neuronal, muscle, epithelial, intestinal and gonadal cells undergo cell death in the developing invertebrate nematode, *C. elegans* (Sulston and Horvitz, '77; Sternberg and Horvitz, '81; '82; Horvitz et al., '82; Sulston et al., '83; Ellis and Horvitz, '86; Hengartner and Horvitz, '94). Muscle cell death (Lockshin, '81; Schwartz and Truman, '82; Haas et al., '95) and neuronal cell death (Truman, '84; Fahrbach et al., '94) has been detected in moths as well. Cell death in mammalian embryogenesis occurs as early as inner cell mass differentiation (El-Shershaby and Hinchliffe, '74; Copp, '78; Pierce et al., '89; Hardy et al., '89). It has also been shown to be involved in the differentiation of the gut mucosa (Harmon,'84; Pipan, '79; 86; Williams and Bell., '91), the retina (Young, '84; Penfold, '86), and in the immune system (Duvall and Wyllie, '86; Williams, '94). Secondary palate formation occurs by the fusion of two opposing palatal shelves in which redundant epithelium undergoes

degeneration and death (Shapiro and Sweeny, '69; Chaudhry and Shah, '73; Pratt et al., '75; Kerr, '87; Mori et al., '94). The developing nervous system provides an excellent example of cell death involving architectural organization by establishing a definitive pattern of neuronal connections and major axonal pathways (Hamburger and Oppenheim, '82; Hurle, '88; Oppenheim '91; Lo et al., '95). In addition, the involvement of cell death in the remodelling of tissues and organs during development is best exemplified by removal of interdigital webs during limb development (Hinchliffe, '80; '81; Hinchliffe and Gumpel-Pinot, '81; Kerr, '87; Zakeri et al., '94; Zakeri and Ahuja, '94; Coucouvanis et al., '95).

B. Function of cell death

From a functional or evolutionary standpoint, dying cells have been divided into several categories (Glucksmann et., '51; Ellis et al., '91; Coucouvanis et al., '95): some cells may have served some evolutionary purpose but provide no current function and undergo a phylogenetic cell death (Fallon and Simandl, '78). Morphogenetic cell death may modify the tissue to permit differentiation of its final form (Glucksmann et al., '51; Hinchcliffe et al., '81). Histogenic cell death can modify a tissue so that it is able to function or to function differently from a similar tissue type (Glucksmann et al., '51). For example, three different patterns of cell death occur in the somites of the developing embryo. These variations are dependent on the stage of the embryo and on the path that migrating neural crest cells will take (Jeffs and Osmond, '92; Coucouvanis et al., '95). Cell death may also occur in one sex as a

means of differentiating sexually dimorphic traits. For example, sexual differentiation in vertebrates involves hormonal control of cell death of reproductive structures such as the Mullerian and Wolffian ducts (Scheib, '63). In developing neurons, cell number may exceed the amount required and therefore some can be removed (Hamburger and Levi-Montalcini, '49, Cowan et al., '84; O'Leary, '87). Cells may be necessary at one stage of development but are no longer required such as the tadpole tail during metamorphosis (Kerr, '74; Lockshin, '81). Some cells may be defective in shape or function such as lymphocytes that have failed to produce functional antigen specific receptors (Cohen, '91; Golstein et al, '91). Other cells may yield an autoimmune attack on the organism such as thymocytes that carry self-recognizing T-cell receptors (Cohen, '91; Golstein et al., '91; McCarthy et al., '92; Osborne, '95). Therefore, cell death accounts for the deletion of cells that takes place in normal tissues. Where it occurs pathologically, it may serve an adaptive or homeostatic role (Walker et al., '88).

C. Cell death and disease

Organismic homeostasis is provided by a balance between the processes of cell proliferation and cell death (Thompson, '95). The importance of the correct spatial and temporal incidence of cell death is demonstrated by the onset of a variety of diseases when the occurrence of cell death is inappropriately activated or inhibited. If normal occurring cell death is repressed, this may result in cancer, autoimmune disorders, and viral infections (Kerr et al., '94; Singh and Anand, '95). The tumor

suppressor gene p53, reported to inhibit cell proliferation and mediate apoptosis, has been shown to be mutated or deleted in many human malignancies (Levine et al., '91: Lowe et al., '93; Singh and Anand, '95). Interestingly, the bcl-2 (B cell lymphoma) oncogene induces cell transformation by enhancing cell survival rather than cell proliferation (Hockenberry et al., '90; Gregory et al., '91). In reference to autoimmune disease, the surface protein Apo-1 or Fas antigen has been shown to be an integral player in deletion of self reactive T cells (Itoh et al., '91; Wantanabe-Fukunaga et al., '92; Mountz et al., '94). Recognition of this cell surface antigen by a monoclonal antibody initiates apoptosis in several cell lines (Trauth et al., '89; Watanabe-Fukunaga et al., '92). The mouse mutants lpr (lymphoproliferation) and gld (generalized lymphoproliferative disease) result from loss-of-functions of Fas and Fas ligand, respectively (Cohen and Eisenberg, '91). These disorders may be the mouse counterparts of the human systemic lupus erythematosus (Cohen and Eisenberg, '91; Cheng et al., '94). In reference to viral infections, a defective cell death mechanism can allow virally infected cells to multiply and spread the infection (Thompson, '95). On the other hand, if the death program is prematurely or abnormally activated, this may result in acquired immunodeficiency (AIDS; Ameisen et al., '95), degenerative disorders of the central nervous system (Lo et al., '95), or ischemic stroke (Raff et al., '03; Martinou et al., '94). In AIDS, depletion of CD4 T cells has been associated with induction of the cell death program in both HIV infected and uninfected cells (Groux et al., '92; Singh and Anand, '95). This is thought to result from the activation of the complex formed by CD4 antigen and HIV-1 gp120

complex (Banda et al., '92). In the neurodegenerative disease Alzheimer's, accumulation of the ß-amyloid protein has been implicated in abnormal cell death of neurons (Mattson et al., '92; Cotman et al., '94). Every disease state that has been associated with cell death has provided direct or indirect evidence that key mediators are involved and demonstrate that the genetic control of cell death is as crucial as cell proliferation for normal existence.

D. Types and morphology of cell death:

Initial characterization of the morphology of cell death described two distinct patterns, necrosis and apoptosis (Kerr, '69; Kerr et al., '72; Searle, '75; Wyllie, '87; '93). In necrosis, there is marked swelling of mitochondria followed by cell lysis. In contrast, apoptosis (originally termed shrinkage necrosis -Kerr, '65; '71) is characterized by rapid condensation of the cytoplasm and nuclear chromatin as well as blebbing of the cell surfaces. These subsequently "fall off" or apoptose as membrane bounded bodies (Kerr, '72). The morphology and incidence of cell death has been further distinguished into three different types (Schweichel and Merker '73; Clarke, '90; Zakeri et al., '95). Type I or classical apoptotic cell death is defined by a specific sequence of condensation and breakdown of cell-cell contact, DNA fragmentation, phagocytosis, and secondary lysosomal degradation of fragments by neighboring cells. This type is seen, for instance, during regression of the rat ventral prostate after castration (Kerr and Searle, '73; Colombel and Buttyan, '95) and in the interdigital zones of the developing mouse limb (Hinchliffe, '80; Zakeri et al., '94; Zakeri and

Ahuja, '94; Zakeri et al., '95). In both model systems, margination and condensation of the nucleus is a prominent event with endonuclease cleavage of the DNA into nucleosomal fragments. Destruction of the cytoplasm is carried out by cell fragmentation. Type II or lysosomal cell death is recognized by primary formation of lysosomes and late condensation and fragmentation, as exemplified by the rat mammary gland and the *Manduca* labial gland (Halaby et al., '94; Zakeri et al., '93; '95). The labial gland provides a synchronous system in which most of the cells die, and the cytoplasm is ingested by the lysosomal system prior to nuclear breakdown. Type III or necrotic cell death has been characterized by disintegration of the cell (without an active role by the lysosomal system or neighboring cells) into fragments that are not subsequently detectable (Schweichel and Merker '73; Clarke, '90; Zakeri et al., '95). In necrosis, degradation of DNA occurs via nucleases as well as lysosomal proteases (after disintegration of the cellular membranes (Kyprianou and Isaacs, '88; Berges and Isaacs, '93). In such instance, cells experience extreme conditions of temperature, pH, or toxic concentrations of a variety of agents. In every case of necrosis there is clear damage to the plasma membrane (Wyllie, '87). Recent work in Monica Driscoll's laboratory ('94) has shown that mutations in particular mechanosensory genes in *C. elegans* that are required for touch sensitivity result in great swelling and death of neurons. This death is distinctly different from apoptotic cell death. As there is disruption of membrane integrity, along with swelling it is thought that these neurons may be undergoing a necrotic death (Lints and Driscoll, '96). These results are interesting in that they suggest that necrotic cell death may be under genetic control.

Whereas necrotic cell death is a degenerative phenomenon that follows irreversible injury (Trump, '84; Farber et al., '90), apoptotic cell death has features that suggest self-destruction rather that degeneration. Evidence for this is seen by an increase in specific RNA synthesis prior to apoptosis in certain cell types (Wadewitz and Lockshin, '88; Buttyan et al., '89; Schwartz et al.,'90; Tenniswood et al., '92; Wong et al.,'93). In addition, inhibitors of protein synthesis have been shown to suppress the occurrence of apoptosis of thymocytes and chronic lymphocytic leukemia cells treated with glucocorticoids (Galili et al., '82; Cohen and Duke, '84; Wyllie, '84). In other cell types, inhibitors of macromolecular synthesis do not inhibit cell death suggesting that the cell death effectors are already present (Edwards et al., '91; Sellens and Cohen, '91; Dutz et al., '92; Vaux, '93). Furthermore, apoptosis is biochemically defined by dependence on energy and double-stranded cleavage of nuclear DNA at the linker regions between nucleosomes. This cleavage produces oligonucleosome fragments (Yamada et al., '81; Duke et al., '83; Cohen and Duke, '84; Cohen et al., '85; Wyllie, '80; '85). An enzyme considered to be a likely candidate for causing this cleavage of DNA is dependent on Ca^{2+} and Mg^{2+} (Wyllie, '80), is active in neutral pH conditions (Duke et al., '83; Wyllie , '87) and is strongly inhibited by zinc (Cohen et al., '85; Duvall and Wyllie, '86).

It is important to note that certain cells may undergo a type of cell death that has not been defined as yet or follow a pattern that has combined features of the three morphological deaths described here (Zakeri et al., '95). In summary, physiological cell death is described in several overlapping terms. "Programmed cell

death" (PCD) is characterized by a specific and predictable sequence of developmental or hormonal stimuli that initiate a cascade of events in the targeted cells (Lockshin and Zakeri,'92). PCD is best exemplified in developmental systems and is most clearly seen in embryos and metamorphosing animals (types 1 and 2 cell death, respectively). The term PCD infers genetic control in that specific cell death genes have been isolated (Ellis et al.,'91; Shi et al., '92; Yuan et al., '93) and the upregulation of several genes has been reported in dying cells (Lockshin and Zakeri, '90; '91; Osborne and Schwartz, '94).

E. Genetic Regulation

The genetic analysis of cell death has been best studied in *C. elegans* where several genes have been isolated that, when mutated, cause alteration in the pattern of cell death (Ellis et al., '91). In these transparent animals, cell death is easily observed. Of the 1090 somatic cells formed during its development, 131 cells undergo a precisely controlled programmed death (Ellis et al., '91). The nematode has provided an excellent model for the study of genes that function in various aspects of cell death. Two genes have been identified as crucial players, ced-3 and ced-4 (cell death abnormal). Mutant animals carrying these genes retain cells that would normally die (Ellis and Horvitz, '86). These genes are negatively regulated by the ced-9 gene (Hengartner et al., '92).

The vertebrate homologs of these genes have also been identified. ced-3 is homologous to interleukin 1ß converting enzyme or ICE (Yuan et al., '93; Patel et

al., '96). ICE is a cysteine protease that cleaves pro-interleukin-1ß into active interleukin 1ß (Thornberry et al., '92; Cerretti et al., '92). It is a member of a growing family of cysteine proteases including CPP32, Nedd-2, Ich-2, Mch-2, and $ICE_{rel}III$ (Wang et al., '94; Fernandez-Alnemri et al., '94; Nicholson et al., '95; Kumar, '95). Several studies involving the overexpression and inhibition of ICE have demonstrated the importance of this protease in cell death (Wang et al., '94; Miura et al., '93; '95; Tewari et al., '95). ced-9 has sequence homology to the mammalian bcl-2 gene (described below, Hengartner et al., 94; Vaux et al., '92).

Some genes are upregulated during cell death include heat-shock protein 70 (Buttyan et al., '88), α prothymosine (Berges and Isaacs, '93), testosterone repressed message-2 (Montpetit et al., '86), cathepsin B (Narvaez et al., '96), transforming growth factor-$ß_1$ (Kyprianou and Isaacs, '89; Selvakumaran et al., '94), tissue transglutaminase (Piacentini et al., '91), ubiquitin (Schwartz et al., '90) and calmodulin (Dowd et al., '91) all of which have been implicated as cell death markers. Other genes have been shown to specifically induce cell death. For example, the adenovirus E1A protein induces p53 mediated apoptosis (Hashimoto et al., '91; Groux et al., '92; Rao et al., '92). The binding of tumor necrosis factor-α to its receptor also causes apoptosis (Itoh et al., '91; Wright et al., '92; Singh and Anand, '95). As mentioned previously the binding of Fas ligand to its surface antigen Fas allows cytotoxic T lymphocytes and natural killer cells to destroy their target cells (Rouviere et al., '93).

The expression of several immediate early genes including c-fos, c-jun, and c-myc, as well as the tumor suppressor p53 has been directly associated with the

activation of cell death (Buttyan et al., '88; Evan et al., '92; Ferguson, '93; Schlingensiepen et al., '94; Packham and Cleveland, '95). Usually involved in cell division, the proto-oncogene c-myc induces apoptosis of fibroblasts in the absence of growth factors (Askew et al., '91; Shi et al., '92). The importance of p53 in apoptosis was shown in studies where mutated p53 prevented the onset of cell death (Levine et al., '91; Lowe et al., '93; Clark et al., '93). The fact that these genes also play important roles in the cycling of cells has opened a new area of investigation in the role of the cell cycle genes during cell death. Therefore, cell death is a result of a complex interaction of direct and indirect effectors as well as suppressors.

For our studies, we have focused on specific genes that have been reported as cell death markers, cell death inhibitors, and cell cycle genes that have been implicated in cell death. These are discussed in more detail below:

Clusterin:

Testosterone Repressed Prostate Message-2 (TRPM-2) was originally identified in dying cells of the rat ventral prostate upon castration (Montpetit, '86; Léger et al., '87; Wong et al., '93). It has been cloned and characterized from several other species as well and is now referred to as clusterin (see Jenne and Tschopp, '92 for review). The mature protein is a secreted glycoprotein that is a product of its cleaved 70 kDa precursor (Collard and Griswold, '87). Its upregulation during prostatic involution has associated clusterin with the process of active cell death (Léger et al. '87; Betuzzi et al. '89; Buttyan et al. '89; Kyprianou and Isaacs '89;

Rosenberg and Silkensen et al., '95). The protein has also been characterized from human serum as apolipoprotein J (de Silva et al. '90), serum protein 40, 40 (SP-40,40, Kirszbaum et al. '89), and complement lysis inhibitor (CLI, Jenne and Tschopp, '89). In relation to cell death, clusterin has been reported to be abundantly expressed in various tissues undergoing cell death including the developing limb (Buttyan et al., '89), regressing mammary gland after weaning (Guenette et al., '94), and in the kidney following ureteral obstruction. These results suggest that clusterin may specifically be involved with cellular death.

Transglutaminase:

Transglutaminases are Ca^{2+}-dependent enzymes that catalyze the crosslinking of polypeptide chains by the formation of insoluble $\epsilon(\gamma$-glutamyl) lysine networks (Folk and Finlayson, '77; Folk et al., '80; Lorand and Conrad, '84). There are three forms of transglutaminase, specifically the blood coagulation factor XIIIa; the keratinocyte enzyme; and tissue transglutaminase (Fesus and Thomazy et al., '88). Tissue transglutaminase is found in a variety of cultured cells and tissues, and body fluids (Chung et al., 72). The level of tissue transglutaminase is induced and activated during involution of liver following hyperplasia induced by lead nitrate (Fesus and Thomazy, '87; Fesus et al., '91; Piacentini, '95). It is believed that the cross-linking is responsible for maintenance of the apoptotic state by preventing internal cell contents from leaking out (Fesus and Thomazy, '88; Fesus et al., '91). Furthermore, retinoic acid (RA) has been shown to induce tissue transglutaminase

activity in macrophages as well as in ovary and melanoma cells (Chiocca et al., '88; Scot et al., '82). Jiang and Kochhar ('92) have reported an increase in transglutaminase activity associated with the induction of cell death in the mesenchymal cells located in the central core of RA-treated limbs. Hence, transglutaminase may be used to distinguish apoptotic cells from normal cells.

bcl-2:

The bcl-2 gene was identified at the breakpoint (t14;18) translocation in human follicular lymphomas (Bakhshi et al., '85; Tsujimoto et al., '85; Cleary et al., '86; Silverman et al., '91; Nunez et al., '90). Its role in oncogenesis is inhibition of cell death inducing signals rather than promotion of proliferation (Vaux et al., '88; Hockenbery et al., '90. The membrane associated protein inhibits apoptosis induced by a variety of stimuli including growth factor withdrawal, irradiation, glucocorticoids, heat shock, and a variety of chemotherapeutic drugs (Vaux et al., '88; Nunez et al., '90; Garcia et al., '92; Miyashita and Reed, '92; Cuende et al., '93; Dole et al., '94; Sentman et al., '91). bcl-2 is expressed in a number of proliferating and differentiating cells in cultured cell lines and during development (Hockenberry et al., '91; Amati et al., '93; Le Brun et al., '93; Veis-Novak and Korsmeyer, '94). In the adult bcl-2 is specifically distributed in stem cells, postmitotic neurons and proliferating zones (Hockenbery et al., '91). bcl-2 knockout studies have shown that although mice die after birth due to extensive cell death in the lymphoid tissues, other organs that express bcl-2 seem to develop normally (Veis et al., '93; Nakayama et al., '94). It is

suggested that the normal development may be due to the fact that bcl-2 is a member of a multigene family and that other members may provide redundant functions in some tissue types (Williams and Smith, '93; Nunez and Clarke, '94). The family now includes bad, bak, bax and bcl-xs which promote cell death and bcl-xl, mcl-1, and A1 which prevent it (Boise et al., '93; Kozopas et al., '93; Lin et al., '93; Oltvai et al., '93; Chittenden et al., '95; Yang et al., '95). bcl-2 and bax have been shown to interact as homo and heterodimers (Hockenberry, '95). Overexpression of bax can activate cell death whereas overexpression of bcl-2 protects the cell from death (Oltavai et al., '93). Sequence analysis has determined the presence of three conserved regions in the different members of the bcl-2 family, BH1, BH2, and BH3 which have been shown to be involved in protein-protein interaction and in the regulation of cell death (Sato et al., '94).

cyclin dependent kinases:

The set of cyclin dependent kinases (Cdks), related in structure and function to the yeast cell division control kinase Cdc2, allow eukaryotic cells to progress through DNA replication and cell division (Elledge and Spottswood, '91; Koff et al., '91; Meyerson et al., '92). The Cdks are catalytic subunits that require activation by specific regulatory subunits or cyclins. In contrast to the simpler *Saccharomyces cerevisiae*, where cell cycle progression depends on one kinase (CDC28) and a number of G1 and mitotic cyclins (Nasmyth, '93), the multicellular system is more complex. Cell cycling in mammalian cells involves the employment of a family of

Cdc2 related kinases (Cdk 1-6) along with a family of cyclins (A-E) that have been implicated in DNA synthesis (Cdks 2, 4, 6 and cyclins A, C-E) and mitosis (Cdk1 and cyclin B, Girard et al., '91; Pines and Hunter, '91; Pagano et al., '92; Zindy et al., '92; Matsushime et al., '92; Sherr, '93; Hartwell and Kastan, '94).

Some of the Cdks may play a role in apoptosis such as Cdc2 (Cdk1) and Cdk2, which are activated in *Tat* (HIV-1 transactivator protein) expressing cells that die (Li et al., '95). In addition, *bcl-2* expression has been shown to suppress the levels of Cdk2 as well as apoptosis (Meikrantz et al., '94). These results suggest that deregulation of the molecular factors crucial for cell cycle progression may result in apoptosis. The idea that Cdks could be regulators of both mitosis and apoptosis is very attractive. However it also increases the complexity of their regulation. For example, it has been shown that the inactivation rather than the premature activation of Cdc2 also increases the level of apoptosis (Ongkeko et al., '95). The expression of another kinase, Cdk5, provides further confusion. Originally identified by its structural homology to human Cdc2, Cdk5 is highly conserved among vertebrates, with over 99% identity at the protein level (Lew et al., '92; Hellmich et al., '92). Although it is a member of the Cdk class of catalytic subunits which are involved in cell cycle regulation, Cdk5 has been shown to be expressed in the embryonic nervous system, in cells that are not proliferating but rather are differentiating (Tsai et al., '93; '94; Ino et al., '94). Cdk5 is also expressed in adult tissues, with highest levels detected in the brain and testis and lower levels detected in ovary and kidney (Meyerson et al., '92; Ino et al., '94). The unique expression pattern of Cdk5 has inspired search for

its regulatory subunit. Cyclin E has been shown to form complexes with Cdk5 (Miyajina et al., '95) and several neural-specific proteins including p23 (Uchida et al., '94, Ishiguro et al., '94), p35 (Tsai et al., '94), and p67 (Shetty et al., '95) have been identified, and shown to display a regulatory activating function to Cdk5 (Tsai et al., '94). Uchida et al. ('94) have reported that the tau protein kinase II is a complex composed of Cdk5 and p23. However no specific correlation was made of Cdk5 to apoptosis in these studies and the suggestion that apoptosis occurs via a Cdk-dependent pathway remains indirect.

Chapter 2. Cell Death During Normal Limb Development

A. Normal pattern of limb development

B. Cell death during patterning of the limb

C. Cell death and genetic control of pattern formation

Cell death during normal limb development

A major question in developmental biology has been the control of cell fate. The developing limb provides an excellent system to study pattern formation in vertebrates as it is accessible throughout development and permits experimental manipulation and specification of interactions that lead to the formation and spatial organization of this structure. The limb also serves as a biological tool to observe and elucidate deviations in ontogeny (Kochhar, '77). Therefore, the limb serves as a complete model as its regulation involves cell movement, division, differentiation, interaction and death (Zakeri, '93).

A. Normal pattern of limb development

In all vertebrates, formation of the developing limb bud is initiated by interaction of two sources of the mesenchyme (Tabin, '91). Cells migrating from the somites give rise to limb muscle cells whereas cells from the lateral plate mesoderm give rise to connective tissue and cartilage (Chevallier et al., '77; Christ et al., '77), which will eventually determine the primary limb pattern. Although the lateral plate mesenchyme initiates limb bud formation, the limb bud ectoderm plays an important role as well. At the tip of the growing limb bud a pronounced epithelial thickening produces a ridge along the anteroposterior axis. This ridge is known as the apical ectodermal ridge (AER) and further limb development is dependent on the signals produced by it (Saunders, '77; Hurle et al., '96). Removal of AER stops limb bud

growth and leads to limb truncation (Rowe et al., '82; Tabin, '91). On the other hand, a graft of the AER to an ectopic site of the limb bud leads to extra limb growth in the ectopic location (Tabin, '91). Yet another function of the AER is to keep the underlying mesenchyme undifferentiated (Globus and Vethamany-Globus, '76). In turn, inductive signals from the subjacent mesenchyme maintain the AER by what is thought to be an "AER maintenance factor" (Searls and Zwilling, '64; Zwilling, '64; Coucouvanis, '95).

As the limb grows, it develops asymmetrically along the anterior-posterior axis (A-P), the dorsal-ventral axis (D-V), and the proximal-distal axis (P-D). Positional information for specifying the A-P axis is already present in the lateral plate mesoderm prior to limb bud formation (Harrison, '18; Hamburger, '38; Zwilling, '56). The D-V axis is defined by the ectoderm and formed later (MacCabe et al., '74). The P-D axis is determined during the growth of the limb bud in rapidly dividing mesenchymal cells underneath the AER in a region that has been identified as the progress zone (Summerbell et al., '73). As the bud grows out, cells left further from the AER start to differentiate and as cells leave the progress zone, their proximal-distal values become defined (Summerbell et al., '73; Wolpert et al., '75). Thus, the AER promotes mesenchymal growth, maintains undifferentiated mesenchyme, and possibly provides positional information along the P-D axis.

The A-P axis consists of a combination of repetitive patterns (the digits) and variation in pattern (each digit being morphologically different from the others- Tabin, '91; Hurle et al., '96). Specific zones regulate the A-P axis. One model

describes a zone of polarizing activity (ZPA) that controls the pattern formation along the A-P axis and stimulates cell division, differentiation, and cell death in the distal mesoderm (Saunders, '72; Schluter, '73; Copp, '78; Hinchliffe and Gumpel-Pinot, '81; Thaller and Eichele, '87; Riddle et al., '93). This model assumes establishment of a gradient of a diffusible morphogen that is present in the posterior part of the limb and that maintains the anterior mesoderm and anterior ectodermal ridge (Saunders, '72; Hinchliffe and Gumpel-Pinot, '81). The molecular controls are however unknown.

B. Cell death during patterning of the limb:

One of the most dramatic features of limb development is cell death, which shapes and contours the digital palettes (Scheib, '63; Saunders, '72; Schluter, '73; Copp, '78; Hinchliffe and Gumpel-Pinot, '81; Thaller and Eichele, '87; Ingham and Martinez Arias, '92; Lee et al., '93; Riddle et al., '93; Zakeri et al., 94; Zakeri and Ahuja, '94; Coucouvanis et al., '95; Mori et al., '95; Hurle et al., '96). The specific spatial and temporal pattern of cell death is species-dependent. During limb formation, cell death is first observed in the anterior and posterior marginal zones (AMZ and PMZ) of the developing limb bud. In later stages, there is massive cell death in almost all of the interdigital mesenchymal tissue (IMT) located between the chondrifying digits (Hurle et al., '96). The ability of the IMT to undergo cell death is dependent upon the overlying ectoderm as well as the presence of adjacent digits (Hurle and Ganan, '86; Ganan et al., '94). In experiments performed by these

researchers, removal of the ectoderm or the adjacent digit inhibited cell death and produced extra digits. During normal development, the digits are formed from an initial foot or hand palette, which consists of a core of mesenchymal cells that form condensations (Hurle et al., '96). These condensations provide the first morphological indication of where the cartilaginous primordia will occur (Newman et al. '81). The IMT, AMZ and PMZ, which usually fail to condense, instead undergo programmed cell death (Saunders and Fallon, '67). Cell death is also detected in the developing AER; this death is thought to limit the size of the AER and subridge and therefore prevent polydactyly (Scott et al., '77; Naruse and Kameyama, '86; Milaire and Roze, '83; Hurle et al., '96). Thus, cell death accompanies the formation of free and independent digits of birds (Saunders and Fallon, '67; Hinchliffe and Thorogood, '74) and mammals (Ballard and Holt, '68). However, in species with webbing between adult digits (ducks), little or no cell death occurs in the webbed regions of the interdigital areas (Saunders and Fallon, 1967; Fallon and Cameron, '77; Hurle and Colvee, '82).

C. Cell death and genetic control of pattern formation:

The formation of the limb encompasses an intricate patterning program involving complex interactions at both the cellular and molecular level. Recent work has identified a number of molecules that may be involved in the regulation of pattern formation of the limb. The homeobox-containing genes (*Hoxa* and *d* and *Msx*) have been proposed to be involved in cell growth, movement and establishment of

positional information (Dolle et al., '89; Hill et al., '89; Oliver et al., '89; Robert et al., '89; Izpizua-Belmonte et al., '91; Yokouchi et al., '91; Haack and Gruss, '93; Dolle et al., '93; Tickle and Eichele, '94). Fibroblast growth factor 4 has been shown to be a key factor in the signaling between epithelial cells of the AER and subjacent mesenchymal cells (Niswander and Martin, '92; 93). Members of the transforming growth factor-ß superfamily, the bone morphogenetic proteins may also be involved in epithelial-mesenchymal signaling, as they too are expressed in the AER and mesenchyme of the early limb bud (Lyons et al., '90; Jones et al., '91; Francis et al., '94). Another signaling molecule, retinoic acid has also been implicated in positional information (Eichele, '89; Tamura et al., '90; Tickle and Eichele, '94). Most recently, great interest has been taken in sonic hedgehog, as its expression coincides with the zone of polarizing activity and therefore may be involved in the anterior-posterior patterning of the limb (Riddle et al., '93).

Given that cell death serves to define the pattern of the limb, it may therefore derive from the genes that direct and determine pattern formation. As mentioned above, several pattern forming genes are known to act in the limb. For our studies we have examined one set of genes, the *Msx*, which may be involved in pattern formation provided by cell death.

Msx genes

One class of genes whose products serve as determinants of embryonic cell fate (Lewis, '78) and whose expression pattern plays a crucial role in limb develop-

ment is the class of homeobox-containing genes. The genes within the complex share a 183 bp sequence that contains a 61 amino acid helix-turn-helix, DNA binding homeodomain (Scott, '92). Among these are *Msx* genes, as they are related to the *Drosophila* muscle segment gene (Hill et al. '89; Robert et al. '89; MacKenzie et al. '91; Monaghan et al. '91; Scott, '92). The homeobox-containing genes are thought to regulate pattern formation by controlling the transcription of other genes. One such regulatory event is the formation of the limb bud in which cell death is an important part, and these genes are expressed in the regions that show cell death. *Msx-1* and *Msx-2* homeobox-containing genes are expressed in both the AER and the progress zone (Morgan and Tabin, '93) in the early limb bud. Cells in the anterior third of the early limb bud, a smaller region of the posterior margin, and interdigital regions later express *Msx-1* and *Msx-2* as well. It has been suggested that while *Msx-1* and *Msx-2* may allow cells to respond to growth signals, they also suppress differentiation of the limb mesenchyme (Morgan and Tabin, '93).

Expression of *Msx-1* and *Msx-2* in the anterior and posterior margins and interdigital zones of the limb has implicated these genes in cell death. Coelho et al. ('92) have found that *GHox-7* and *Ghox-8* (chicken *Msx-1* and *Msx-2*, respectively) are not expressed in the proximal anterior and posterior margins of mutant polydactylous diplopodia-5 and talpid^2 chick limb buds, which lack proximal anterior and posterior necrotic zones. These genes are also not expressed in the proximal posterior margin of normal developing chick hindlimb buds, which lack a posterior necrotic zone (Yokouchi et al., '91). This evidence provides support for the hypothesis that *Msx-1*

and *Msx-2* may help to define the regions of programmed cell death in the developing chick limb bud (Coelho, '93). However, while *Msx-2* is more precisely expressed in the future necrotic zones, *Msx-1* expression expands beyond these regions. Therefore *Msx-2* may be a more interesting candidate gene for a role in cell death. Further analysis is required to determine the roles that the *Msx* genes play in limb morphogenesis.

Chapter 3. Cell Death During Abnormal Limb Development

A. Genetic analysis of mammalian limb development and cell death

B. Hammertoe limb deformity mutant

Cell Death During Abnormal Limb Development

A. Genetic analysis of mammalian limb development and cell death:

During limb development, cell proliferation, cell differentiation or cell death may be altered by a defective gene. Kochhar ('77) has discussed four possible mechanisms of abnormal development due to defective genes: a defect in the behavior of the limb mesenchyme; a defect in or absence of cellular interactions between the AER and the mesoderm; a defect in the molecular events related to chondrogenic differentiation; and a defect in the pattern of cell death in normal sites or abnormal occurrence of it in an ectopic site. Evidence that normal cell death in animals is caused by the activation of a suicide program comes from studies of mutants in the nematode (Ellis et al., '86), the chick (Hinchliffe et al., '81; Hurle et al., '96), and the mouse (Kochhar, '77; Zakeri and Ahuja, '94).

There are several limb mutations in the avian system with alteration in the pattern of cell death (Saunders, '72; Hurle and Ganan, '86; Hurle, '88). In *ws* (sex-linked wingless mutation), variable expression of the gene results in variable mutant phenotypes including embryos with no forelimbs and normal hindlimbs to embryos without limbs at all (Hinchliffe and Ede, '73; Hurle et al., '96). Usually the mutant wing buds lack an AER and early and increased cell death occurs in the preaxial mesenchyme. In contrast, the talpid^3 (*ta*^{3}) chick mutant is autosomal recessive and displays suppression of cell death in the anterior necrotic zone, the posterior necrotic zone, the opaque patch of the central limb mesenchyme, and in the interdigital zone

(all of which are zones of cell death in the normal chick limb- Hinchliffe and Ede, '67; Hinchliffe and Thorogood, '74). This lethal mutation displays an extended AER, shortened limbs, polydactyly, and failure of bone formation (Kochhar, '77; Hurle et al., '96).

There are at least 70 established genetic loci affecting the development of the limb in the mouse (Lyon and Searle, '89). In mammals, however, the study of limb mutations and their relationship to cell death is more limited. One group of interesting established mouse limb mutants that displays similar phenotypic features disrupting limb formation is referred to as the luxoid group. The luxoid mutants include Dominant hemimelia (Dh), Luxoid (Lu), Luxate (Lx), Strong's luxoid (LST), Oligsyndactyly (O), Postaxial hemimelia (Px), Extra toes (XT), and Hemimelic extra toes (Hx) with an adjacent gene Hammertoe (Hm) (Gruneberg, '63; Johnson, '67). Except for Px, all display semidominant genetics (phenotypic abnormalities in heterozygotes with a more severe condition in homozygous condition). Characteristic of this group is interference with correct formation of long bones and the number and pattern of the digits (Gruneberg, '63; Johnson, '67). The importance of this group is that they all affect a single congenital phenotype. Among all of these mutations, Dominant hemimelia (Dh) and Hemimelic extra toes (Hx) have been shown to produce an altered pattern of cell death (Rooze, '77; Knudsen and Kochhar, '81). Dh mutant limbs are devoid of physiological necrosis and many will develop a preaxial polydactyly (Rooze, '77). Hx results in polydactyly of all four feet of heterozygotes. The effect is more severe in the hindlimbs than in the forelimbs, with shortening of

the radius and tibia, and supernumerary metacarpals, metatarsals, and digits (Knudsen and Kochhar, '81). During embryogenesis in Hx. extended PCD occurs in the opaque patch resulting in resorption of the tibial precartilage; and suppressed PCD occurs within the basal layer of the AER resulting in outgrowth and protrusion (Knudsen and Kochhar, '81). Recently, van der Hoeven et al., ('94) described the mouse mutant *Fused toes* which was caused by a transgenic insertion. This mutation affects cell death in the forelimbs and in immature thymocytes in the heterozygous condition and embryolethality in the homozygous condition.

The phenotypic abnormalities reflect the variety seen in human limb disorders, making the mouse a good model to study limb morphogenesis in humans. Several developmental genes that are mutated in the mouse have apparent homologs to humans, such as the small eye mutation which is equivalent to the human aniridia syndrome (Hill et al., '91). The Extra toe mutation in mouse is similar to the human Grieg's cephalopolysyndactyly syndrome (a disease affecting limb and craniofacial development- Vortkamp et al., '91). The human disorder Apert's syndrome, where patients have spoonlike hands due to extensive webbing, correlates with the Hammertoe mouse mutant (Robert Hill, personal communication; Zucker, '91). Recently, Tsukurov et al. ('94) have described the mapping of human complex bilateral polysyndactyly gene to a region that is homologous to chromosome 5 in the mouse. The limb deformity mutant Hm has been mapped to mouse chromosome 5.

B. Hammertoe Limb deformity mutant

Observations of mutant mouse strains with syndactyly and webbed limbs, in which cell death appears to fail, and the mapping of the sequence of events in interdigital segregation, have led to the idea that cell death is under direct or indirect genetic control (Johnson, 1969; Hinchliffe and Thorogood, '74; Ingham and Martinez Arias, '92; Zakeri et al., '94).

Hammertoe is a semidominant mutation. Homozygous Hammertoe mutant mice show webbing between digits 2, 3, 4, and 5. In heterozygotes, webbing does not reach the distal end of the toes. This condition is detectable in homozygous embryos on day 14.5 and in heterozygotes on day 15.5 (Green, '81). The homozygotes are viable and fertile. Little is known about the nature of the limb deformity (M. Green, Jackson Labs, Bar Harbor, personal communication). Specifically, it has not been shown if the persistence of webbing in this mutant limb correlated with a defect in cell death, and so nothing is known about the regulation of cell death in this mutant. The webbing affects the pattern of digit formation, but this relationship is also not yet understood. This mutant therefore provides an ideal strain to study the pattern of cell death as well as genes responsible for cell death. It is known that the Hammertoe gene has been mapped to chromosome 5 (Green, '81). It is interesting that *Hx* and *Msx-1* are also mapped to chromosome 5.

Chapter 4. Chemically Manipulated Cell Death During Limb development

A. Retinoic acid as a teratogen

B. Retinoic acid as a morphogenetic agent

C. Retinoic acid and cell death

Chemically Manipulated Cell Death During Limb Development

A great number of chemical agents have been shown to have teratogenic effects on the developing embryo by inducing extensive cell death in various tissue types (Warkany, '69; Russell and Russell, '54;). For example, treatment of pregnant females in postimplantation stages with 4-hydroperoxycyclophosphamide causes prolonged G2/M cell cycle arrest leading to abundant levels of cell death and embryotoxicity (Little and Mirkes, '92). Other agents including pentistatin, hypothermia, and the DNA alkylating agent, N-acetoxy-2-acetyl aminofluorene have been shown to cause high levels of cell death in developing neural tissues as well as other tissues (Knudsen et al., '92; Little and Mirkes, '95; Thayer and Mirkes, '95). Several teratogens have a specific effect on the developing limb if administered during a specific stage of embryonic development (Summerbell, '73; Kochhar, '73; 76a; '77). Administration of chlorambucil, hydroxyurea, and retinoic acid (RA) to pregnant mice on gestational day 12 produces limb deformities including micromelia (Kochhar 75; Sadler and Kochhar, '76; Sadler et al., '76).

A. RA as a teratogen

RA serves as a unique teratogenic agent in that, although it is necessary for a variety of physiological functions, treatment of pregnant animals with RA results in a variety of facial, neural tube, cardiovascular and limb abnormalities depending on the stage of treatment (Kochhar et al. '84; Lammer et al., '85; Alles and Sulik, '89;

Granstrom et al. '90; Yasuda et al. '90; Rizzo et al., '91; De Luca et al., '95). In fact, RA treatment is teratogenic effect in a variety of species including rats, hamsters, rabbits, and humans (Pinnock and Alderman, '92; Armstong et al., '94). Whereas low doses of RA (milligram/kilogram body weight) to the mother yield teratogenic effects in a variety of tissue types depending on the time of treatment, high doses to the mother result in embryolethality (Shenefelt, '72; Armstrong et al., '94). It is thought that the levels of RA are regulated by a variety of proteins that bind and transport RA to specific compartments of the cells (Favennac and Cals, '88). However, the teratogenic effects of RA may result from easy passage through the placenta and the absence of these proteins or inability of these proteins to handle the excess levels in the early embryo (Geelen, '79; Underwood, '89; Pinnock and Alderman, '92). These abnormalities may then result from the ability of excess RA to directly affect important processes including cell migration, proliferation, differentiation, interaction, and death. Use of this teratogen is expected to extend the understanding of pathogenetic mechanisms to the molecular level as well as its role during morphogenesis.

B. RA as a morphogenetic agent during normal development

RA is one of the natural derivatives of vitamin A, a member of the family of retinoids. All-trans-RA is an active form of retinol which is stored in the liver (Ross, '93; Miller and Warrell, '96). It has been found to influence the differentiation of many cell types such as epithelia, melanoma, neuronal, endothelial, myeloid stem cells

and embryonic stem cells (Ross, '93). In addition, it is clinically important for the prevention and treatment of cancers such as acute promyelocytic leukemia (Lotan, '86; Shapiro, '86; Favvennec and Cals, '88; De Luca, '91; Parkinson et al., '92).

Morphogenesis can be influenced by several nongenetic factors, such as RA. Most dramatic is its role as a regulator of pattern formation during embryonic development. Evidence for this role comes from RA's ability to induce mirror image duplication of the limb's distal elements when it is applied locally to the anterior border of the chick limb bud (Summerbell, '84; Tickle et al., '82; '85; Tabin, 1991). RA has also been shown to result in limb and lower body duplication in the mouse (Rutledge et al., 1994). Further evidence for its importance in limb development is provided by the fact that RA is present in limbs of developing chick (Thaller and Eichele, '87) and mouse (Schluter, '73; Scott et al., '94). Although it was previously thought to be a natural morphogen, several results now demonstrate that RA is not a morphogen (Niazi and Saxena, '78; Maden, '83; Honig and Summerbell, '85; Thaller and Eichele, '90). None-the-less, RA is still considered to be an important signal the activity of which affects pattern formation in the limbs.

The mechanism by which RA acts during pattern formation may relate to its effect on the expression of cellular binding proteins and retinoic acid receptors as well as on other genes involved in pattern formation. A cytoplasmic receptor is cellular retinoic acid binding protein (CRABP), which binds a single molecule of retinoic acid (Brown and Tickle, '92). Whether the action of CRABP is to anchor RA in the cytoplasm or to transport it to the nucleus remains to be proven. Retinoic acid

receptors (RARs) are members of the family of nuclear receptors affecting transcriptional responses to steroid and thyroid hormones (Evans, '88; Green and Chambon, '88). When activated by the ligand all-trans-RA, RARs form heterodimeric complexes with another family of receptors (retinoid X receptors) and bind to upstream regulatory DNA sequences, known as response elements of their target genes (de The et al.,'90; Sucov et al.,'90; Leroy et al., '91; Rowe et al., '91). Where all-trans-RA only binds RARs, its isomer, 9-cis-RA binds both RARs and RXRs (Thaller and Eichele, '87; Levin et al.,'92; Heyman et al., '92). The receptor for a third isomer, 13-cis-RA, is not known. Three genes of the RAR family (RARα, RARß, and RARγ) all have different isoforms (18) that result from differential promoter usage and alternative splicing (De Luca et al., '95). The receptors are differentially expressed in the limbs (Dolle et al., '89) and have different sensitivities to RA (Leroy et al., '91). One of the receptors, RA receptor ß, is expressed in regions of cell death in the interdigital mesenchyme (Dollé et al., '89; Leroy et al., '91). RA also affects the expression of homeobox-containing genes. Yokouchi et al. ('91) have shown repression of *Ghox-8* expression in ectodermal and mesenchymal regions of chick limb after treatment with sufficient RA to result in extra digit formation. Thus it is clear that RA plays a role in limb development and may also direct one pattern of cell death.

D. RA and cell death

The events leading to cell death in the limb are under tight regulation and strongly influence the shape of the limb. Several investigators have demonstrated that interference with stages of embryonic development in which interdigital cell death occurs will result in severe malformation of the limbs (Kochhar, '73; Hurle, '88; Ingham and Martinez Arias, '92). It has been proposed that the teratogenic effect of RA results from the induction of cell death. RA induces cell death in the developing limb (see Schweichel, 1971; Kochhar, 1977; Sulik and Alles, 1991; Zakeri and Ahuja, 1994). These reports described the appearance of dying cells in the interdigital necrotic zone (Schweichel, 1971) and the mesenchymal core of the embryonic limb after RA administration (Kochhar, 1977), and associated it with sites of natural programmed cell death (PCD, Sulik and Alles, 1991). This induction in cell death in zones of normal embryonic cell death is perhaps the origin of the pathogenesis of subsequent malformations (Sulik and Dehart, 1988; Sulik et al., 1988; Alles and Sulik, 1989, 1990). On the other hand, given that RA has been shown to enhance cell death in regions of normal programmed cell death in the embryonic limb (Alles and Sulik, '89) and that it can regulate genes that are responsive to it, one can assume that RA may direct the pattern of digit formation by specifically affecting cell death.

FOREWARD TO THE RESEARCH UNDERTAKEN

The importance of cell death in the maintenance of homeostasis in the organism has been a major focus of developmental biology, immunology, aging and teratology. Recently molecular biology has provided several molecular markers for physiological cell death, revealing the different instances of cell death and identifying different regulatory signals in these instances. To investigate the signals, mode of action, and outcome of their transmission we have used several approaches to study cell death. We examined the expression of a putative cell death marker, clusterin, in a number of model systems to better assess its role in cell death. This work is discussed in the first chapter. The second approach was to examine in detail cell death in one model system, that of the developing mouse limb. In the limb there is a precise pattern of anterior, posterior, and interdigital cell death. We first characterized the nature of cell death during normal limb development with methodology that displays the morphology and biochemistry of cell death. We investigated the importance of cell death by examining a limb deformity mutant which we showed was a result of an altered pattern of cell death in the interdigital regions of the limb. We were also able to manipulate the pattern of cell death by use of retinoic acid which has been shown to enhance selective cell killing. These results are reviewed in chapter two. To address the specific question of what determines the targeting of specific cells for cell death in the presence of surviving neighbors, we have examined specific genes that have been directly or indirectly associated with cell

death. The genetic regulation of cell death in normal and abnormal circumstances is covered in chapter three. Given the effect of RA on the developing limb, we investigated the phenotypic manifestation of the effect of RA as well as the regulation of RA in the developing limb. This is described in chapter four.

PART II. RESULTS and DISCUSSION

Chapter 1: Analysis of the Relationship Between Clusterin Gene Expression and Cell Death

A. Objective

B. Materials and methods

C. Results:

a. Expression of clusterin during implantation and embryogenesis

b. Expression of clusterin in the mouse male reproductive tract

c. Expression of clusterin in the mouse female reproductive tract

D. Discussion: The role of clusterin in cell differentiation and cell death

Chapter 2. Characterization of Normal, Abnormal, and Chemically Induced Cell Death during Limb Development

A. Objective

B. Materials and methods

C. Results:

a. Morphological and biochemical analysis of cell death in the developing limb

b. Morphological and biochemical analysis of cell death in the Hammertoe mutant limb

c. Morphological and biochemical analysis of retinoic acid induced cell death in the normal and mutant limb

D. Discussion: The nature of normal and abnormal cell death in limb development

Chapter 3. Analysis of Cell Death Implicated Genes in the Normal and Abnormal Developing Limb

A. Objective

B. Materials and methods

C. Results:

a. Expression analysis of cell death associated genes in relation to cell death in the limb

b. Expression analysis of limb patterning genes in relation to cell death in the limb

c. Expression analysis of cell cycle genes in relation to cell death in the limb

D. Discussion: The genetic regulation of cell death in the developing limb

Chapter 4. Analysis of the effect of RA induced cell death on the limb phenotype and on components of the RA signaling pathway

A. Objective

B. Material and methods

C. Results:

a. Phenotypic manifestation of RA-induced cell death in the normal and mutant limb

b. Analysis of RA responsive factors in the normal and mutant limb

D. Discussion: The genetic regulation of RA in the limb

Chapter 1

Analysis of the Relationship Between Clusterin Gene Expression and Cell Death

A. Objective

B. Materials and methods

C. Results

a. Expression of clusterin during implantation and embryogenesis

b. Expression of clusterin in the mouse male reproductive tract

1. Localization of clusterin during testicular development

2. Regional and intracellular localization of clusterin in the caput of the epididymis of adult mice

3. Clusterin mRNA expression and germ cell maturation in the epididymis

c. Expression of clusterin in the mouse female reproductive tract

D. Discussion: The role of clusterin in cell differentiation and cell death

A. Objective

Clusterin has been reported to be expressed in a number of different systems undergoing cell death (Jenne and Tschopp, '89). Its upregulation during prostatic involution has suggested that its expression is associated with active cell death (Leger et al., '87; Betuzzi et al., '89; Kyprianou and Isaacs, 89). In addition, its expression was reported in the interdigital tissue of the developing limb (Buttyan et al., '89). However, clusterin is also expressed in a number of situations in which cell death does not occur (Garden et al., '91; Zakeri et al., '92; French et al., '93; Ahuja et al., '94; Ahuja et al., in press). Therefore the role of clusterin in cell death is unclear. To address these inconsistencies and to ask if clusterin expression is specifically associated with cell death, we have investigated the expression of clusterin both at the level of message and protein in a number of embryonic and adult tissues in which we can identify cell death. We first examined the expression of clusterin in the implanted and developing embryo. The embryo provides a number of tissues that are undergoing cell death. We paid close attention to the developing limb to determine which cells were specifically expressing clusterin. We then examined the expression of clusterin in the male gonads to confirm previous results from the rat testis in our mouse model system and to again determine if clusterin expression specifically co-localized with dying cells. In the female reproductive system, cell death is more pronounced and easily detected in the cell types that are expressing it, ovarian follicles undergoing atresia. Our results indicate that the expression of clusterin in the

male reproductive tract and in the developing embryo is confined to normal and differentiating cells. In the female reproductive system, the gene is expressed in both surviving and dying cells. Our results suggest that clusterin may be involved in membrane remodeling during cellular differentiation, stress, or death.

B. Materials and Methods

a. Animals

Swiss Webster mice (Charles River; Wilmington, MA) and germ cell deficient atrichosis mutant (*at*) and dominant spotting mutant (*W*) mutant mice, along with their control littermates were obtained from Jackson Laboratories (Bar Harbor, Maine). Animals were sacrificed by CO_2 and cervical dislocation and the reproductive organs from adult male and female mice were excised. For embryonic studies, mice were mated overnight and females were checked for the presence of a vaginal plug. The time of plug detection was designated gestational day 0.5. Embryos were removed from pregnant females at different days of gestation and embryonic limbs were excised in cold 1X phosphate buffered saline (PBS, 0.85% NaCl, 0.02% KCl: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Tissues were either fixed in 4% paraformaldehyde for frozen sections or frozen in liquid nitrogen for RNA isolation.

b. Tissue fixation and preparation for frozen sections

Tissues were fixed overnight in 4% paraformaldehyde (Fisher). For 50 mL of fixative, 2 g of paraformaldehyde (Fisher) were added to 25 mL of dep-treated H_2O (1 mL of diethyl pyrocarbonate (Sigma) is added to 1 L of H_20, mixed, allowed to stand for 1 hour, and autoclaved for 1 hour). The paraformaldehyde-dep H_2O mixture was stirred while heating to 65°C at which time it was removed from the heat and 5 μL of 10M NaOH and 5 mL of 10X PBS are added. The solution was filtered through Whatman paper and dep H_2O was added to make a final volume of 50 mL.

Paraformaldehyde was prepared fresh and used the same day. After overnight fixation, tissues were placed in 20% sucrose in 1X PBS overnight (filtered and stored at 4°C), embedded in OCT (Tissue Tek; Miles Inc.) embedding compound, and frozen in isopentane/liquid N_2. Special care was used in the orientation of the mouse limb samples during the embedding procedure. Frozen sections (6μm) were cut, placed on poly-L-lysine coated slides and stored at -70°C prior to use.

c. Slide preparation

Slides were placed in autoclaved metal racks and dipped in 0.2M HCl, dep H_20, and acetone for 30 seconds each. Slides were allowed to dry for 2 hours in a hood, dipped in poly-L-lysine (Sigma) in 0.01M Tris, pH 8.0 for 5 minutes and were allowed to dry overnight. Slides were used within 4 weeks of treatment.

d. Immunocytochemistry

Tissue sections were rehydrated through serial graded solutions of 20%, 10%, and 5% sucrose in 1X PBS for 5 minutes each, followed by 2 washes in PBS for 5 minutes. Sections were blocked with 3% goat serum in PBS for 20 min at 37°C to prevent non-specific binding prior to incubation overnight at 4°C with anti-S35, anti-45, anti-301 (1/100 dilution in 1X PBS), or anti-SGP-2 (1/200 dilution in 1X PBS). The protein was localized by using horseradish-peroxidase-linked secondary antibody and stable diaminobenzidene (DAB, Research Genetics, Inc.) to develop a brown chromophore at the site of antibody binding.

Protocols e and f were done in collaboration with Johnathon Lakins and Dr. Martin Tenniswood at the Alton Jones Cell Science Center: Protocols were approved by the ACUCS at WAJCSC

e. Preparation of antisera to clusterin synthetic peptides

Four peptides corresponding to sequences from the α subunit of TRPM-2 (residues 1-205 of the proprotein) were selected on the basis of their antigenic index (Jameson and Wolf, '88) and synthesized using a method for simultaneous multiple peptide synthesis as previously described (Houghten, '85). These included residues 1-17 (EQEFSDNELQELSTOGSC), residues 32-48 (KHIKTLI EKTNAERKS), residues 52-72 (LEEAKKKKEGALDDTRDSEC), and residues 133-148 (NGDRIDSLLESDRQQSC). In all but peptide 32-48 a non-encoded cysteine residue was included as the C terminal amino acid to facilitate covalent coupling of the peptide to myoglobin in a separate preparation. Each peptide (0.45 to 0.5 mg) was mixed and dissolved in 125 μL of Freund's complete adjuvant. The primary injection was intramuscular at two sites in a New Zealand White Rabbit. At 2, 4, 9, and 14 weeks following the primary injection the animal was given two subcutaneous booster injections. Each injection consisted of 400 μL of immunogen containing a mixture of 0.45 to 0.5 mg of each peptide in PBS emulsified in Freund's incomplete adjuvant. Test sera were prepared from between 5 to 10 mL of blood sampled from the ear vein one or two weeks following each injection and titered by ELISA using a mixture of the peptides as antigen as described previously. Twelve days after the fourth booster shot the animal was exsanguinated by cardiac puncture. The resulting antisera

were stored at -20°C. Antiserum 301, used in this study was prepared from a test bleed obtained from the ear vein 1 week after the fourth booster shot.

f. Comparison of reactivities of different antibodies with recombinant TRPM-2 malE fusions

Recombinant α,ß subunits of TRPM-2 as well as the pre-protein have been synthesized as maltose binding fusion proteins using the *E. coli* expression vector, pMalcRI. The fusion proteins containing the TRPM-2 α, ß subunits and preprotein were purified from *E. coli* lysates by amylose affinity chromatography (New England Biolabs) followed by elution with 10mM maltose. Two μg of each of the fusion proteins was heated for 10 minutes at 100°C in 140 μL SDS reducing buffer (31 mM Tris-HCl, 1.25% w/v SDS, 2.5% v/v ß-mercaptoethanol, pH 6.8), loaded on spin columns of Sephadex G-25 (Pharmacia, Montreal) equilibrated in SDS non reducing buffer (31 mM Tris-HCl, 1.25% w/v SDS, 2.5% v/v, pH 6.8) and centrifuged for 2 minutes at 500xg. Eluted samples were combined with 8 μL of glycerol and 14 μL of 0.2% w/v bromophenol blue. Aliquots of 25 μL of each eluate containing approximately 300 ng of total protein were electrophoresed on 10% SDS-polyacryl-amide gels at 100 V for 1.5 hours. The gels were equilibrated by three 20 minutes incubations in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, 0.1% w/v SDS) and electroblotted to nitrocellulose membranes at 250 mA for 2 h at 4°C. Transfer was verified, and the position of molecular weight standards run in parallel marked, following reversible staining of the membranes with 0.2% w/v Ponceau S in

3% w/v trichloroacetic acid. Each membrane was incubated for 1.5 hours at room temperature in 10 mL of PBS pH 7.4 containing 0.1% v/v Tween 20® and 10% w/v skim milk (Difco) to block non-specific reactions, and then washed four times with PBS, 0.1% v/v Tween 20 for 5 minutes. The membranes were incubated with each of the antisera diluted 1 to 8000 in 4 mL PBS, pH 7.4, 0.1% v/v Tween 20 and 10% w/v skim milk for 1.5 h at room temperature, washed 4 times with PBS, 0.1% v/v Tween 20 for 5 minutes, and incubated for 1.5 h at room temperature with 5 mL of a 1:20,000 dilution of horseradish-peroxidase-linked-goat-anti-rabbit IgG (Caltag Laboratories, in PBS 0.1% v/v Tween 20). The membranes were washed 4 times with PBS 0.3% v/v Tween 20 for 5 minutes; 4 times with PBS 0.1% v/v Tween 20 for 5 minutes and once with PBS for 5 minutes. The immunoreactive bands were detected using enhanced chemiluminescence using the protocols described by the supplier (Amersham) except that 1 mL of detection reagent mix was typically used on an 8.5 cm x 5.5 cm membrane sealed by surface tension to Parafilm. The membranes were exposed on Kodak X-OMAT AR film between intensifying screens for various times between 2 and 300 seconds.

g. Transformation

To prepare competent DH5α (GIBCO BRL) cells, 5 mL of LB (10g bacto-tryptone, 5g bacto-yeast extract (Difco), 10g NaCl in a final volume of 1L, pH 7.0) were inoculated with 200 uL DH5α cells and grown up overnight at 37°C. The next morning, 50 mL of LB broth were inoculated with 0.5 mL of the overnight culture

and grown at 37°C for 3 hours (OD_{550} was approximately 0.5 -0.7) and then placed on ice. After 20 minutes the cells were centrifuged at RT. 2500 RPM for 15 min. The pellet was resuspended in 25 mL of ice cold, sterile 0.1 M $CaCl_2$ and left on ice for 20-30 minutes with occasional swirling. The solution was centrifuged again for 10 minutes to pellet cells. The pellet was resuspended in 3 mL 0.1M $CaCl_2$ and placed on ice or stored at 4°C overnight. DNA was diluted to a concentration of 10 ng/μL and 20 ng (2 μL) of DNA were added to 300 μL competent cells in a 15 mL conical tube. As a control, 300 μL of cells were placed in a second tube without DNA. The conicals were gently mixed and left on ice for 1 hour with occasional shaking. The cells were then heat shocked at 42°C for 90 seconds to enhance transformation. 1 mL of LB broth was added and cells were grown in a 37°C shaking incubator for 45 minutes and then placed on ice. 100 μL and 200 μL of cells were plated out onto separate LB + amp plates (LB broth, 15 gms agar/L, 50 μg/mL ampicillin (Boehringer Mannheim). On a third plate, 100 μL of cells only were plated out. Plates were incubated overnight at 37°C. The next day a single colony was picked and 5 mL LB + amp broth was inoculated and incubated in shaker at 37°C overnight. 250 mL of TB (Terrific broth 3 gms tryptone (Difco), 6 gms yeast extract (Difco), 1 mL glycerol (Fisher) in a volume of 225 mL with H_2O were autoclaved and allowed to cool. To this 50 μg/mL of ampicillin and 25 mL of autoclaved 10X potassium salt buffer (0.17M KH_2PO_4, 0.72M K2HPO$_4$) were added.

h. Plasmid preparation

Bacteria from a 250 mL culture of TB broth + amp were pelleted at 5000 RPM for 15 minutes at 4°C. The pellet was resuspended in 10 mL ARS I (alkaline rapid screen, 4.5 gms glucose, 12.5 mL 1M Tris HCl (pH 8.0), and 10 mL 0.5M EDTA (pH 8.0) in a total volume of 500 mL) and one scoop of lysozyme (Jersey Lab and Glove Supply) and incubated on ice for 30 minutes. 20 mL ARS II (2 mL 10N NaOH, 10 mL 10% SDS, and 88 mL H_20) was added and the mixture was swirled and incubated on ice for no more than 5 minutes. 15 mL ARS III (204. 12 gms NaOAc in 500 mL H_20, pH 4.8) were added and the mixture was inverted and incubated on ice for 45 minutes. This mixture was then centrifuged at 12000 RPM for 20 minutes and the supernatant was transferred to a clean tube with equal volume (45 mL) of isopropanol. This solution was mixed well and placed at -70°C. After 20 minutes, it was centrifuged at 12000 RPM for 20 minutes and the pellet was dissolved in 7 mL of 1X TE and 4 mL of 7.5 M NH_4OAc. 2 volumes 100% ethanol were added, mixed well and the tube was placed at -70°C for 20 minutes. This was centrifuged at 12000 RPM for 20 minutes and the pellet was resuspended in 7 mL TE and 10 uL RNase A (10 mg/mL)was added for a 30 minute incubation at 37°C. 3 mL of 5M NaCl, 2.5 mL 30% PEG/1.5 M NaCl were added, mixed well, and the tube was left on ice for 30 minutes. This was centrifuged at 12000 RPM for 20 minutes and the pellet was dissolved in 560 µL H_20. To this mixture, 70 µL of 10X PK buffer and 70 µL Proteinase K (5 mg/mL) were added for an overnight incubation at 37°C. The next day, 70 µL 3M NaOAc were added and phenol/CH Cl_3 extractions were performed

until the interface was clean (~ 4 times). Two and a half volumes of 100% Ethanol were added and DNA was precipitated at -70° for 20 minutes. This solution was centrifuged at 13000 RPM for 15 minutes at 4°C and the pellet was washed with cold 70% ethanol. The pellet was then dried in a Savant speed vac, dissolved in 400 µL of dH_20 and the optical density was read at 260nm.

i. In situ hybridization

1. Non-radioactive technique: Sections were postfixed for 5 minutes with 4% paraformaldehyde, washed in 10 mg/ml proteinase K (Jersey Lab and Glove Supply) in 1X SSC (0.15 M NaCl, 0.015 M Na citrate, pH 6.8) for 5 minutes, 2X SSC for 2 minutes; 1.41% triethanolamine, pH 8.0 for 2 minutes; 1.41% triethanolamine (Fisher)/0.25% acetic anhydride (Sigma), pH 8 for 10 minutes, and 0.2X SSC for 10 minutes. Dehydrated slides were incubated in prehybridization buffer (50% formamide, 0.6M NaCl, 10 mM Tris, 1mM EDTA, 500µg/ml *E. coli* tRNA, 50µg/µL salmon sperm DNA, 1X Denhart's solution, 0.5% SDS) and then hybridized in the same buffer with the labeled probesy for 16-18 hours at 50°C. Post hybridization washes included 2X SSC for 2 minutes followed by 25 µg/ml RNase A in RNase buffer (500mM NaCl, 1 mM Tris, 1 mM EDTA, pH 7.5) for 30 minutes at room temperature; RNase buffer for 30 minutes at 50°C; 2X SSC for 1.5 hours at 50°C, and at 0.2X SSC for 2 hours at 50°C. The Nucleic Acid Detection Kit was used routinely as described by Boehringer Mannheim.

Probe Preparation:

The pG21-04 plasmid (containing the TRPM-2 cDNA (Léger et al., 1987) was linearized with Eco RI for the anti-sense probe and with Hind III to generate the sense probe. Digoxigenin-labeled cRNA probes were generated using SP6 polymerase and T7 polymerase respectively with the Genius RNA Labeling Kit (Boehringer Mannheim, Illinois). Specifically, 2 μg of DNA (1 μL), 2 μL of NTP mixture, 2 μL of 10X transcription buffer, 10 μL dep-H_2O, 2 μL of RNA polymerase, and 1 μL of RNAse inhibitor were added to a tube and incubated for 2 hours at 37°C. Another μL of RNA polymerase was added and the mixture was incubated at 37°C for an additional 30 minutes, after which 2 μL of 0.2M EDTA, 2.5 μL 4M LiCl, and 75 μL of 100% ethanol were added. The probe was precipitated overnight at -20°C. Each reaction was used for 20 sections.

2. Radioactive technique: In situ hybridization experiments were performed essentially as described by Sassoon and Rosenthal ('93). Sections were washed in 0.85% NaCl and 1X PBS for 5 minutes each, followed by fixation in 4% paraformaldehyde for 20 minutes and two 5 minutes washes in 1X PBS. Sections were then incubated in 2 mg proteinase K in PK buffer (1 M Tris, 0.5 M EDTA) for 7.5 minutes, washed in 1X PBS for 5 minutes, in 0.1 M triethanolamine containing acetic anhydride for 10 minutes, in 1X PBS for 5 minutes, and in 0.85% NaCl for 5 minutes. Slides were dehydrated in graded ethanol solutions, air dried and hybridized with 9 parts hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl (pH 7.4), 5 mM

EDTA, 10 mM $NaH_2PO_4 \bullet H_20$ (pH 8.0), 10% dextran sulfate, 1X Denhardt's solution, 0.5 mg/ml total yeast RNA) and 1 part [^{35}S]UTP labeled probe in 10 mM DTT for 16-18 hours at 50°C. Posthybridization washes included 5X SSC, 10 mM DTT at 50°C for 30 minutes; followed by 50% formamide, 2X SSC at 65°C for 20 minutes; 1X washing solution (0.4 M NaCl, 10 mM Tris (pH 7.5), and 5 mM EDTA) at 37°C twice for 10 minutes each; 20 mg/ ml RNAse A in 1X washing solution at 37°C for 30 minutes; 1X washing solution at 37°C for 5 minutes; 2X SSC at 37°C for 15 minutes; and 0.1X SSC at 37°C for 15 minutes. The slides were dehydrated, allowed to dry and dipped in photographic NTB-2 emulsion (Kodak) and exposed for two weeks. The slides were developed (1:1; Kodak Dektol developer: H_2O) for 2 minutes, washed for 10 seconds, fixed (Kodak) for 5 minutes, washed with dH_2O for 5 minutes, counterstained with 0.2% toluidine blue for 10 minutes, dehydrated in 50%, 75%, 85%, 95%, 100% (twice) ethanol for two minutes each and xylene (twice) for 5 minutes each. The slides were mounted with Permount® and coverslipped.

Probe Preparation

To prepare radioactive probes, 4 μL 5X transcription buffer (Stratagene), 2 μL 0.1 M DTT (Jersey Lab Supply), 3 μL cold 10 mM CTP/ATP/GTP (combined; Stratagene), 5 μL ^{35}S-UTP (Amersham), 1 μL DNA template (300 ng), 2.5 μL H_2O, 1 μL RNAse inhibitor (Boehringer Mannheim), and 1.5 μL RNA polymerase were combined in a tube and incubated for 2 hours at 37°C. At this time 1 μL polymerase was added and the mixture was incubated at 37°C. After 30 minutes, 1μL of RNAse

free DNAse RQ1 (Promega) was added and the mixture was incubated for 15 minutes at 37°C. 3 μL of total yeast RNA (extracted three times with phenol/chloroform and ethanol precipitated several times to make a stock solution:10 mg/ml; Boehringer Mannheim) and 25 μL dep H_2O water was added. Alkaline hydrolysis was performed by adding 50 μL of solution A (10 μL 1M DTT, 80 μL 1M $NaHCO_3$, 120 μL 1M Na_2CO_3, and 790 μL H_2O) for 15-30 minutes depending on the length of DNA template. After hydrolysis was completed, the reaction was neutralized with 50 μL solution B (200 μL 1M NaOAc, 10 μL acetic acid, 10 μL 1M DTT, 780 μL H_2O) and run on a 1:1 ratio of fine: medium Sephadex G50 column (Pharmacia Biotech, Sambrook et al., '89) to isolate the labeled probe from unincorporated nucleotides and very small labelled fragments. The column was prepared by using a pasteur pipet which was plugged with siliconized glass wool (Supelco) with the tip broken off. The column was equilibrated prior to loading the probe with running buffer (10 mM Tris-HCl, 5 mM EDTA, 0.1% SDS (sodium dodecyl sulfate) and 10 mM DTT added just prior to use), followed by 50 μL total yeast RNA (10 mg/ml), and 500 μL running buffer. Probe containing 1 μL of saturated phenol red to follow free nucleotides was run through and fractions were collected until the phenol red was about 1 inch from the bottom. Tubes containing labeled probe were monitored by geiger counter. Probes were ethanol precipitated with one tenth volume of 3M KAc and 2 volumes of 100% ethanol overnight at -20°C. The next morning, the probe was dried and resuspended in 10 μL of 1M DTT

and 1 µL was counted in CytoScint scintillation fluid (Fisher). A final concentration of 35000 cpm/µL in hybridization buffer was used.

j. RNA isolation

Isolation of RNA was performed by a modified version of the protocol described in Cathala et al. ('83). Tissue was frozen in liquid nitrogen and ground with a mortar and pestle containing liquid nitrogen. This was then homogenized in 7 volumes (per gram of tissue) of GTC-lysis buffer (5M guanidine monothiocyanate, 10 mM EDTA, 50 mM Tris-HCl pH 7.5, and 8% v/v ß-mercaptoethanol). Five volumes of 4M LiCl were added per volume of GTC buffer and this mixture was stored overnight at 4°C. The next morning, lysates were centrifuged at 4°C for 90 minutes at 10,000 RPM. The supernatant was discarded and the pellet was resuspended in 3 ml of cold 3M LiCl and this was centrifuged at 4°C for 60 minutes at 10,000 RPM. The supernatant was discarded again and the pellet was resuspended in 1 ml of solubilization buffer (10mM Tris-Cl pH 7.4, 1 mM EDTA, 0.2% SDS). Samples were repeatedly frozen in a dry ice-ethanol bath and vortexed until thawed, until the pellet was resuspended. RNA was extracted by phenol, phenol/chloroform, and chloroform/isoamyl alcohol extractions. RNA was precipitated with 0.4M LiCl and 2 volumes of 100% cold ethanol at -20°C overnight.

k. Northern blot hybridization

RNA was separated using a high resolution gel (1.5% agarose and 2.2M formaldehyde (Fisher) in 1X MOPS (10X MOPS: 200mM MOPS, 50mM NaOAc, 10mM EDTA, pH 7.0). RNA samples were centrifuged for 30 minutes at 4 °C , dried, and suspended in water to measure optical densities. 20 μg of RNA were placed in separate tubes and ethanol precipitated once again. The dried pellet was resuspended in 10 μL of sample buffer (50% formamide, 1X formaldehyde, 1X MOPS buffer). Samples were heated at 70°C for 5 minutes and quenched on ice. To each sample, 0.5 μL of ethidium bromide (10 mg/ml) and 0.5 μL of bromophenol blue (10 mg/ml in glycerol) were added and mixed. Samples were loaded immediately and the gel was run at 40V for approximately six hours.

To transfer RNA onto membrane, the gel was then pre-soaked in 10X SSC while shaking for 1 hour. The gel was transferred onto Hybond (Amersham) overnight by capillary action, after which it was crosslinked in a stratalinker (Stratagene) at 120,000 microjoules. The blot was prehybridized overnight at 42°C in prehybridization buffer (50% formamide, 5X SSC, 5X Denhardt's, 50 mM $NaPO_4$ pH 7.0, 0.2% SDS, 500 μg/ml salmon sperm DNA). The blot was hybridized in hybridization buffer (50% formamide, 5X SSC, 1X Denhardts, 20 mM $NaPO_4$, 10% Dextran sulfate, and 100 μg/ml salmon sperm DNA (boiled for 5 minutes and quenched on ice just prior to use)) containing 2 X 10^7 cpm probe overnight at 42°C. After hybridization, the blot was washed in 2X SSC, 0.1% SDS for 15 minutes at room temperature, twice in 2X SSC, 0.1% SDS for 20 minutes each at 65°C, in 1X

SSC, 0.1% SDS for 20 minutes at 65°C, in 0.1X SSC, 0.1% SDS for 20 minutes at 65°C, and in 0.1X SSC alone for 20 minutes at 65°C. The blot was placed in a plastic bag and exposed to film overnight with intensifying screens at -70°C.

Probe Preparation

To prepare the random prime probe, 200 ng insert, 5 μL primer, and 27 μL H_20 were boiled for 5 minutes and placed on ice for 2 minutes. To this 10 μL of labeling buffer, 5 μL ^{32}P-dCTP (Amersham), and 2 μL Klenow were added. This was incubated at 37°C. After 1 hour, 13 μL H_20 and 7 μL 10X STE were added. A push column was hydrated with 70 μL of 1X STE, after which the probe was pushed through. Another 70 μL 1X STE was pushed through to wash the column and 1 μL was taken to count. $2X10^7$ counts were used for hybridization.

C. Results

a. Expression of clusterin during implantation and embryogenesis

In the mouse, implantation occurs between days five and seven of gestation. During implantation, cell death is detected in the uterine epithelial cells surrounding the blastocyst (El-Shershaby and Hinchliffe, '75). Cell death begins at approximately day 5. Expression of clusterin mRNA was first examined during implantation at days 5.5 (Fig. 1A-C) and 6.5 (Fig. 1D-F) of gestation. Clusterin was not detected in the dying cells surrounding the implanted embryo. However, clusterin transcripts were detected in specific cells of both the uterine glands and ducts of day 5.5 (Fig. 1A-B) and 6.5 (Fig. 1D-E) of implanted uteri. Our results indicate that clusterin is expressed in normal cells during implantation.

The developing embryo provides a number of tissue types in which cell death is an active process. These include the developing nervous system, the secondary palate, and the limb. We examined the expression of clusterin to determine if it co-localized with these sites of cell death. We detected clusterin in several tissue types (Fig. 2). We were unable to detect clusterin message during the peak of cell death in the interdigital zones of the developing limb. However clusterin was expressed at low levels in the cartilage forming cells of the day 15.5 limb (Fig. 2A-D). The most abundant expression of clusterin is found in three embryonic structures (Fig. 2). The simple nonneuronal epithelium of the choroid plexus that is found in the third and fourth ventricles (Fig. 2A-B), the specialized epithelium of the inner ear (ductus

cochlearis (Fig. 2E-F), and in the retinal epithelium all expressed high levels of clusterin (Fig. 2A,C). These three tissues are all secretory epithelia facing a blood/non blood interface.

b. Expression of clusterin in the mouse male reproductive tract

1. Localization of clusterin during testicular development

Clusterin expression is found in rat Sertoli cells during germ cell development (Smith et al. '92; Zakeri et al. '92). This pattern of expression was also seen in the adult mouse testes. During testicular development, premeiotic germ cells including primitive type A and B spermatogonia are present in 7-8 day old testes (Nebel et al., '61; Bellve et al., '77). Cells undergo the first meiotic division and late pachytene spermatocytes are present in 17 day old testes. Adult testes include stages from the stem cell to the fully differentiated spermatozoa stage. Interstitial Leydig cells and supporting Sertoli cells are found at all developmental stages.

Cell death plays an active role during spermatogenesis. In fact, germ cells have been shown to die at every stage during spermatogenesis (Blanco-Rodriguez and Martinez-Garcia, '96). We examined the expression of clusterin in the testis and found the mRNA to be expressed exclusively in the Sertoli cells as localized by *in situ* hybridization (Fig. 3). Sertoli cells expressing clusterin mRNA were found throughout the seminiferous tubules in the testis of 7 day old mice (Fig. 3A). Sertoli cells expressing clusterin mRNA are located peripherally during germ cell maturation and testicular development as seen in testis from 17 day old (Fig. 3B) and adult (Fig. 3C)

mice . To confirm these results we investigated the expression profile of clusterin in the testes of two mutant mouse strains: the *at* (Fig. 3D), and W^v (Fig. 3E) strains. The *at* mutant is virtually devoid of germ cells (Handel and Eppig, '79), and the W^v has limited immature germ cells similar to 7 day old and 17 day old wild type mice. The absence of germ cells does not affect clusterin mRNA expression in the Sertoli cells of adult *at* mice. In addition, expression of clusterin in the adult W^v testes resembles that seen in the testis of 17 day old wild-type mice. Therefore the clusterin mRNA is clearly expressed in the Sertoli cells and the presence of germ cells does not affect the expression of clusterin in these cells.

To determine if the clusterin mRNA was a functional transcript, we examined the expression of clusterin protein in the male germ cells. We used a variety of antisera raised against different subunits of the protein. The specificities of the different antisera were first analyzed by reactivity to recombinant clusterin protein sequences. In-frame fusions of the coding regions for the α and ß subunits and proprotein with the *E coli* MalE protein (Fig. 4A) were prepared in the expression vector, pMalcRI, expressed in DH5αf hosts, purified by amylose affinity chromatography and used as antigens on Western blots (Fig. 4B). Anti-S45 recognizes the ß subunit (69 kDa) and proprotein (91 kDa) fusions but not the α subunit (67 kDa) (Fig. 4B). In contrast, anti-S35 recognizes specifically the α subunit and proprotein fusions but not the ß subunit (Fig. 4B). The anti SGP-2 recognizes both subunits and the pre-protein (Fig. 4B). The anti-301 antiserum, which was raised against a peptide epitope located in the α-subunit, recognizes the full length α-subunit and proprotein

fusions (Fig. 4B), showing the same specificity as anti-S35 antibody. In this assay the anti-SGP-2 antibody shows the greatest avidity for clusterin peptide epitopes as suggested by the strong signal (Fig. 4B) with a 5 second exposure to film. In contrast the exposure times for the other antibody preparations in Figure 4B were either 5 sec (anti-S35), 1 min (anti-301) or 2 min (anti-S45).

These antibodies were used to localize the protein in the male reproductive system by immunohistochemistry. Anti-S35 antibody showed strong reactivity to the sperm protein in the testis (Fig. 5B). These results are consistent with those reported by Kierszenbaum et al ('88). Anti-S45 antibody, which recognizes the ß subunit, does not stain in the adult testis. Anti-SGP-2, which recognizes both the α and ß subunit with much stronger reactivity to the ß subunit, produces a signal that is slightly above background in the adult testis. In comparison to the above antibodies, anti-301, raised against a synthetic epitope of the α subunit, recognizes an epitope on the sperm, with more specific reactivity to the sperm head in the adult testis (Fig. 5C-D). As such these antibodies recognize different subunits as well as epitopes of the clusterin protein.

2. Regional and intracellular localization of clusterin mRNA in the caput of the epididymis of adult mice

Clusterin mRNA was not confined to the Sertoli cells of the testes in the male reproductive tract, since the transcript was also detected at high levels in the head of the epididymis (caput, Fig. 6B) as detected by northern blot analysis. Given the

intense expression in the caput, we specifically analyzed the epididymal cell types expressing clusterin.

Segmental expression of clusterin mRNA was detected in the caput of the adult epididymis using both radioactive (Fig. 7A) and non-radioactive (Fig. 7B) *in situ* hybridization techniques. The latter method uses digoxygenin labeled anti-sense probes and specifically localizes clusterin mRNA without the scattering problems inherent in ^{35}S-labeled probes. Both radiolabeled and digoxygenin labeled sense probes were used to confirm the segment specific expression pattern of clusterin. Segment 1 of the adult caput is highly vascularized, has a wide diameter and lacks detectable spermatozoa. Segment 2 is less vascularized, and has a smaller lumen that contains some sperm. In segment 1, clusterin mRNA is expressed at low levels in only a few cells (Fig. 7A-B). Clusterin mRNA expression is slightly increased in segment 2. With a progressively decreasing diameter and increased volume of sperm, the two lobes of segment 3 express high levels of clusterin mRNA. As the lobes of segments 4 widen in diameter, expression of clusterin mRNA declines slightly. The localization of the protein in different segments of the epididymis correlates with the expression of the message (Fig. 7C).

In addition to the segmental expression of clusterin mRNA, there seem to be differences in its subcellular localization within the epithelial cells expressing the gene. The few epithelial cells that express clusterin mRNA in segment 1 show localization in the basal and perinuclear regions (Fig. 8B-C). The mRNA is restricted primarily to the perinuclear region of the cell in segments 2 (Fig. 8C). Clusterin expression is

detected in the perinuclear region and toward the lumen in the lobes of segment 3 (Fig. 8D). The majority of the clusterin mRNA is located above the nucleus and subjacent to the luminal membrane in segment 4 (Fig. 8E-F).

3. Clusterin mRNA expression and germ cell maturation in the epididymis

Clusterin mRNA is expressed in all the epithelial cells of the 7 day old mouse epididymis. The specific putative segments are not clearly defined in the 17 day old epididymis. However, clusterin expression is clearly segment specific and distinct from that found in adult epididymis (Fig. 7D). The efferent ductules can be easily distinguished at this time, and similar to the adult epididymis, clusterin mRNA is not expressed in the efferent ductules. However, clusterin mRNA appears to be expressed at high levels in segment 1, and in the segments that will become segments 3 and 4 but have yet to differentiate. These data suggest that the expression of clusterin mRNA may be regulated by the appearance of mature germ cells in the epididymis. To specifically address the relationship between germ cells and clusterin expression, we examined the epididymides of the mutant strains described above. Epididymides of *at* and $\underline{W^v}$ mutant mice showed no expression of clusterin mRNA in the efferent ductule (Fig. 7E and F, respectively). Similar to day 17 wild type mice, the epididymides of both mutant strains showed abundant expression of clusterin mRNA in segment 1, and in the remaining segments of the caput. It therefore seems that clusterin expression is regulated by the presence of mature sperm in the epididymis.

c. Expression of Clusterin in the Mouse Female Reproductive Tract

During the development of ovarian follicles, most of the follicles are destined for atresia, with only a few undergoing ovulation. Atretic follicles are morphologically identified by pyknotic nuclei within granulosa cells, surface hemorrhages or a collapsed deformed appearance (Byskov, '78; Gilbert et al., '83). Tilly et al. ('91) found apoptotic fragmentation in atretic follicles of the pig and suggests that apoptosis is a basic mechanism associated with ovarian follicular atresia in mammalian as well as avian species. In the ovary, clusterin transcript was localized by *in situ* hybridization to the granulosa cells of developing and atretic follicles (Fig. 9A-C). There is a pronounced difference in the steady state level of clusterin mRNA expression in developing versus atretic follicles. Expression increases several fold in atretic follicles compared to that of normal developing follicles.

We also examined the expression of clusterin in the ovary at the level of protein. All three antibodies that recognize the α subunit showed similar localization of the protein, on the zona pellucida, although the intensity of the signal varies according to the antibody used (anti-S35: Fig. 9D; anti-301-4: Fig. 9E; anti-SGP-2: Fig. 9F). Anti-S45, which is specific for the ß subunit, did not produce any detectable signal. It appears that the granulosa cells make clusterin and secrete it for binding to the oocyte. The protein was also expressed in atretic follicles (Fig. 9G).

Similar to the ovary, we detected clusterin mRNA in normal and dying cells of the mouse uterus. Some uteri displayed expression in selected cells of the glands alone (Fig. 10A-B). In other uteri, expression was confined to selected epithelial cells

of the ducts. This expression in the ducts was found only in cells displaying pyknotic nuclei (Fig. 10C-D). These results were interesting in that the presence of such cells has not been reported in the uterus. The varied expression of clusterin in the different uteri may reflect the different cycling phases that the uteri were in at the time of dissection (for example, estrus versus proestrus). The expression of clusterin in the female reproductive system is complex in that clusterin is found in both normal and dying cells.

D. DISCUSSION

Clusterin is expressed in many tissues types and under different physiological conditions (Rouleau et al, '90; Wong et al, '93; Aronow et al, '93; Witte et al, '94; Jordan-Starck et al, '94; Bursch et al, '95). Clusterin has been postulated to promote cell-cell interactions, to mediate aggregation of Sertoli cells and red blood cells (Fritz et al. 1983; Blaschuk et al. 1983); to assist spermatogenesis (Zakeri et al. '92), and to modulate complement formation (Jenne and Tschopp, 1989), where it is claimed to stabilize membranes during apoptosis. Hence, the control of clusterin expression is clearly complex and not yet understood in any detail.

Interestingly, we found no direct correlation of clusterin expression with sites of programmed cell death in the developing limb. We have specifically studied the interdigital area, for which we have several markers, and we detect no expression of clusterin in these areas. It is difficult to explain the inconsistencies between our results to the one report that detected the expression of clusterin in the interdigital mesenchyme (Buttyan et al., '89). Since we can detect low levels of clusterin the differentiating cartilage of the limb, we do not believe that a technical problem exists. In addition, our findings are consistent with Garden et al.,('91) who did not detect clusterin in the interdigits of the limb but did detect it in the differentiating skeletal muscle.

Rather, the expression of clusterin suggests that it functions in some manner to condition non-blood fluids. The uterine glands and ducts, in which expression is high, surround the early embryo, a foreign body protected from cytolysis. Clusterin

message is also abundant in the developing inner ear, choroid plexus, and eye. At all of these sites, the epithelial cells maintain a blood/non-blood fluid boundary. The hypothesis that clusterin acts as an anti-inflammatory agent would be consistent with this argument that clusterin may function to maintain the membrane integrity of barrier cells that are adjacent to a non-blood surface.

In the male reproductive tract, clusterin expression has been shown to be developmentally regulated in the rat testes and epididymis (Grima et al. '90; Sylvester et al. '84; '91). The distribution of clusterin mRNA along Sertoli cell processes adjacent to elongating spermatids correlates with similar localization of the protein determined by immunohistochemistry. In line with the supportive and nutritive roles provided by Sertoli cells, our data suggest that clusterin may be involved in the regulation of these functions during testicular development. To determine if developmental timing of clusterin expression correlated with the presence of germ cells, we examined the differential expression of clusterin mRNA in the testis of immature wild type mice and two mutant mouse strains. Clusterin expression in the testes of both the *at* and *W^v* mutant mice is apparently normal. Since the steady state level of clusterin mRNA in Sertoli cells of the wild type, *at* and *W^v* mutants is similar, the expression of clusterin mRNA in Sertoli cells appears to be independent of either immature or mature germ cells.

Immunohistochemical staining with anti-SGP-2, anti-S35 and anti-301 antibodies (all of which recognize the α subunit) has demonstrated that the protein is secreted into the lumen of the seminiferous tubules and that it associates with the

surface of the spermatozoa. The demonstration that anti-S45, which was raised against the β subunit, does not cross-react with the sperm membrane suggests that either only the α-subunit is present on sperm, or that the interaction between the β-subunit and the membrane masks the epitopes on the β-subunit. Furthermore, it is of interest that the anti-S35 and anti-301 antibodies, both of which recognize the α-subunit, do not localize the protein to the same region of the sperm. Anti-S35 localizes the protein to the sperm tail, whereas anti-301 preferentially recognizes an epitope in the head of the sperm. It has previously been suggested that the clusterin gene is expressed by sperm, but that the post-translational modification and proteolytic processing of the gene product is distinct in the sperm (Smith et al., 1992). The results reported here suggest that anti-S35 recognizes a protein that is related to, but distinct from the α-subunit, which does not contain the epitope recognized by anti -301. Thus the sperm tails bind the α subunit, while the sperm head may bind the full length or proprotein clusterin.

Numerous studies have examined the developmental regulation of clusterin expression in the testes and epididymis of several species including the rat (Grima et al, '90; Sylvester et al, '91; Grima et al, '92; Cyr and Robaire, '92; Wong et al, '93; Rosemblit and Chen, '94) and the ram (Tung and Fritz, 1985; Rosenior et al, 1987; Cheng et al, 1988), and have noted that the most abundant expression of clusterin at the mRNA and protein levels occurs in the caput of the epididymis (Hermo et al, '91; Zakeri et al, '92; Cyr and Robaire, '92; Sensibar et al, '93). We also detect message expression in selected segments of the mouse epididymis with the protein

product bound to sperm within those segments. With the exception of the efferent ductule, clusterin mRNA is expressed throughout the caput epididymis during development. It is downregulated in segments 1 and 2 of the caput epididymis in the adult animal, but remains elevated in segments 3 through 5. At this time spermatozoa are present in the lumen of the ducts, suggesting that germ cells may play a role in the repression of clusterin expression in segment 1 and 2 of the caput. Our studies on immature and mutant epididymides indicated that mature germ cells are required to repress clusterin expression in segments 1 and 2 of the mouse epididymis as clusterin mRNA expression is also high in segment 1 and throughout the caput epididymis of the adult $\underline{W^v}$ mutant mouse in which although germ cells are present in the testis of $\underline{W^v}$ mutant mice, they have only proceeded as far as the first mitotic division.

In addition to the segmental regulation of clusterin expression, there are also differences in the cellular localization of clusterin mRNA between segments. In the adult epididymis, clusterin mRNA is found in the basal and perinuclear regions of the epithelial cells in segment 1 and 2, and progressively localizes to more luminal locations in more distal segments. In the $\underline{at}$ and $\underline{W^v}$ mutant mice, clusterin mRNA is localized predominantly in the perinuclear region of the cells, as it is in the 7 and 17 day old wild type mice. This suggests that in addition to influencing the absolute level of clusterin mRNA levels, the developmental program may also specify the localization of the mRNA in the cell to enhance the synthesis and secretion of the protein.

Constitutive expression of clusterin in the Sertoli and epididymal epithelial cells argues for a wider role for clusterin than in cell death alone. The female reproductive tract, however provides a model in which both developing and dying cell types can be observed. Expression of clusterin is induced in the granulosa cells both during maturation and atresia of the ovarian follicle. Protein localization on the sperm may protect them from complement lysis in the vagina (Garden et al. '91), while its localization on the zona pellucida may serve to protect the ovum. In the nonpregnant uterus, expression was found selectively in normal cells of glandular epithelium. Expression was also detected in pyknotic epithelial cells of the uterine duct. However, during implantation clusterin is expressed selectively in cells of both the uterine glands that are actively secreting hormones and the ducts that transport them. Expression of clusterin in both normal and dying cells again suggests for a common role for this gene under these situations.

Our findings of the differential distribution of clusterin lead us to consider the latter group of hypotheses more probable. Clusterin expression was most abundant in tissues that face a barrier between blood and external fluid; and clusterin protein was associated with cells that may consequently face immunologic challenge or are undergoing specific changes such as differentiation. The protein appears to bind to the plasma membrane of many cells undergoing membrane remodelling, and may therefore play a central role in maintaining membrane integrity by protecting the cells from complement fixation (Tenniswood et al., 1992). Thus clusterin may serve to play an immunoprotective role for privileged sites or a supportive role for these cells.

With this working hypothesis of a supportive/protective role, it is still possible for clusterin to be associated with cell death, especially if it helps to suppress an inflammatory response to cell death, for example in the dying cells of the involuting prostate upon hormone withdrawal. Yet, it would seem to be more of a result of the process rather than a cause. As a defense mechanism, certain tissues may increase the levels of clusterin transcription to protect against events leading to cell death, but this response is probably too late to prevent it.

Chapter 2

Characterization of Normal, Abnormal, and Chemically Induced Cell Death During Limb Development

A. Objective

B. Materials and methods

C. Results:

a. Morphological and biochemical analysis of cell death in the developing limb

1. Morphological detection of cell death

2. Engulfment and removal of dead cells

3. Disintegration of dead cells

4. DNA fragmentation of dead cells

b. Analysis of cell death pattern in abnormal limb development

c. Morphological and biochemical analysis of retinoic acid induced cell death in the normal and mutant limb

D. Discussion: The nature of normal and abnormal cell death in limb development

A. Objective

Cell death is a prominent aspect of embryogenesis. In the developing mouse limb, cell death begins at day 10.5 and peaks at day 14.5. During this period, enough of the tissue undergoes cell death to allow molecular analysis. The state of cell differentiation, level of proliferative activity, differential morphogen distribution or other cellular characteristics may mediate the selective sensitivity suggested by the highly localized and differentially controlled cell death. We have used several approaches to determine the nature and pattern of cell death in the developing limb as well as to investigate the effect of altering this pattern by a genetic mutation or a chemical compound.

We first examined the pattern of cell death in the normal mouse limb by establishing specific markers that would indicate the location and potentially apoptotic nature of cell death. Most authors have assumed, based on morphological evidence, that the death is apoptotic (Schweichel and Merker, '73; Hinchliffe, '81). The concept of apoptosis, however, encompasses several aspects, including a characteristic DNA fragmentation, cell shrinkage and fragmentation, and removal of dead cells by active phagocytic cells. The process is cell-specific, non-inflammatory, and, in programmed cell death, under genetic control (Hinchliffe, 1981; Zakeri et al., '93).

Having established the nature of cell death in the normal mouse limb, we employed two tools to further characterize the role and regulation of cell death in the

developing limb. In one approach we used mutant animals with limb deformity to demonstrate the importance of cell death in the correct formation of the limb pattern. We showed that abnormal phenotype in the Hm limb was the result of inadequate cell death in specific zones of the developing limb.

In a second approach, we used an agent, RA, which has been shown to enhance cell death in regions of normal programmed cell death in the early embryonic limb (Alles and Sulik, '89). RA can also regulate genes that are responsive to it. Given these facts, one can assume that RA may direct the pattern of digit formation by affecting cell death. Several investigators have reported on the teratogenic effect, and some have argued that the teratogenic effect is the result of an increase in the amount of cell death. If this is true, the effect should result from indiscriminant cell killing. We have examined the effect of all-trans-RA in normal and mutant limbs during the peak of cell death in limb development and have characterized the nature of RA-induced cell death to be apoptotic.

B. Materials and Methods

a. Animals

Hammertoe (Hm, C3 He B/FeJLe-ala Ca^J SlHm, Jackson Labs) homozygous normal (+/+) and homozygous abnormal (Hm/Hm) mice were mated overnight. These matings were parallel non-hybrid crosses (+/+ X +/+) and (Hm/Hm X Hm/Hm). For heterozygous offspring, Hm/Hm X +/+ matings were performed. Females were checked for the presence of a vaginal plug. Matings and retrieval of tissues were performed as described in Chapter 1.

b. Retinoic acid treatment

Pregnant females were gavaged with 200 mg/kg body weight of all-trans RA at day 14 of gestation (Sigma, 20 mg/ml suspended in sesame oil) using biomedical animal feeding needles (Perfektum). These doses were consistent with those determined previously by Alles and Sulik (1989) to be teratogenic. Control dams were treated with sesame oil only. The treated dams were sacrificed by cervical dislocation approximately 12 hours after the treatment time, and the fetuses were removed.

c. Tissue fixation and preparation for frozen sections

As described in Chapter 1

Limbs were laid down in order to be able to cut longitudinal sections of the ectodermal ridge and all the interdigital zones.

d. Slide preparation

As described in Chapter 1

e. Light and electron microscopy

These studies were performed in collaboration with Daniela Quaglino at the University of Modena. For a more detailed analysis limbs were processed for semithin sections and for transmission electron microscopy (TEM, Zakeri et al., 1993). Samples were fixed in 2.5% glutaraldehyde in 1X PBS at 4°C for several days, and postfixed in 1% osmium tetroxide in 1X PBS at 4°C for 1 hour. They were then dehydrated in graded ethanols, in propylene oxide, and finally embedded in Spurr resin. Semithin sections were cut with a Reichert Ultracut ultramicrotome and then stained with toluidine blue and observed in a Zeiss Axiophot light microscope. Pictures were taken at 6, 25 and 157X magnification. Selected areas were cut in thin sections, collected on copper grids and stained with uranyl acetate (5% in 70% ethanol) for 15 minutes and then in lead citrate for 10 minutes. Observations were done with a Philips 400T and with a JEOL 1200 XII electron microscope.

f. Hematoxylin staining

Slides were brought to room temperature from -70°C and stained in filtered Harris Hematoxylin (Sigma) for 3 minutes. Slides were then washed in tap H_2O three times for one minute each and mounted with Crystal Mount (Fisher).

g. Supravital staining

For assessment of the extent of cell death, fetuses were divested of extra embryonic membranes and supravitally stained for 30 minutes at 37°C with a 0.01% solution of nile blue sulphate (NBS, Sigma) in 1X PBS. The exact timing of the staining is very important in order to avoid nonspecific staining.

h. Lysosomal activity

To measure lysosomal activity, we analyzed acid phosphatase by use of a kit (Sigma, 180-A) as in Zakeri et al. (1994). Tissues were fixed and sectioned as above. Sections were post-fixed with citrate (pH 3.6) -acetone - 37% formaldehyde (13:33:4) for 30 seconds and then washed in dH_2O for 1 minute. Sections were treated with naphthol AS-BI phosphate and fast garnet stain for 1 hour at 37°C (0.6 ml $NaNO_3$ and 0.6 ml fast garnet stain were mixed and allowed to stand for 5 minutes. 22.8 ml of prewarmed dH_2O , 3 ml of acetate solution and 3 ml of napthol were added). Slides were washed in running tap H_2O for 2 minutes and then air dried for 15 minutes. The slides were counterstained with methylene blue (1:100 dilution), rinsed in dH_2O two times for 1 minute each and mounted with Crystal Mount (Fisher). The acid phosphatase activity is detected as a distinct red focal precipitate.

i. DNA analysis for fragmentation

1. Gel electrophoresis: For DNA analysis by gel electrophoresis the distal portions of the hand and foot palettes from day 14.5 embryos, containing the interdigital webs

and the developing digits, were dissected out and treated immediately. Tissue was digested with 0.6 µg/ul proteinase-K (Jersey Lab and Glove Supply. in 50mM Tris buffer with 100mM EDTA and 0.5%SDS) overnight at 37°C. The mixture was treated with 33 µg/ml RNase A (Jersey Lab and Glove Supply), extracted with saturated phenol (Boehringer Mannheim), and loaded onto 2% agarose gels together with ethidium bromide. After electrophoresis the gels were viewed and photographed under UV light. DNA was also obtained from apoptotic thymocytes and analyzed in an identical manner.

2. In Situ end labeling: To identify cells with fragmented DNA we used a nonisotopic DNA end labeling in situ technique, employing digoxigenin-11-dUTP and terminal transferase (ApopTag™ Peroxidase Kit, Oncor, Gaithersburg MD, Zakeri et al., 1994 and Zakeri and Ahuja, 1994). Sections were post-fixed in ethanol: acetic acid (2:1) for 5 minutes at -20°C and washed in 1X PBS twice for 5 minutes each. Endogenous peroxidase was quenched with 0.1% hydrogen peroxide in 1X PBS for 20 minutes and then rinsed in 1X PBS twice for 5 minutes. Sections were equilibrated in equilibration buffer for 20 minutes before the addition of reaction buffer containing TdT (terminal deoxynucleotidyl transferase) enzyme and digoxigenin-11-dUTP for 90 minutes at 37°C. The reaction was stopped by incubating sections in stop/wash buffer for 30 minutes at 37°C followed by three washes in 1X PBS for 5 minutes each. The digoxigenin-11-dUTP containing oligonucleotide extensions were detected by anti-digoxigenin-peroxidase for 30 minutes followed by four washes in 1X PBS for 5

minutes each. Color development was performed by staining sections in stable diaminobenzidene (DAB, Research Genetics, Inc.) for 4 minutes. Slides were then washed in tap H_2O three times for one minute each, distilled H_2O for 5 minutes. Sections were counterstained with methyl green (0.5% methyl green in 0.1M sodium acetate, pH 4.0) for 3 minutes and washed in dH_2O three times for 1 minute each. Sections were then rinsed in 100% butanol three times for 2 minutes each, followed by three rinses in toluene for 2 minutes each, and mounted with Permount®.

j.Immunocytochemistry

For detection of phagocytic cells, frozen sections were brought to room temperature, rehydrated in a graded series of 20%, 10% and 5% sucrose in 1X PBS for 5 minutes each and washed in dH_2O for 5 minutes. Endogenous peroxidase was inactivated by treating sections with hydrogen peroxide (1 H_2O_2 (30%, Sigma) : 2 H_2O) and washed in dH_2O and 1X PBST (PBS with 0.1% Tween 20 (Polyoxyethylenesorbitan Monolaurate, Sigma) for 5 minutes each. Non-specific binding sites were blocked with 5% dry milk (Carnation) and 0.1% gelatin (Fisher) in 1X PBST for 30 minutes at 37°C. Slides were washed with 1X PBST-gel for 5 minutes and primary antibody F4/80 (recognizes the surface antigen F4/80 specific to mature macrophages, Serotec, Hume et al. ('84), diluted at 1:10 in 1X PBST-gel was applied to the slides for overnight incubation at 4°C. After three washes with 1X PBST-gel for 5 minutes each, a 1:50 dilution of secondary antibody: peroxidase labeled $F(ab)_2$ fragment of goat anti-rat IgG (H+L) (Jackson Immunological) in 1X PBST-gel was applied and slides

were incubated for 2 hours at room temperature (RT). Slides were washed three times in 1X PBST-gel for 5 minutes each and immunoreactivity was visualized by DAB staining for 2 minutes. Slides were counterstained with hematoxylin (Sigma) for 30 seconds, washed in dH_2O three times for one minute each and mounted with Crystal Mount.

C. Results

a. Morphological and biochemical analysis of cell death in the developing limb

1. Morphological detection of cell death

We began our studies with one of the most-used indicators of cell death, that of morphological examination of the dead cell by light and electron microscopy (Hinchliffe, '81). The day 10.5 embryonic limb bud displays a homogeneous population of cells along with the presence of a few blood islands (Fig. 11A). A few dead cells are occasionally observed in the marginal area of the day 10.5 limb. At day 11.5 of gestation different zones of the limb begin to be specified (Fig. 11B). The areas of condensed cells and separate cells became more prominent in day 12.5 embryonic limb (Fig. 11C). Some regions consist of condensed cells that will give rise to cartilage and bone of digits (Fig. 11E). Other regions display separated cells associated only by thin extracellular connections (Fig. 11F). The regions specifying digits and the interdigital regions are clearly defined in the day 13. 5 limb (Fig. 11D). The interdigital cells of the limb begin dying at this stage and engulfed apoptotic cells are detected (Fig. 11G).

2. Engulfment and removal of dead cells

Dead cells are easily detected in three dimensions using the vital dye Nile blue sulfate (NBS). NBS stains the acidic compartments of cells, such as lysosomes. Lysosomes are not initiators of apoptotic cell death (Schweichel and Merker, '73; Zakeri et al., '95). However large secondary lysosomes of phagocytes do participate

in the disintegration of engulfed apoptotic fragments. The dead cells are revealed by NBS in the anterior and posterior marginal zones in day 12.5 limbs (Fig. 12A). As limb development progresses, the amount of cell death expands in day 13.5 limbs and peaks in the interdigital regions of the limb by day 14.5 (Fig. 12B). Subsequently, the process of cell death in the interdigital regions is reduced and completed by gestational day 15.5 (Fig. 12C).

Having established the importance of phagocytic cells in apoptotic cell death, we attempted to determine the origin of these cells. The early literature suggested that neighboring mesenchymal cells may take on phagocytic characteristics and be involved in the engulfment of and disintegration of apoptotic bodies (Schweichel and Merker, '73). On the other hand, several blood islands may be the source of true macrophages (Hinchliffe, '81). We therefore asked if the phagocytic cells would express a macrophage-like surface protein. We used the F4/80 antibody, which specifically recognizes mature macrophages and monocytes (Hume et al., '84). We found that these phagocytic cells did indeed express this macrophage-like surface protein (Fig. 13A-C). This expression was seen as early as day 12.5 when cell death is confined predominantly to the lateral sides of the developing limb (Fig. 13A). The amount of staining increased and was detected strongly in limbs of day 14.5 limbs (Fig. 13B-C). Thus, although the phagocytes are postulated to be neighboring mesenchymal cells, they are stained by F4/80 antibody. Our results indicate that these cells have properties of mature macrophages, but their origin is still unclear. However, the evidence suggests that they are macrophages derived from the blood

cells have properties of mature macrophages, but their origin is still unclear. However, the evidence suggests that they are macrophages derived from the blood system since they stain for F4/80. Since there are blood islands in close location to regions where cell death occurs, macrophages could easily migrate to the site of cell death.

3. Disintegration of dead cells

Following the phagocytosis of the dead cells the cell fragments and cell debris must be destroyed. Along with presence of these phagocytic cells, we also wanted to test if they were actively involved in the breakdown of apoptotic bodies. This destruction is accomplished by lysosomal enzymes in the phagocytosing cell. With a choice of several lysosomal enzymes, we chose to examine the activity of acid phosphatase as a marker for locating and evaluating cell death in the limb. For these studies, we used sections serial to the ones used for F4/80 staining to test for acid phosphatase staining. The expected low level of staining, indicative of the basal level of lysosomal activity, was observed in all cells of the limb. However, denser staining, and therefore more lysosomal activity, was detected in the areas where there was active cell death as indicated by both macrophage staining and light microscopy. Consistent with the results described above, lower intensity of staining was detected in the day 12.5 limb (Fig. 13D) as compared to the day 14.5 limb (Fig. 13E-F), directly in correlation with the amount of cell death. We know that acid phosphatase is not the only enzyme that is active in dying cells of the limb, as abundant staining

of another lysosomal enzyme, ß-glucuronidase is also observed (Zakeri et al., '95). Although this methodology does not conclusively separate the lysosomal activity within dying cells from that of the phagocytic cells, the fact that the staining extends beyond the dying cell and in some cases surrounds the boundary of the apoptotic body suggests that the activity is from a secondary source. Therefore, it seems that the primary source of acid phosphatase staining is provided by the secondary lysosomes of the phagocytic cell.

4. DNA fragmentation as a marker of cell death

A biochemical hallmark of glucocorticoid-induced apoptosis in thymocytes has been the formation of a ladder when DNA is electrophoresed due to internucleosomal DNA cleavage caused by the induction of specific endonucleases. We therefore asked if one could detect DNA ladders in areas of the limb that display of cell death. We used several DNA isolation methods to enrich for fragmented DNA. The conventional methods, involving analysis of isolated DNA from carefully dissected regions of the limb by gel electrophoresis, showed no DNA ladder (Fig. 14A). No fragments were detected with a more sensitive method involving radioactive end labeling of isolated DNA which easily detects DNA fragmentation in glucocorticoid treated thymocytes (Fig. 14B-C).

The lack of detection of DNA fragmentation may have been due to the fact that dead cells represent a small fraction of cells in the limb samples even with our careful dissection of the specific regions. To more specifically examine DNA

fragmentation in the limbs, we used *in situ* end labeling (Fig. 13G-I). With the use of terminal transferase and digoxigenin-11-dUTP we identified DNA fragmentation in day 12.5 limbs (Fig. 13G) and even more fragmentation was detected in day 14.5 limbs (Fig. 13H-I) in areas that corresponded with the presence of macrophages and acid phosphatase. Not all the apoptotic nuclei were stained. We attribute this to the fact that the cells may be at different stages of cell death and nuclear condensation.

The early and prominent nuclear condensation in addition to cytoplasmic changes of the affected cells is indicative of type I apoptotic cell death and is similar in morphology to what has been reported for thymocyte cell death (Schweichel and Merker, '73). Thus, cell death on the borders and in the interdigital areas of the limbs may be identified, quantified, and confirmed as apoptosis by the criteria of morphology, DNA fragmentation, phagocytosis, and lysosomal activity.

b. Analysis of cell death pattern in abnormal limb development

The argument for genetic control of cell death comes from isolation of mutants with abnormalities in cell death, found mainly in the nematode *C. elegans* (Ellis et al., '91). Mutant mice with apparent webbing in the toes, postulated to derive from abnormalities in cell death, have only recently begun to be studied. Knowledge of the normal pattern of cell death during mouse limb development allowed us to analyze the pattern of cell death in an abnormal situation.

As described by Green ('89), the Hammertoe defect is detectable as a continued presence of webbing between the digits starting at day 14.5 of gestation in abnormal homozygotes. This timepoint is also crucial for our studies as the peak of cell death occurs in normal limbs at this stage. By crossing the presumptive Hm/Hm resulting from crosses of Hm/+, we produced an F_2 generation showing 100% limb abnormalities. Day 14.5 embryos were investigated for the pattern of cell death using the established cell death markers. By NBS the pattern of cell death is clearly altered as seen by the diminution of stain in the Hm/Hm limbs at day 14.5 between digits 2-5 (Fig. 15B). However, dead cells were seen in the anterior and posterior necrotic zones and between digits 1 and 2.

Having established the altered pattern of cell death in the Hm/Hm mutant limb grossly, we then confirmed these results by the use of the established markers described above. By morphological and biochemical analysis we showed that this defect correlates with an altered pattern of cell death between these digits.The suppression of cell death between digits 2-5 was confirmed in semi-thin plastic sections (Fig. 16A). Similarly, the lack of cell death between digits 2-5 (Fig. 16B-C) and its normal appearance in the anterior and posterior marginal zones (Fig. 16E-F), as well as between digits 1 and 2 of the hand palette was confirmed by F4/80 antibody and DNA fragmentation staining. This suppression of cell death detected between digits 2 and 5 correlates with the lack of regression of cells that results in webbing between digits 2 and 5.

c. Morphological and biochemical analysis of retinoic acid induced cell death in the normal and mutant limb

We first used the vital dye NBS to confirm the ability of RA to induce cell death in our system. To be able to recover embryos and examine the effect of RA on developing limbs with respect to the time that cell death is detected, pregnant mothers were treated with all-trans RA by gavage (200 mg/kg body weight) at day 14 of gestation and embryos were retrieved 12 hours later. NBS staining in embryos treated with sesame oil (control) clearly showed normal cell death occurring in the interdigital webs of the digits as well as in the AMZ and PMZ (Fig. 17A). RA treatment enhanced the amount of cell death in comparable staged embryos (Fig. 17B). We have assumed that NBS stains the lysosomes of phagocytic cells. These results would suggest therefore that RA treatment would result in an increased number of phagocytes, a major element of apoptotic cell death required for the removal of dead cells (Hume et al., '84). To test this hypothesis, we examined serial sections from the entire foot plate for the experiments described below. Phagocytic cells, which engulf dying and dead cells in the limb express surface proteins specific to mature macrophages (Hume et al., 1984;). F4/80 antibody reacted with many phagocytic cells in the enhanced cell death regions after RA treatment (Fig. 17D-E). Although it is difficult to quantify the number of dead cells it appears that the phagocytes (as detected by F4/80 antibody) filled with apoptotic bodies were more extensive in RA-treated animals. Sections sequential to the ones used for phagocytic cell localization with F4/80 immunostaining were used for analysis of lysosomal

activity via acid phosphatase staining. Dense staining and therefore more lysosomal activity was detected in the limbs treated with RA (Fig. 17F). These findings suggest that more phagocytic cells are active in RA-treated limbs.

In RA-treated limbs, there was also a marked increase in the number of cells with fragmented DNA in the areas of PCD (Fig. 17G). These results further supported the argument that RA-induced cell death is apoptotic.

Once we determined that RA induces apoptotic cell death in normal developing limbs, we asked if this crucial regulator of pattern formation would induce cell death in the Hm mutant limb. To examine the effect of RA, the appearance of cell death was first measured by NBS at day 14.5 of gestation. The homozygous abnormal limb treated with oil continues to exhibit interdigital webbing at the peak of cell death (day 14.5) in the developing limb (Fig. 18A). Similar to the +/+ limb, RA selectively increased cell death in the AMZ, PMZ and between digits 1 and 2 of the Hm/Hm foot (Fig. 18B-C). More interestingly, we found that RA induced cell death between digits 2 and 5 of the mutant limb, which originally displayed a defect in cell death (Fig. 18B-C). In addition, the induction of cell death in the interdigital regions that originally displayed a suppression of cell death was further confirmed by F4/80 staining (Fig. 18D), lysosomal activity (Fig. 18E) and DNA fragmentation (Fig. 18F). Thus RA induces apoptotic cell death in previously cell death defective regions of the Hm limb.

D. DISCUSSION

Cell death is an important aspect of embryogenesis and serves a role in the normal form and function of the organism. However, little is known about the mechanisms involved in cell death during development. The mammalian limb serves as an excellent tool to elucidate the regulatory events regarding cell death as well as cell movement, division and differentiation. We have examined cellular death during normal limb development by use of markers that display the morphology of cell death, the presence of phagocytic cells and lysosomal activity. In addition *in situ* labeling confirms fragmentation of DNA in the mammalian limb. By these criteria, cell death in the developing limb can be categorized as type I or apoptotic cell death. However, the signal(s) responsible for cellular destruction and activation of phagocytosis by neighboring cells or recruited macrophages remain to be identified.

We show that the mouse mutant Hm, provides an abnormal system in which the pattern of cell death is specifically altered in the interdigital regions of the limb. The defect is quite distinct from other limb deformity mutants including the nearby mapped, homozygous lethal Hx where both induction and inhibition of cell death takes place and is more global (Knudsen and Kochhar 1981). The Hm defect in cell death is partial in the heterozygote and complete in the homozygote. Furthermore, the prevention of regression of these cells is manifested in flexure of the digits seen in the adult. Timing seems to be play an important role in this defect, since normal and mutant limbs are indistinguishable at day 12.5 of gestation and easily identified at day 14.5. In addition, the forelimb (approximately 12 hours ahead of hindlimbs in

development) are not as severely affected as the hindlimb in the Hammertoe mutant. The fact that this defect is not a generalized failure of cell death in the embryo or the limb since some cells do die in the mutant limb is very interesting. Thus, the defect in Hm might be an alteration in local cell-cell signaling resulting in altered patterning of the limb cell death.

We have studied the morphological and biochemical characteristics of RA-induced cell death compared to that of physiological cell death in the developing mouse limb. The several markers we used illustrate that RA induced cell death is apoptotic and that it appears to be identical to that of the naturally-occurring cell death in the normal developing limb. These markers also detect an increase in the amount of cell death in the zones of naturally- occurring cell death in the limbs of day 14.5 normal embryos whose mothers were treated with RA at day 14 of gestation. First, these results suggest that RA may have a part in the process of cell death. Stimulation of the same apoptotic criteria, such as the presence of apoptotic bodies. presence and activation of phagocytic cells, and DNA fragmentation by exogenously administered RA suggests that endogenous RA may serve a key role in the signaling mechanism of cell death. Second, they clearly demonstrate the temporal and spatial sensitivity of cells to RA. Although RA is required for many biological processes. elevated levels of RA have been shown to be teratogenic (Lammer et al., '85; Gudas et al., '93). Previous studies have shown that mesomelic defects result from administration of RA on day 11 of gestation (Kochhar, '77; Alles and Sulik, 1989). These researchers have demonstrated that the limb reduction anomalies correlated

to increased cell death in sites of naturally occurring cell death (mesenchymal core of the limb) of RA-treated embryos retrieved 12 hours later. We have developed a window in which specific doses of RA given to the pregnant mother would target cell death during normal limb development, without any obvious malformations resulting. The occurrence of malformation however depends on the time at which RA is administered during embryogenesis. In the day 11 limb bud, cell proliferation is highly active (Janners and Searls, '70; Kochhar, '77). Enhanced cell killing at this stage may destroy cells that will give rise to other cells in the limb, hence the deformities at this stage. At day 14, cell death is the most dramatic feature involved in shaping the limb. Enhanced cell killing in the interdigital regions probably removes cells that will die any way or may kill cells that will not give rise to other cells. As such the effect of RA as a teratogenic agent at this latter stage is not easily clarified. The lack of excessive cell death in other regions of the limb or the embryo does however argue against a toxic response of the tissue to RA, hence the altered phenotype.

The data obtained from the RA-treated Hm limb are consistent with these conclusions and provide further support of the importance of RA in limb development, in that it may serve a regulatory role in directing cell death. RA selectively enhanced apoptotic cell death in the AMZ, PMZ, and between digits one and two. Additionally, it selectively induced cell death in regions where normal PCD is suppressed, between digits two and five. This selective induction of mesodermal cells in regions predisposed to cell death suggests that these cells possess the cell death machinery and require or are sensitive to a signal such as RA that acts on its own or

in concert with other factors to trigger the sequence of events that lead to cellular demise. Therefore, in the Hm mutant limb, the capability of cells to die is preserved and upon a key environmental signal (possibly RA), cells will enter the cell death pathway. Evidence for this is provided by the fact that the effect seen in RA-treated Hm/Hm limbs is more drastic than that in RA-treated +/+ limbs. That is, since the cells in the INZ, AMZ, and PMZ are already dying in the normal limb, and the other cells are not sensitive to RA, little if any effect would be expected during this specific time. The cells of the INZ display a defect in cell death in the day 14.5 Hm/Hm limb and require exogenous RA to initiate cell death.

Chapter 3

Analysis of Cell Death Implicated Genes in the Normal and Abnormal Developing Limb

A. Objective

B. Materials and methods

C. Results:

a. Expression analysis of cell death associated genes in relation to cell death in the limb

b. Expression analysis of limb patterning genes in relation to cell death in the limb

c. Expression analysis of cell cycle genes in relation to cell death in the limb

D. Discussion: The genetic regulation of cell death in the developing limb

A. Objective

The markers we used illustrate the pattern of cell death in the normal limb and define the altered pattern in the mutant limb. However, they do not provide information regarding the genetic regulation of cell death. The association of a particular gene with cell death would give insight into the mechanisms that govern cell death. Although no single gene has served as a universal marker for cell death, several genes are reported to be putative markers. To ask what determines the targeting of specific cells for cell death in the presence of surviving neighbors, we have examined the expression profile of specific genes in relation to cell death at the level of message or protein in the developing limb. The Hammertoe mutant limb, displaying suppression of cell death, provides an excellent tool for comparison to test the association of these genes, if any, to cell death.

For our studies, we first analyzed the expression of genes that have been specifically associated with cell death in a number of situations. Tissue transglutaminase is upregulated in cells that are dying. bcl-2 has been shown to inhibit cell death. We first asked if these genes were expressed in the developing limb and then if their pattern was altered in the mutant limb.

The second set of genes we examined were of interest for two reasons. The Msx genes are found in the developing limb and they are expressed in the interdigital regions, and are therefore implicated in cell death. We first investigated the cell-specific expression of these genes and again asked if the pattern would be altered in the mutant limb. Since these genes are homeobox-containing genes, they are

considered important transcription factors in the patterning of the limb we also examined their expression before the onset of cell death at day 12.5 of gestation as well as after enhancing cell death in the interdigital regions at day 14.5 by treatment with RA.

It is thought that apoptosis may result from an improper activation of a family of protein kinases normally involved in the regulation of mitosis (Ucker, '91; Baserga and Rubin, '93). Most recently, members of cell cycle family have been investigated for their possible role in cell death. Several reports have identified the premature activation of various members of the cyclin dependent kinase family in apoptosis (Meikrantz et al., '94; Shi et al., '94). We have examined the expression of two Cdks that have been upregulated during cell death in in vitro model systems (Cdk1 and Cdk 2) and a third which has found to be expressed in terminally differentiated cells, rather than proliferating cells (Cdk 5). The expression pattern of these genes has been studied in the normal, Hm mutant, and RA-treated limbs.

B. Materials and Methods

a. Animals

As described in Chapter 2

b. Retinoic Acid treatment

As described in Chapter 2

c. Tissue fixation and preparation for frozen sections

As described in Chapter 1

d. Slide preparation

As described in Chapter 1

e.Immunohistochemistry

1. cyclin dependent kinases: Sections were rinsed in 1X PBST (phosphate buffered saline with 0.1% Tween) twice for 10 minutes each and incubated in 100% methanol containing 0.4% hydrogen peroxide for 20 minutes to remove endogenous peroxidase activity. 1X PBST washes were repeated and sections were incubated for 1 hour in blocking solution (Vector ABC kit) and overnight with primary antibody (0.1 μg/ml of anti-Cdc2 (Cdk1), anti-Cdk2, or anti-Cdk5 from Santa Cruz). After three washes with 1X PBST for 10 minutes each, sections were incubated with secondary

biotinylated antibodies (ABC kit) overnight, followed by three washes 1X PBST again. ABC reagent was applied for 2 hours and slides were rinsed again three times in 1X PBST for 10 minutes each. Finally, slides were incubated with DAB solution for 2 minutes, washed with dH_2O three times, counterstained with methylene blue (1:100 dilution), dehydrated and mounted.

2. transglutaminase (gift of P. Davies): To unmask hidden epitopes, two procedures were used. In the first procedure, as suggested by DAKO labs, slides were placed in a glass Coplin jar filled with 0.01M citrate buffer, pH 6.0. The jar was covered with Saran wrap® and a small hole was made in it. The jar containing the slides was microwaved twice for 5 minutes each (Dako Labs). In between heatings, dH_2O was added to prevent slides from drying out. After heating, dH_2O was added to the jar again and slides were allowed to cool for 20 minutes. The slides were then rinsed with dH_2O several times. In the second procedure (Harlow and Lane, '88), slides were incubated in 0.1% trypsin, 0.1% $CaCl_2$, 20 mM Tris (pH 8.0) solution for 10 minutes at room temperature. The digestion was stopped by rinsing slides with running cold tap H_2O for 5 minutes. The slides were then processed as described in "c" above.

f. Transformation and plasmid preparation

As described in Chapter 1

g. Radioactive in situ hybridization

As described in Chapter 1

Probes:

1. pbluescript KS bcl-2 (840 basepair insert EcoRI/HindIII, a gift of S. Korsmeyer)

2. pbluescript SK Msx-1 (237 basepair insert SacI/XbaI, a gift of D. Sassoon)

3. pCR II Msx-2 (270 basepair insert BamHI/EcoRI, a gift of D. Sassoon)

i. Cdk5 immunoprecipitation and kinase assay

Limbs at gestational days 12.5 (minimal cell death) and 14.5 (peak of cell death) were quickly frozen in liquid nitrogen. Tissue extracts were placed in extraction buffer (5 mL of RIP A Buffer per gram of tissue: 50mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.05% Na deoxycholate, 0.1% SDS, 0.1mM Na_3VO_4, 1mM DTT, 20 mM glycerophosphate, 2mM EDTA, 1 μg/ml aprotinin, 100 μg/ml methylsulfonylfluoride (in isopropanol), 10 μg/ml leupeptin) and manually homogenized on ice. The lysates were run through a syringe (26.5 gauge needle) until they flowed through easily and were incubated on ice for 15 minutes. Lysates were then centrifuged at 4°C for 15 minutes at 14000 rpm to remove cellular debris. Supernatants were kept on ice. Protein quantitation was performed by use of the Biorad protein assay dye reagent (diluted 1 to 4 in H_2O) with the addition of BSA (1 mg/ml) in different quantities for standards or 1-2 μL of each sample. Optical densities of all the standards and samples were read at 595nm. Concentrations were determined by a protein assay standard curve. To 500 μg of tissue extract, 1 μL of anti-Cdk5 was added and tubes

were placed on a rocker for 1 hour at 4°C. For the control, 1 μL rabbit IgG (0.1 μg/μL) was added instead. After one hour, 15 μL beads (GIBCO BRL Protein A Agarose) were added and tubes were placed on a rocker at 4° for an hour once again. The mixture was centrifuged briefly to pellet the Protein A agarose complex and the pellet was then washed three times in RIP buffer. The beads were then incubated in eb buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 20 mM EGTA, 80 mM B-glycerophosphate, 0.1 mM Na_3VO_4) on a rocker at 4°C for 5 minutes. Tubes were centrifuged briefly and buffer was removed with a syringe (28.5 gauge needle). The beads were then incubated with kinase buffer (20μL/ reaction: eb buffer + 2.5 Histone HI (Gibco BRL), 5 μM cAMP (Sigma), 10 μm ATP (Boehringer), 2.5 μCi γ-P32 ATP (Amersham) at 30°C for 30 minutes. Twenty μL of 2X Laemmeli buffer (0.1M Tris, 4% SDS, 20% glycerol (Fisher), 10% ß-mercaptoethanol (Sigma), 0.2% bromophenol blue (Fisher)) was added to samples and they were boiled for 2 minutes. Samples were centrifuged for 1 min at 3000 rpm at 4°C and 20μL were loaded into each lane. The gel was run at 13 mA for three hours. The gel was then dried at 80°C for 2 hrs and exposed to film (Dupont Cronex) overnight. To prepare the gel, a 12% separating gel (20 μL of 0.5M EDTA, 2.5 ml of 1.5M Tris pH 8.8, 50 μL of 20% SDS; 7.5 μL of 100% Temed, 4 ml of 30% acrylamide/0.8% bis sulfate, 100 μL of 10% ammonium persulfate and 3.3 ml H_2O) and 4.5% stacking gel (10 μL of 0.5 M EDTA, 625 μL of 10mM Tris, pH 6.8, 25μL of 20% SDS, 5 μL of 100% Temed, 750 μL of 30% Acrylamide/0.8% bis sulfate, 50 μL of 10% ammonium persulfate and 3.5 ml H_2O) were made.

C. Results

a. Expression analysis of cell death associated genes in relation to cell death in the limb

1. tissue transglutaminase

Tissue transglutaminase (tTG) is a cytoplasmic protein whose expression has been associated with cell death (Fesus et al., '91b; Piacentini et al., '94). In our system, we found tTG to be expressed in the apoptotic bodies of the interdigital regions (Fig. 19A-C). We also detected transglutaminase protein at high levels in the blood cells and at low levels surrounding the plasma membrane of all cells (Fig. 17B and C, respectively). It has been shown that tTG is constitutively expressed in specific cell types including endothelial cells, mesengial cells, and smooth muscle cells (Thomazy and Fesus, '89). Our data indicates that apoptotic cells express tTG at higher levels than do the surviving cells. In the Hm/Hm limb we detected the basal level of tTG in the mesenchymal cells and again the more abundant expression of the protein was detected in the dying cells of the AMZ, PMZ, between digits 1 and 2. However little if any expression was detected in interdigital regions (Fig. 19G) in the Hm limb. Given the association of tTG expression to cell death we asked if tTG would be increased in correlation to the increase number of dying cells after RA treatment. We found the expression of tTG to be upregulated in both normal (Fig. 19F) and mutant (Fig. 19H) limbs after RA treatment.

2. bcl-2

bcl-2 has been shown to inhibit cell death in a number of in vitro model systems, and has been shown to be responsible for survival of cells. We wanted to investigate the expression pattern of bcl-2 in relation to the dying interdigital regions of the limb. In situ hybridization analysis revealed abundant levels of bcl-2 mRNA in the differentiating pre-chondrogenic cells of the digital rays (Fig. 20A, C-D). As expected bcl-2 expression was not detected in the dying mesenchymal cells of the interdigit. Interestingly, its expression was also absent in the interdigital cells that do not show any signs of cell death. We then asked if bcl-2 would be expressed in the interdigital zones of the Hm/Hm mutant limb in which there is a suppression of cell death. Surprisingly, we found the expression pattern of bcl-2 to be identical to that of the normal developing limb. (Fig. 20B). It therefore seems that the absence of cell death does not imply the presence of bcl-2 gene in the developing limb.

b. Expression analysis of limb patterning genes in relation to cell death in the limb

The expression of the homeobox-containing genes is central to limb development, and two genes in particular, *Msx-1* and *Msx-2*, are of key interest as they have been implicated in cell death. We examined the expression of pattern forming genes in relation to cell death in the limb. During the development of the normal mouse limb, Msx-1 (Fig. 21A, C-E) and Msx-2 (Fig. 22A, C-E) are expressed in the interdigital regions, consistent with previous studies (Robert et al., '89; Hill et al., '89). We then examined the expression of these genes specifically in apoptotic

cells. We found that Msx-1 expression extends throughout the interdigit and is not detected in the apoptotic cells (Fig. 21E). Although the pattern of Msx-2 is more restricted than Msx-1, it also is not specifically associated with dying cells (Fig. 22E). To further confirm this lack of association, we examined the expression pattern of these genes Hm mutant limb. Similar to the normal limb, we find interdigital expression of both Msx-1 (Fig. 21B) and Msx-2 (Fig. 22B) in the mutant limb. The pattern of Msx-2 was restricted compared to Msx-1 in the mutant limb as well. Since these genes have been speculated to play an integral role in the early pattern of the limb, we wondered if their pattern might be altered just before the onset of cell death. We therefore compared the expression patterns of these genes at an earlier stage (day 12.5) of development in normal (Fig. 23A,C) and mutant (Fig. 23B,D) limbs, just before interdigital cell death. Msx-1 and Msx-2 were expressed in the limb bud in both normal (Fig. 23A,C) and mutant (Fig. 23B,D) limbs, respectively. The lack of association between these genes and cell death was further confirmed by the fact that we were unable to detect a difference in the expression pattern of these genes in normal (Fig. 24B,D) or mutant (Fig. 24C,E) limbs after RA treatment.

c. Expression analysis of cell cycle genes in relation to cell death in the limb

In collaboration with Drs. Debra Wolgemuth and Qi Zhang (paper submitted), we have found Cdk5 to be expressed and exclusively associated with a number of dying cell types. Cdk5 was easily detected in the dying cells of the developing limb (Fig. 25A). We have also shown Cdk5 to be expressed in other apoptotic cells of

developing embryo including cells of the retinal epithelium and mesenchymal cells of the eye (Fig. 25B) and the trigeminal ganglion (Fig. 25C). Cdk5 was also found in the atretic follicles of the adult ovary (Fig. 25D).

The expression of Cdk5 in the apoptotic cells of the anterior and marginal zones and in the interdigital areas of the foot plate was unique to Cdk5 and not representative of other members of the Cdk family (Fig. 26A). Serial sections of day 14.5 embryonic mouse hindlimbs were stained for the presence of Cdk 1, Cdk 2, and Cdk5 using antibodies. We were unable to detect Cdk1 (Fig. 26B) and Cdk2 (Fig. 26C) in any of the cells of the developing limb, surviving or dying.

Although Cdk5 is highly expressed in dying cells, we cannot conclude that it also has kinase activity. To determine whether Cdk5 kinase activity correlated with the intense immunoreactivity in dying cells, we examined its activity in embryonic limbs. Lysates of embryonic limbs at days 12.5 (minimal cell death) and 14.5 (peak of cell death) were examined for Cdk5-associated kinase activity in an *in vitro* assay using histone H1 as a substrate (Tsai et al., '93). The immunoprecipitates revealed an activity in the day 12.5 limb. As expected, the levels of Cdk5 kinase activity increased in the day 14.5 limb (Fig. 27).

The correlation of Cdk5 expression and cell death was further confirmed by the use of the Hm/Hm limb. The mutant limb exhibited Cdk5 expression in the dying cells of the AMZ, PMZ, between digits one and two, as expected. In agreement with previous results, only a few dying cells of the interdigital region expressed Cdk5 (Fig. 28C). Since the expression of Cdk5 was so closely associated with dying cells in the

AMZ, PMZ, and between digits 1 and 2, and reduced between digits two and five, we asked if it would be expressed in the extra cells that die upon RA treatment. Our results indicate that Cdk5 expression was indeed enhanced in RA treated normal limbs (Fig. 28B) and induced in RA treated mutant limbs (Fig. 28D). Unlike tTG, Cdk5 expression was specific to apoptotic cells only. These observations provide new insight into the possible function of this novel Cdk during apoptotic cell death.

F. Discussion

Commitment to cell death during development, and regulation of cell death once the decision is made, are key questions in developmental biology. We have analyzed the expression of a number of genes in the developing normal limb, the mutant limb, and the RA-treated limb to address the question of the mechanisms.

Numerous examples of alteration of pattern forming genes that correlate with altered phenotypes exist in the literature. However, we found no clear association between the *Msx* genes and cell death. Although these genes are expressed in the interdigital regions, they were not expressed in the dying cells, and in the case of Msx-1, extended beyond the interdigits. We performed *in situ* hybridization to examine differences in the expression pattern of these genes between the Hm and normal limb. A similar pattern was found in the mutant limb, suggesting again that these genes are not directly involved in cell death. They may still regulate positional identity. The *Msx* genes may prepare or allow the interdigital cells to respond to the death signal(s) in the normal limb and may serve the same function in the mutant limb, where the cell death signal is defective or absent.

As described by Novak and Korsmeyer ('94), bcl-2 was expressed in the developing digits of the normal limb. Consistent with its function as an inhibitor of cell death, its expression was not detectable in the interdigital zones. We thought that, as a promoter of the survival signal, bcl-2 would be expressed in the interdigital areas of the Hammertoe mutant limb that displays a suppression of cell death. We found that the expression of bcl-2 was not altered in the mutant limb. It was expressed

specifically in the chondrogenic regions of the developing digits. bcl-2 may play a role in chondrogenesis of the developing limb by specifying these cells for cartilage formation. Its expression in the digits may serve to protect these very crucial cells from cell death in the normal or mutant limb. The fact that bcl-2 expression is not altered in the mutant limb suggests that the effect of the Hm gene may occur well downstream of it. Yong Zhu in our laboratory has found that the expression of bax (bcl-2 family member which heterodimerizes with bcl-2, and whose overexpression enhances cell death) is distributed at low levels in all the cells of the developing limb. Taken together, these results indicate that if bax is distributed throughout the limb, and bcl-2 is specifically expressed in the digital rays, then in the interdigital cells only bax is present and therefore these cells will die. In the digital zones, bcl-2 is highly expressed compared to bax. Given that bcl-2 heterodimerizes with bax, the abundant levels of bcl-2 may be enough to "cancel" out the effect of bax and still have enough bcl-2 present to perform its specific function, protection of the cartilage cells. This model, however, must be more complex and involve other bcl-2 family members, as not all the interdigital cells (tissue surrounding developed digits) die. These possibilities are being examined by Young Zhu.

In our analysis of gene expression, we found two proteins, transglutaminase and Cdk5, to be specifically associated with apoptotic cells in the sites of programmed cell death in the normal limb. Further supporting these results, there was a direct correlation in the suppression of their tTG and Cdk5 expression with a suppression of cell death between digits two and five of the mutant limb, and a direct correlation

in their enhanced expression with increased cell death caused by RA treatment. Therefore these genes seem to clearly mark apoptotic cell death during limb development.

Tissue transglutaminase was expressed in apoptotic cells. Its expression was also detected abundantly in blood cells and at much lower levels in all other cells. The crosslinking of proteins that is catalyzed by tTG is thought to prevent leakage of contents of apoptotic cells. The fact that it is an irreversible activity (Piacentini, '95) is inconsistent with its expression in cells that are not dying. However, it has been shown that tTG accumulates in cells that are not actively dividing (Fesus et al., '91; Aeschlimann et al., '93; Piacentini et al, '94; 95). During limb development, all the cells are actively dividing in the day 11 limb (Janners and Searls, '70). By day 12, only 50% of the cells in the proximal mesoderm are dividing, with approximately 10% cell division in the chondrogenic area (Kochhar, '76). Although 90% of the cells are dividing in the distal mesoderm at this time, this amount declines with further limb development and cell condensation. Therefore fewer and fewer actively cycling cells are present with the progression of development. tTg may serve to stabilize the membranes of cells that are differentiated or in senescence. In terms of constitutive expression in cell types including endothelial cells, Thomazy and Fesus ('89) and Piacentini ('95) suggest a preparatory role of tTG during cell death in cell types that are constantly facing environmental stresses. Although tTG expression alone cannot definitely be indicative of dying cells, its expression in the dying cells is clearly visualized and transglutaminase may help stabilize membranes of dying cells.

Cdk1 and Cdk2, which are 58% and 62%, respectively, identical to Cdk5, were not detectable in the apoptotic cells of the limb. Thus, the association of Cdk5 and apoptotic cell death is not a general property of the Cdks. Although Cdk5 has been reported to be expressed in a number of cell types, its combined expression and activity have been found only in the developing and adult nervous systems (Meyerson et al., '92; Tsai et al., '93). We have shown that Cdk5 is not only expressed but also active in the dying cells of the developing limb.

The role for Cdk5 in cell death is unclear and is further complicated by the fact that its expression and activity are involved in neurogenesis (Nikolic et al., '96). These researchers have shown by transfection studies with dominant negative Cdk5 mutants as well as antisense p35 constructs, that Cdk5 along with its neuronal specific activator, p35, are necessary for neurite outgrowth during neuronal differentiation. It is therefore difficult to reconcile the two very different situations in which Cdk5 is highly expressed and active. It is possible that the specific activator may be crucial for defining the role of Cdk5 in neurogenesis vs. cell death. The fact that p35 is a neural specific protein (Tsai et al., '94), suggests that another protein must be involved in the activation of Cdk5 during cell death. The specific regulator for Cdk5 during cell death is unknown but may provide insight into the role of Cdk5 in this process. There might be some similarities for the roles of Cdk5 in cell death and neurite outgrowth. In the latter situation, the Cdk5-p35 complex and actin have been shown to be located in similar subcellular compartments (Nicolic et al., '96). Cdk5 has also been reported to phosphorylate the tau protein (Hosoi et al., '95). These proteins are an

integral part of the cytoskeletal network (Maccioni et al., '95). Again, given the specific activator, and specific situation, Cdk5 may serve a role in the dynamic rearrangement of the cytoskeleton in a cell whether it is required for the outgrowth of a neuronal cell or for the blebbing, or for formation of apoptotic bodies in dying cells. If Cdk5 does serve this function in apoptosis, its role would be to establish the phenotype of the dying cell rather than to be an effector gene that is required for the onset of cell death. Future studies will elucidate the mechanisms by which Cdk5 is regulated during cell death.

It is interesting to note that the several genes that have been actively implicated in the process of cell death (clusterin, transglutaminase, and now Cdk5) all have common elements. They have all been shown to be upregulated during cell death and they all seem to play some role in the structural form of the dying cell. They also seem to play a role in membrane (de)stabilization of the differentiated cell type. As one single feature (lysosomal activity, DNA fragmentation, etc.) does not tell the whole apoptotic story, it is reasonable to assume that more than one gene or family of genes would be required for the highly orchestrated process of cell death. It is also reasonable to assume that the same genes that are required for the daily maintenance of a cell may be upregulated and recruited to perform similar functions during cell death.

Chapter 4

Analysis of the effect of RA induced cell death on the limb phenotype and on components of the RA signaling pathway

A. Objective

B. Material and methods

C. Results:

a. Phenotypic manifestation of RA-induced cell death in the normal and mutant limb

b. Analysis of RA responsive factors in the normal and mutant limb

D. Discussion: The effect of RA on the limb

A. Objective

We have shown that RA selectively enhances cell death in sites of normal programmed cell death in the normal developing limb. More interestingly, we have established that this agent induces cell death in regions of the developing mutant limb that are defective for this process. Based on these results, we asked if an effect of this induced cell death in the mutant limb would be evident in the phenotype of the limb after birth. We found that RA did indeed have an ameliorating effect. We therefore investigated the effect of RA on the limb phenotype by administering it to pregnant females at different times of gestation to determine the most sensitive period and effective dose.

Given the effect of RA on cell death, we investigated the regulation of RA. Despite the discovery of accessory molecules that regulate RA accessibility (Maden et al., '88; '89) and transduce its signal (Petkovich et al., '87; Benbrook et al., '88; Brand et al., '88; Giguere et al., '90; Ishikawa et al., '90; Kastner et al, '90) the mode of the specific effect of RA on the patterning of the limb remains unclear. Even less is known about its part in directing cell death. We attempted to dissect the steps involved in the action of RA in the regulation of the pattern of cell death. We focused on the pattern of expression of RARß since it is expressed in the interdigital zones and implicated in the process of cell death during limb development. To assess the availability of RA as well as its ability to bind receptor proteins we used a transgenic mouse that has a retinoic acid response element upstream of a mouse *hsp68lacZ* construct. The pattern of the receptor expression and transgene expression

were compared in normal and mutant limbs to determine if the presence of the receptor as well as the presence of RA and its activity are altered in the mutant phenotype and therefore related to the pattern of cell death. Our results suggest that RA affects cell death independently of the RARß pathway.

B. Material and methods

a. Animals

As described in Chapter 2

For transgenic studies, homozygous males containing three copies of a 34 base pair sequence encoding the RARß retinoic acid response element were generously provided by J. Rossant ('91). The oligonucleotide was inserted upstream of a mouse heat inducible hsp68 promoter. Homozygous normal and mutant Hm females were crossed with transgenic males. Although the embryos of the Hm mothers were heterozygous for the Hm condition, the semidominant effect allows one to distinguish normal, heterozygous and homozygous animals from one another as early as day 14.5 of gestation. Dissected embryos were washed with phosphate buffer (0.1 M Na_2HPO_4, 3% phosphoric acid pH 7.3) and fixed in 0.2% glutaraldehyde, 2% formaldehyde, 0.01% sodium deoxycholate, and 0.02% NP-40 in phosphate buffer for 30 minutes. Embryos were then washed with wash buffer three times for 15 minutes each and incubated in X-gal (5-bromo-4-chloro-3-indoyl-ß-D-galactoside, Jersey Lab Supply) for approximately 4 hours at 37°C for color development.

b. Retinoic acid treatment

For these studies, pregnant mothers were allowed to come to term. Litters were assigned numbers and phenotypical analyses were performed in blind.

c. Skeletal staining

Skeletal staining was performed as described by Inouye ('76). Tissues were fixed in 95% ethanol for 4 days. After this time, the skin was removed, and tissues were placed in acetone for 1 day to remove fat. Tissues were then stained in skeletal stain (1 volume 0.3% filtered alcian blue (Sigma) in 70% ethanol, 1 volume 0.1% filtered alizarin red (Sigma) in 95% ethanol, 1 volume glacial acetic acid, and 17 volumes 70% ethanol). Following this, tissues were quickly rinsed in water and cleared in 1% aqueous KOH until skeletons were visible (approximately 24 hours), and 20% aqueous glycerin (Fisher) with 1% KOH, 50% aqueous glycerin, and 80% aqueous glycerin for 2 days each. Skeletons were stored in 100% glycerin. Cartilage stained blue and bone stained red.

e. Tissue fixation and preparation for frozen sections

As described in Chapter 1

f. Slide preparation

As described in Chapter 1

g. Transformation and plasmid preparation

As described in Chapter 1

h. Radioactive in situ hybridization

As described in Chapter 1

1. Probes

BSK-5'mRARß2 (374 basepair insert KpnI/SacI. a gift of P. Chambon)

C. Results

a. Phenotypic manifestation of RA-induced cell death in the normal and mutant limb

Compared to the normal limb the second phalanx of the Hm/Hm hindlimb is strongly flexed. This flexure causes the distal portion of the digits to bend inward and gives the Hm/Hm limb its clubbed shape. The fact that RA could specifically induce PCD in the developing mutant limb prompted us to examine if the induction of cell death would have any phenotypic manifestation. RA was administered to pregnant +/+ and Hm/Hm mothers at day 14 of gestation and females were allowed to come to term. Litters were examined after birth.

All litters survived until birth. However, some infants died a few days later. Some litters grew to adulthood without any obvious problems, while a few were transferred to surrogate mothers. These litters either developed successfully or died within 1-2 days. We know that the mammary glands of the mothers whose litters died were not apparent 1-2 days after they gave birth, and milk spots in the postnates were small if detected at all. It seems unlikely that the inability of the mothers to feed their young to weaning stages was a direct effect of RA, as some mothers were able to successfully feed their litters. It also does not seem that RA directly caused a defect in litters' ability to feed from their mothers as again, several did without any problems and others were able to feed from surrogate mothers. In addition, the Hm mutation did not affect the survival rate of litters since both normal and mutant postnates grew to adulthood or died a few days later, regardless of their genetic background. Timing of treatment seemed to be a factor in the survival rate. Animals

treated earlier in development (day 12 of gestation) did not survive after the first day of birth. However, in later stages it didn't seem to have a specific effect since in these stages (for example day 13) the litter survived in some experiments and not others.

Litters were examined for phenotypic variations in the limb after RA treatment on day 14 of gestation. The distal portion of the RA-treated +/+ digit was slightly more curved and extended (Fig. 29B) compared to control normal non-treated animals (Fig. 29A). The RA-treated Hm/Hm limb (Fig. 29D), was altered in the limb pattern in that the syndactyly did not extend to the nails as in the club-shaped Hm/Hm foot (Fig. 29C). The RA-treated Hm/Hm foot showed more separation in the interdigits. In fact, the digits of the RA-treated Hm/Hm limb were more extended and flattened with less flexure, and more comparable in shape to those of the normal limb.

Since there was a partial rescue of the homozygous mutant limb at this stage of development and at this dose, we attempted to achieve a full rescue in three different ways. We first examined the effect of the same dose at different stages of development (days 12, 13, and 15 of gestation). We did not detect any effect in normal (Fig. 30E) or mutant (Fig. 30F) limbs treated at day 15 of gestation (examined 4 days after birth) since cell death is minimal during this period. We also did not detect an obvious effect in normal (Fig. 30A,C) or mutant (Fig. 30B,D) limbs treated at day 12 of gestation (Fig.A-B, examined the first day of birth) or treated at day 13 of gestation (Fig.C-D, examined several days after birth). The lack of a phenotypic effect on mutant limbs treated at these other gestational stages (when

little or no cell interdigital cell death occurs normally) suggests that these cells may not be primed to respond to RA and undergo apoptosis, or that the number of dying cells is too small too cause an obvious phenotypic effect. It is important to note that regardless of the variation in survival rate, the effect or lack of it that RA had on the limb phenotype was consistent whether it was observed three days after birth or three weeks after birth.

We next examined the effect of RA treatment on day 14 in the heterozygous mutant limb which displays a partial defect. Here we found an almost complete rescue (Fig. 29F). The digits of the RA treated Hm/+ limb (Fig. 29F) were almost completely separated and showed less flexure compared to the control Hm/+ limb (Fig. 29E).

Finally, since the greatest effect was observed at day 14 of gestation, we treated Hm/Hm pregnant mothers with a double dose (400 mg/Kg body weight) at this stage of development. Once again, the defect was almost completely rescued (Fig. 31B) suggesting that day 14 is a crucial period in which cell death serves to define the developing digits and that this ameliorating effect is dose responsive.

Along with its effect on soft tissue, RA treatment early in gestation has been shown to influence the skeletal system as well (Kochhar, '77; '85; Alles and Sulik, '89). We were therefore interested to see if the Hammertoe defect and its rescue by RA resulted from a direct effect on the cartilage or the bones of the limb. Limbs were stained with alcian blue and alizarin red and examined for morphological differences. The skeletal pattern of the Hm/Hm limb (Fig. 32A,D), besides the

flexure, shows obvious differences in shape or size compared to that of the normal limb (Fig. 32C, F). Similar to previous studies (Alles and Sulik, '89), we found the bones of the limb in the RA-treated animals to be smaller than controls at two weeks of age (Fig.32B,E). However, these differences were not recognizable after 6 weeks of birth. No other significant changes were observed in the skeleton of RA-treated limbs. Our results indicate that the defect in Hm/Hm limbs is a consequence of soft tissue syndactyly only.

b. Analysis of RA responsive factors in the normal and mutant limb

The Hm defect correlates with an altered pattern of programmed cell death between digits 2-5. Therefore, the Hm gene may affect normal limb formation by altering the cell death pathway. This abnormal effect is prevented by RA. RA elicits its response via its receptors, *RARβ* is of particular interest since it is expressed in the interdigital regions of day 14.5 normal limbs and therefore has been implicated in cell death (Fig. 33). We performed in situ hybridization to examine the possibility that the Hm phenotype might reflect a difference in the expression pattern of this gene in the limb. We observed *RARβ* to be expressed in the interdigital areas of the normal limb (Fig. 33A) as reported by Dollé et al. ('89; '91). There were no obvious changes in the spatial pattern of expression of *RARβ* (Fig. 33B) in the mutant limb. This pattern furthermore not change by the induction of cell death after RA treatment (33E).

To examine the availability of RA and its ability to bind receptors, we examined the expression of the *RAREhsplacZ* transgene in day 14.5 normal and

heterozygous mutant limbs. Mice transgenic for a reporter gene containing a retinoic acid response element fused to a ß-galactosidase gene were used for these studies. As described by Rossant et al. (1991) we found lacZ staining in the interdigital regions in normal limbs (Fig. 33C). The same pattern of expression was found in heterozygous limbs at day 14.5 (Fig.33D). As with the expression pattern of *RARß*, we could not detect an alteration in *lacZ* expression. The expression pattern of *RARß* and RA availability, as measured by the RARE transgene, are similar in limbs treated with RA as well (Fig. 33E). Our observations suggest that *RARß* expression and RA availability are not altered in the Hm mice and therefore may not be directly involved in the regulation of cell death.

D. DISCUSSION

The improved phenotype resulting from the induction of cell death by RA at a specific time argues for the role of this relationship in correct patterning of the limb. It also demonstrates that the importance of the time period in which cell death must occur as well as the specific cells that are responsive to the cell death signal during this period. Hence, the term "programmed cell death" has many implications. In order for a cell to die it requires an activating signal, it requires an ability of the target cell to be sensitive to such a signal, and it requires an ability of the cell to respond to it. Timing and positional identity are important factors as well. An alteration in any or all of these processes would result in abnormality of the form or function or both in a given structure, as found in the Hm limb. Our studies show that the interdigital cells of the Hm limb are not defective in sensing a death-inducing signal or in carrying out a response to such a signal. Both of these activities occur in mutant limbs treated with RA. This suggests that it is the signal itself that may be abnormal or absent. Although we do not know if RA is this endogenous signal or if it substitutes for it, we do know that it has a specific action. To understand the mechanism by which RA acts, we have examined its regulation.

We show that the *Hm* defect is not associated with an altered expression pattern of *RARß* or of the *RARElacZ* transgene in the *Hm* limb. Although *RARß* may regulate positional identity or serve as an inhibitor of cartilage formation (Mendelsohn et al., 1991), its expression does not specifically correlate with the presence or absence of apoptotic cell death. Furthermore, we detect no differential

pattern of gene expression in normal or mutant mice after RA treatment. Our results correlate with the studies regarding *RARβ* knockout mice (Mendelsohn et al., 1994). These researchers have investigated the function of the *RARβ*2 isoform by using the gene knockout approach and found no obvious abnormalities in the embryo or the adult. Thus *RARβ* is not necessarily required for normal development. They also showed that RA induced the same abnormalities in regions of the hindbrain and limbs of *RARβ*2 null mutants as in wildtype animals, demonstrating in this case that *RARβ*2 is not required to transduce the RA signal in these structures. In addition, $RAR\beta_2$ expression levels or the activity of the *RARβ*2 promoter fused to the lacZ reporter gene remained the same in knockout mice (Mendelsohn et al., 1994). They have therefore concluded that *RARβ* is not necessary for the activity of its promoter. It is possible that a functional redundancy exists between different isoforms. Although the results by us and others suggest that *RARβ* is not a key mediator of the RA signal, the expression pattern of this gene may set up the sensitivity of specific cells to respond to cell death inducing signals, hence the similar pattern of expression in normal and mutant limbs.

As was the case with the expression pattern of *RARβ*, we did not detect altered levels of the transgene in normal vs. mutant mice These results were quite confusing in that the correction of the Hm defect by exogenous RA would suggest that the amount of RA in the mutant limb is deficient or absent. As observed by transgene expression studies, this is not the case. Rossant et al. ('91) have stated that although the RARE construct is most probably representative of RA's action in vivo,

this construct may also be responsive to unknown ligand-receptor complexes. One possibility is that the response element can not only be activated by all-trans-RA but by its isomers 9-cis-RA and 13-cis-RA as well. As mentioned before, 9-cis-RA can bind both RARs as well as the distantly related family of receptors known as RXRs, and the receptor for 13-cis-RA is unknown. If this is the case then specific levels of all-trans-RA in normal vs. mutant limbs would be difficult to quantitate. Therefore transgene expression could represent the action of the different RAs in general as well as other unidentified ligands and not that of all-trans-RA alone.

The fact that the effect of exogenous RA also did not alter RARß or transgene expression may suggest that the effect is so subtle or that it is so quick, that it is difficult to observe. In the latter situation, administration of RA could alter transgene expression in a matter of minutes rather than hours. Expression levels could return to normal by the time we look for changes in expression. Nonetheless, an immediate and transient change may be enough to yield the phenotypic effect we observe days later.

Finally, the interaction of RA-*RARß* may not explain the effect of the Hm mutation or its prevention by RA at all. These effects may be modulated well downstream of the RA-RAR interaction. RA functions in a complex manner, given its interaction with a variety of factors that are known, and a number of factors which remain to be discovered. RA may interact with a different RARE altogether or a number of them in order to elicit the specific effect we observe in the mutant limb.

Knowledge of the normal pattern of cell death and of the fact that RA acts by enhancing and inducing cell death allows one to predict the entire spectrum of vulnerable sites that might be induced at any known point during embryogenesis. Several studies have reported on fetal abnormalities that have resulted from excess levels of RA due to exogenous treatment. On the other hand, given the fact that RA is present in the developing limb, improper sequestering of RA or a defect in its mechanism may also result in limb deformities, such as *Hm*. Future studies investigating other nuclear receptors and pattern forming genes that respond to RA will determine if RA is indeed involved in normal signaling events associated with physiological cell death in normal embryos and if a defect in its signaling pathway may lead to deformities in mutant embryos.

CONCLUSION

We have used three models to study cell death during development: the normal embryonic limb, the abnormal Hm limb in which cell death is suppressed in the interdigital zones, and the RA-treated limb, in which cell death is enhanced in the sites of naturally occurring cell death. The predominant themes of this work have been the importance of cell death during development and the complexity of this process. Our studies on the Hm mutant limb demonstrate the importance of cell death in the normal development of the limb. The fact that the pattern of cell death is partially defective in the Hm limb (it occurs normally in the anterior and posterior marginal zones, and between digits one and two) is enough to give rise to an extensive limb deformity. Induction of cell death by a chemical agent, RA, in cell death suppressed areas results in amelioration of the phenotype further confirms the arguement that cell death is an essential component of normal limb development, regulated by local signals. We know that several specific mechanisms are involved in this process: the presence of a death signal; the ability of a cell to receive or be sensitive to such a signal; the induction of a biochemical cascade within the cell involving the production of novel proteins or the employment of pre-existing proteins that will carry out the killing; activation of structural proteins for the reorganization of the cellular membrane and cellular compartments for the formation of apoptotic bodies; and the ability to signal phagocytic cells that will engulf and destroy these bodies in a non-inflammatory manner. Given this complexity, a defect in any one of

these processes could lead to abnormal form and or function of a given structure. By use of the Hm limb, we have shown that given an exogenous signal, RA, the cell death defective interdigital cells can specifically respond to such a signal and die in an apoptotic manner.

These cells can also activate proteins such as transglutaminase and Cdk5 during apoptosis. We believe that these proteins are involved in the design of the apoptotic body. These proteins therefore serve as specific markers for cell death and may be used to characterize normal or abnormal cell death patterns in other instances.

Since the cell death machinery is turned on and functional after treatment of mutant limbs with RA, it seems that the lack of or defect in a cell death inducing signal is the reason for the Hm abnormality. Although the idea that RA may be this signal is very attractive, our studies of RA availability and RA receptor expression suggest that it may not be, and that if it is, it acts independently of the retinoic acid receptor ß pathway. Nonetheless, the relationship of RA and cell death may have clinical relevance for genetic disorders in the limb as well as other tissues in humans.

Cell death is crucial for normal development and complex in that it encompasses several regulatory components that interact with cell survival components. Future studies will elucidate the specific signal(s) and the intermediate factors they trigger which provide the molecular basis for cell suicide.

PART III. FIGURES AND FIGURE LEGENDS

Figure 1. Localization of clusterin in implanted uteri.

Pregnant uteri were retrieved on days 5.5 (A-C) anf 6.5 (D-F) of gestation and prepared as described in Materials and Methods, Chapter 1. Expression with the digoxigenin-labeled antisense clusterin riboprobe was detected in both uterine glands and ducts at both days 5.5 (A-B) and 6.5 (D-E) of implantation. B and E are higher magnifications of A and D, respectively. C and F are sections hybridized with sense probes. White arrow indicates expression clusterin in day 5.5 glands and open arrow indicates expression in day 6.5 uterine glands. Magnifications: A, C, and D: 40X; B, E, and F: 400X.

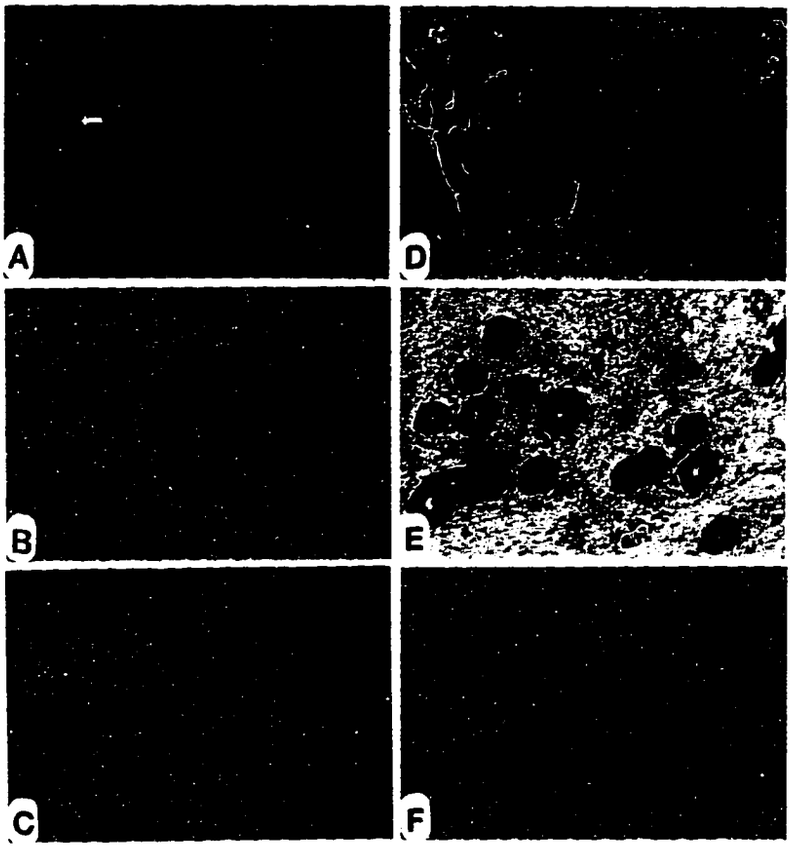


Figure 2. Localization of clusterin message in the developing embryo.

Embryos were removed at days 15.5 (A-D) and 13.5 (E-F) of gestation and prepared as described in Figure 1. Clusterin message was detected in the non neuronal epithelium of the choroid plexus of day 15.5 (A: arrowhead, B), in the retinal epithelium of the eye (A: solid arrow, C), and in the precartilage of the developing limb (A: open arrow, D). Message was also detected in the multilayered epithelium of the ductus cochlearis (E: arrow, F). Magnifications: A and E: 40X; B, C, D and F: 100X.

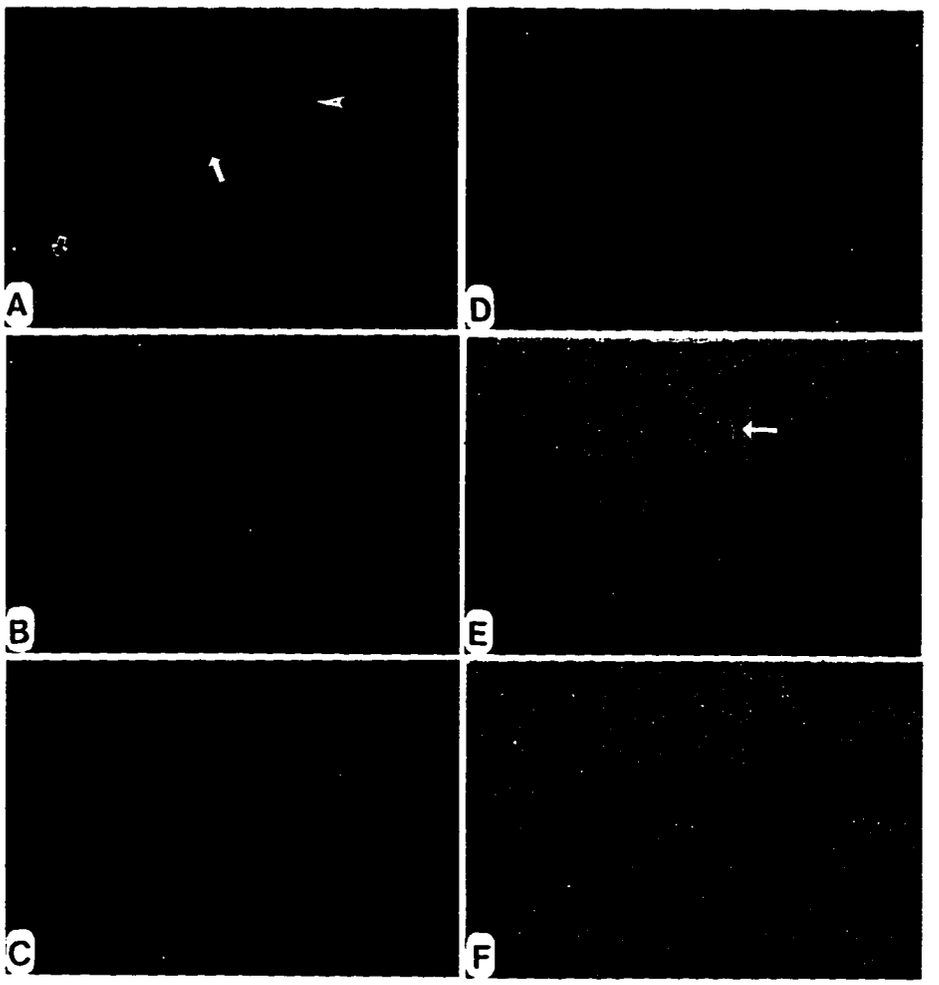


Figure 3. Localization of clusterin in normal and mutant mouse testes.

Testes were removed from 7 day old, 17 day old, and adult males and fixed in 4% paraformaldehyde. Sections were prehybridized and hybridized as described in Materials and Methods, Chapter 1. Cross sections of testes showing message localization in Sertoli cells by nonradioactive in situ hybridization of 7 day old (A), 17 day old (B), adult (C), W^v mutant (limited immature germ cells, D), and *at* mutant (devoid of germ cells, E) testes. Clusterin transcript is abundantly expressed in the Sertoli cells of developing, normal, and mutant testes. Magnification: 100X.

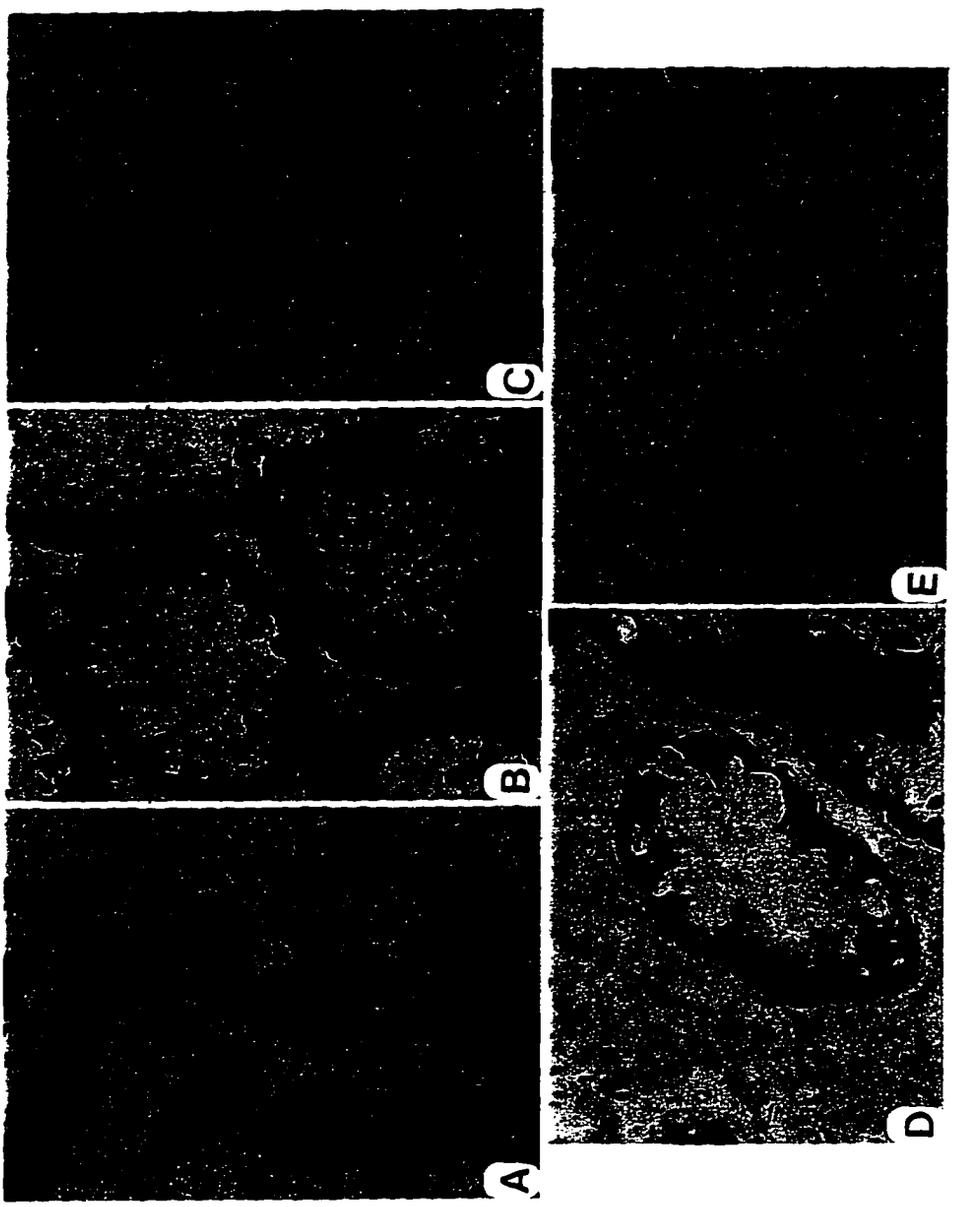


Figure 4. Specificity of antibodies for rat clusterin amino acid sequences.

A: Predicted translation products of MalE clusterin fusions in DH5αf *E. coli* hosts. MalE 2-26, MalE protein with the N terminal secretory signal sequence deleted to direct expression to the cytoplasm. α+β, fusion to the clusterin proprotein, α and β subunits (residues 1-205 and 206-426 respectively, numbering based on the proprotein) less the N-terminal 21 amino acids encoding the clusterin secretory signal. α and β are fusions of the individual clusterin subunits only. Bold arrow head shows the site of limited proteolysis of the clusterin polypeptide expressed in mammalian cells. **B:** Four identical blots of amylose affinity purified MalE clusterin fusion proteins prepared, separated by SDS PAGE, transferred to nitrocellulose and probed with the different antibody preparations as described in Materials and Methods, Chapter 1. A fifth blot probed with detecting second antibody alone did not demonstrate any immunoreactive bands even after long exposure times (data not shown) Lanes designated α, β and α+β are preparations from clones harboring expression plasmids outlined in panel A. Molecular weight markers are rabbit muscle phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa) and hen egg white ovalbumin (45 kDa).

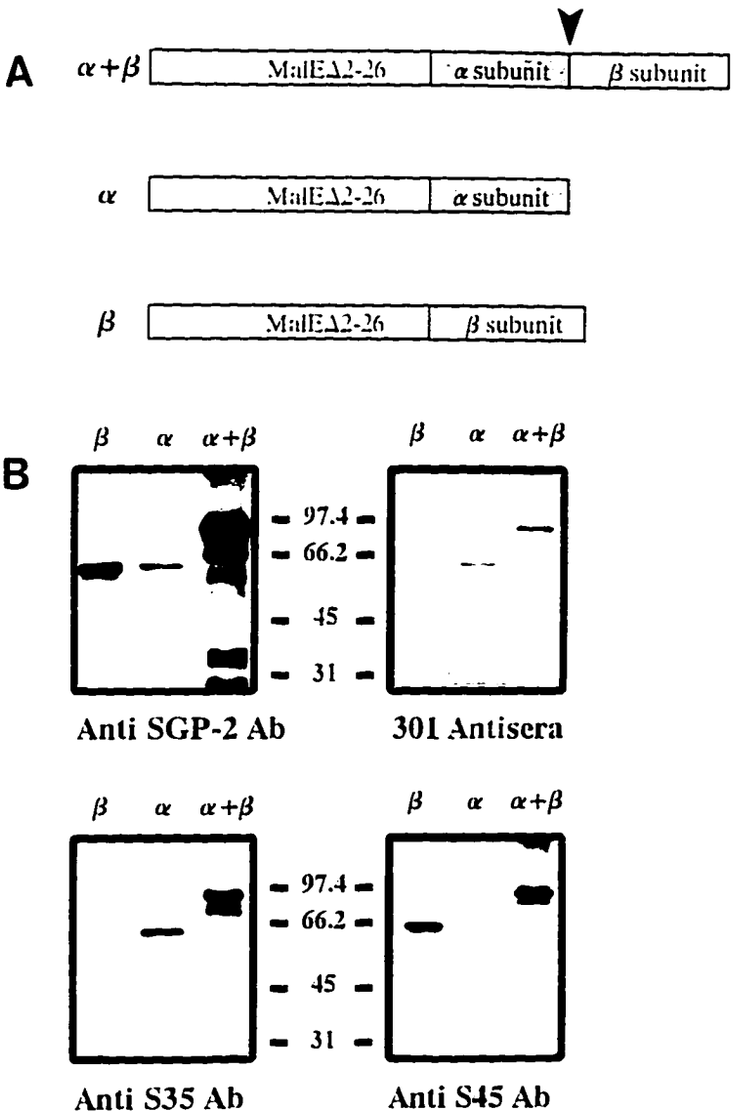


Figure 5. Immunolocalization of clusterin protein in adult testis.

Tissues were fixed overnight and treated as described in Figure 3. Sections of testes showing protein localization by immunohistochemistry. Section incubated with only secondary antibody showed no expression (A). Anti-S35 shows strong reactivity to mouse sperm tail (B, solid arrow). Anti-301-4 reacts specifically to the sperm head (C-D, open arrows). Magnification: A-C: 400X, D: 1000X.

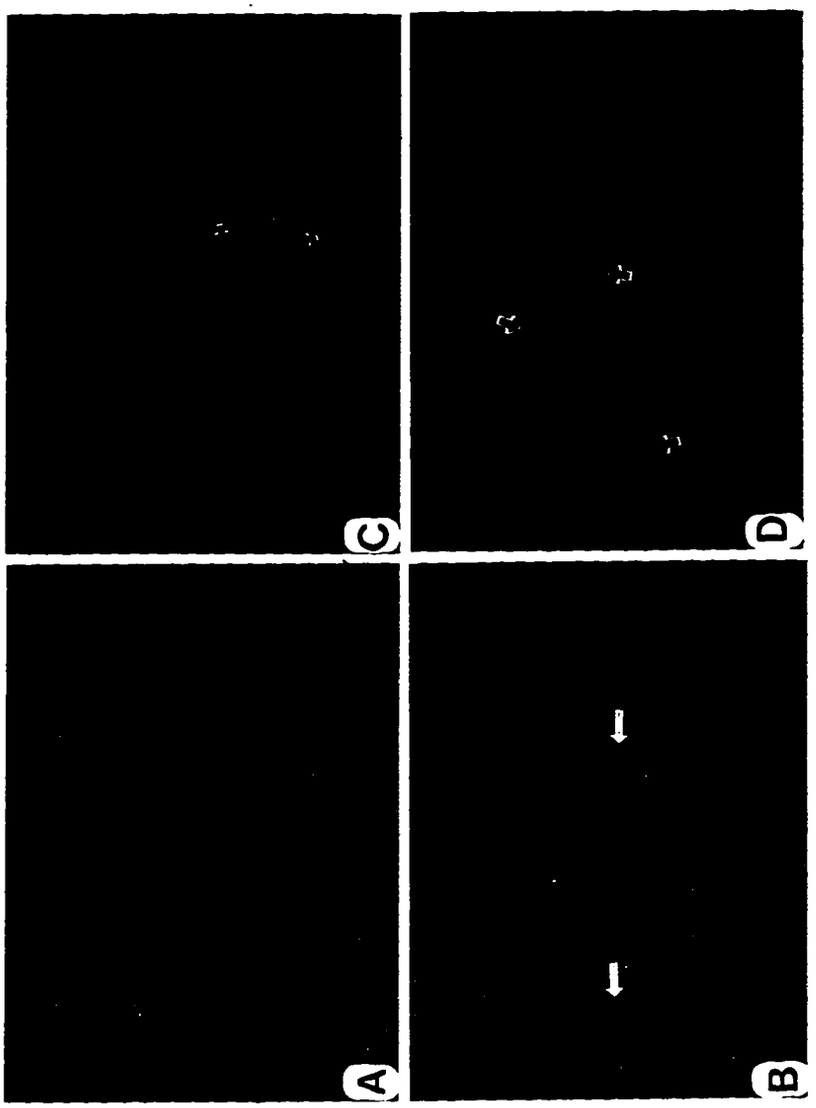


Figure 6. Clusterin RNA levels in mouse testis and epididymis.

A: Whole mount testis (TS), head of epididymis (H), body of epididymis (B), tail of epididymis (T), and efferent ductule (ED). **B:** RNA was isolated and northern blot hybridization was performed as described in Materials and Methods, Chapter 1. A 1.8 Kb clusterin transcript was detected at abundant levels in the head region of the epididymis (H) compared to the body (B) and tail (T) of the epididymis, as well as the testis (TS). **C:** Whole mounts of normal (N) testis and epididymis compared to the *at* mutant (M) testis and epididymis.

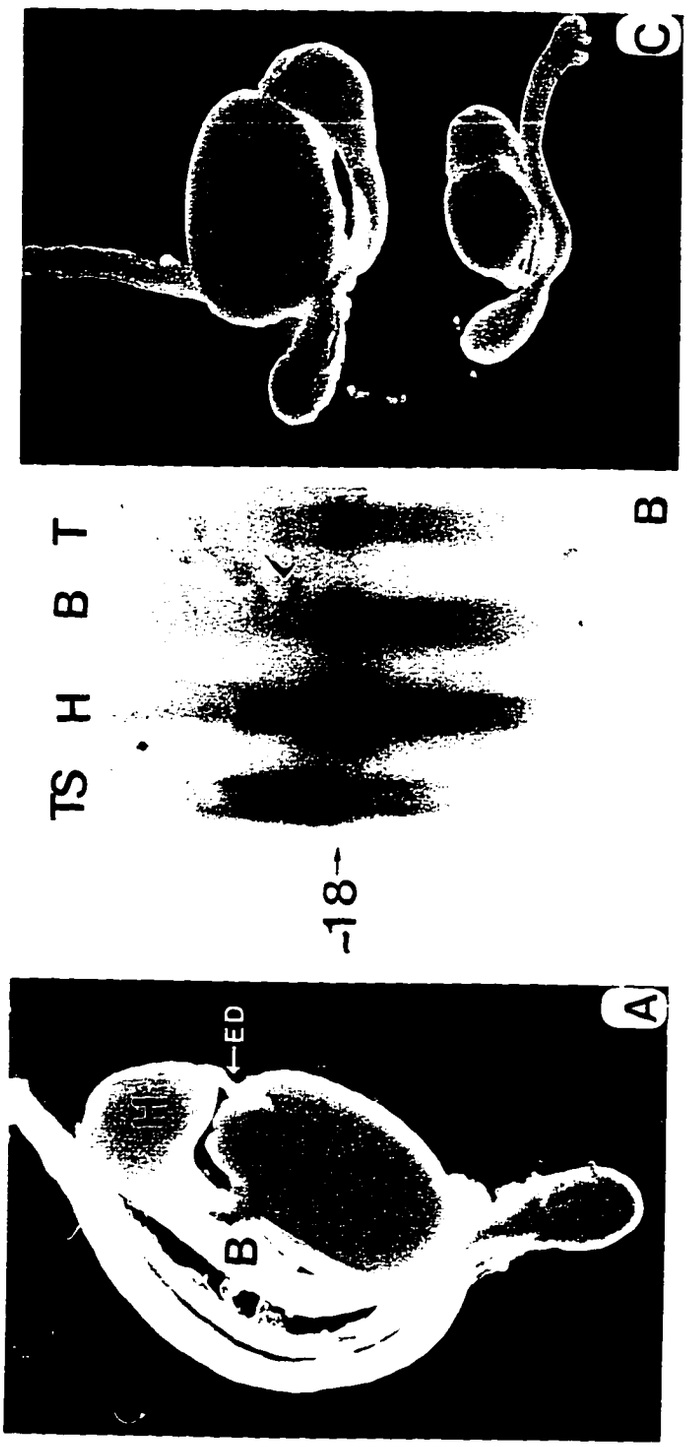


Figure 7. Localization of clusterin transcript and protein in normal, developing, and mutant epididymides.

Epididymides were prepared and used as described in Materials and Methods, Chapter 1. Section of epididymis hybridized with radiolabeled clusterin antisense probe (A). Section hybridized with to non-radioactive, digoxygenin labeled clusterin antisense probe (B). Protein localization of clusterin is detected in secretory vessicles of epididymal tubules and on sperm by anti-SGP-2 antibody (C). Similar to message localization, the protein is not detected in initial segment of the caput (1), expressed at low levels in segment 2, and at abundant levels in segment 3. Clusterin mRNA levels decrease in segment 4. Section of 17 day old epididymis hybridized with clusterin transcript (D). Clusterin mRNA is not expressed in the efferent ductule of the epididymis in the 17-day-old mouse, but is expressed at high levels in segments 1,3 and 4. Section of epididymis from adult *at* mouse (E). Section of epididymis from adult *Wv* mouse (F). The steady state level of clusterin mRNA is substantially increased in segment 1 of the *at* and *Wv* mutants compared to normal epididymides. Magnifications: dissecting microscope A-B: 40X; D-F: 50X. compound microscope C: 40X.

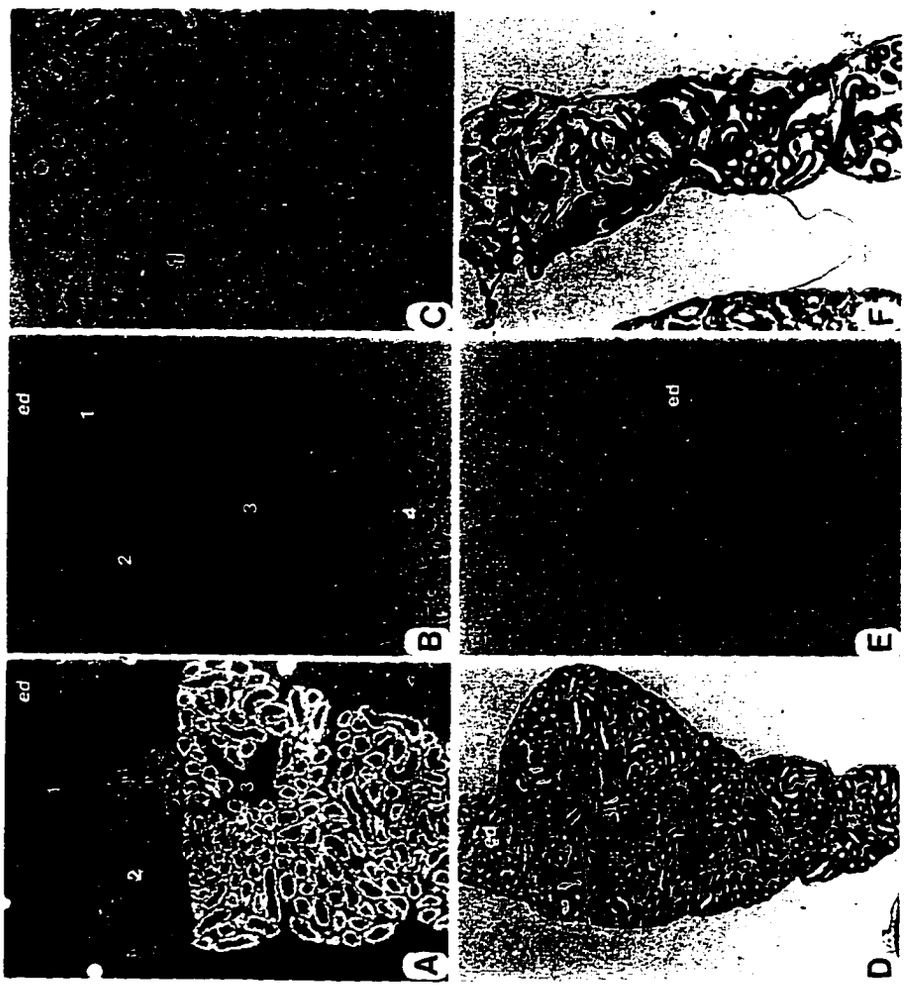


Figure 8. Localization of clusterin mRNA in different segments in adult caput.

Adult epididymides were fixed overnight, sectioned and hybridized to digoxygenin labeled clusterin antisense probe as described in Material and Methods. Section of adult epididymis showing differential expression of clusterin transcript in segments 1-4. The epithelium in segment 1 shows little or no clusterin mRNA staining (A-B, C: left hand side). Expression of clusterin mRNA is seen in the perinuclear region of the cells in segment 2 (C: right hand side). Localization of clusterin mRNA becomes progressively luminal in segments 3 (D) and 4 (E-F). Arrows indicate intracellular localization of clusterin mRNA. lu: lumen. Magnigications: dissecting microscope A: 40X. compound microscope: B-F: 400X.

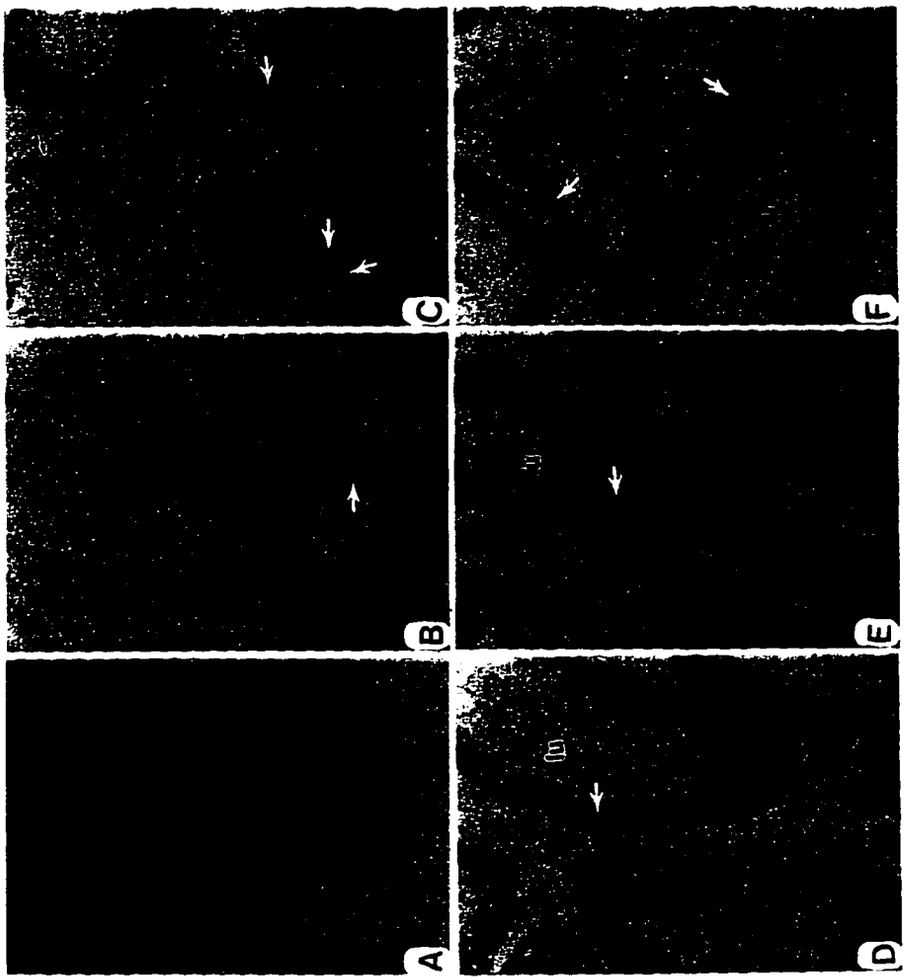


Figure 9. Localization of clusterin in adult mouse ovaries.

Adult ovaries were prepared and examined as in Materials and Methods, Chapter 1 for the expression of clusterin mRNA and its related protein product. Section of ovary showing clusterin mRNA expression as indicated by dark staining in developing (open arrow) and atretic (closed arrow) follicles (A-B). Higher magnification of atretic follicle (C). Note the expression of clusterin in granulosa cells only. Protein localization of clusterin with anti-S35 antibody (D), anti-301-4 (E) and anti-SGP-2 (F) antibodies on the zona pellucida (arrows) of the adult ovary. All three antibodies also reacted with atretic follicles as seen in G (short arrow) with anti-SGP-2. Magnifications: A: 40X; B, D-G: 400X; C: 1000X.

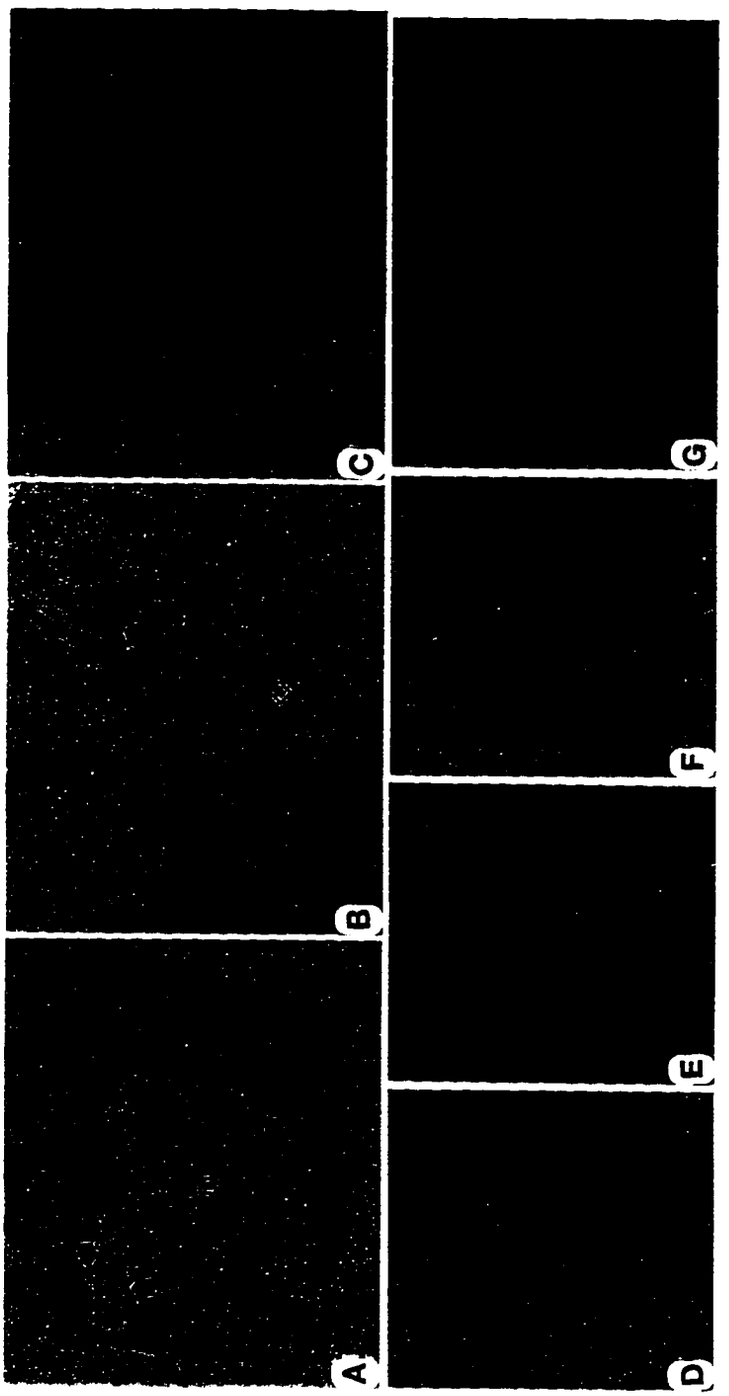


Figure 10. Localization of clusterin transcript in adult mouse uterus.

Adult uteri were prepared and examined as in Materials and Methods, Chapter 1 for the expression of clusterin message. In some non pregnant uteri, expression was detected only in a few cells of the glands (A-B), while in others expression was limited to ductal cells displaying pyknotic nuclei (solid arrows, C-D). Expression was not detected in normal cells of these ducts (D, open arrow). Magnifications: A, C: 100X; B: 400X; D: 1000X.

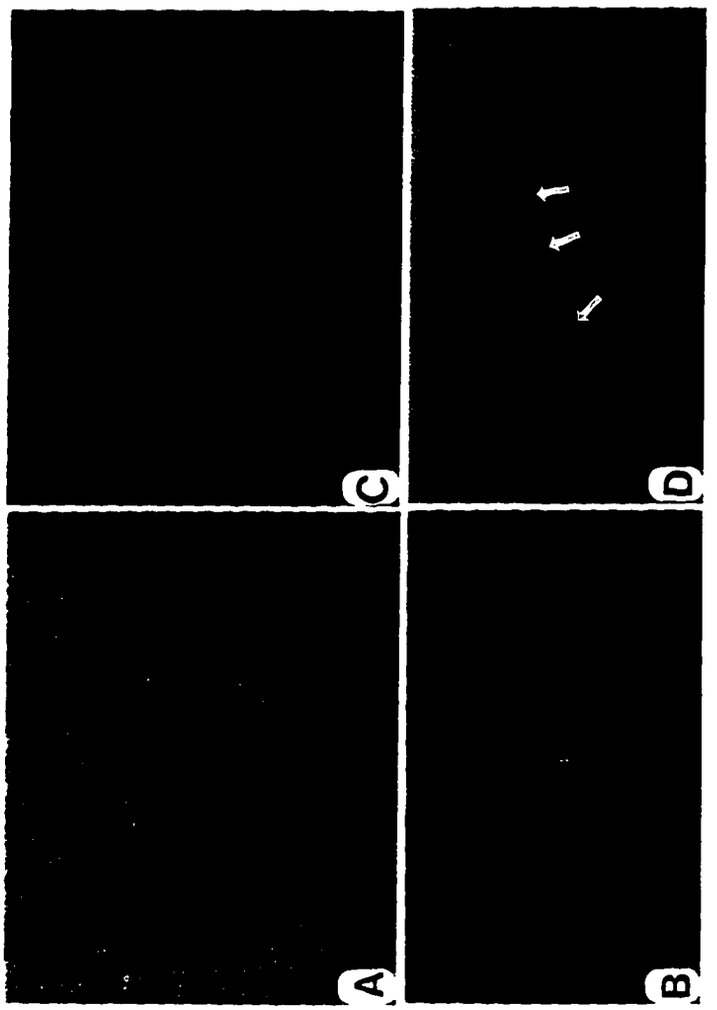


Figure 11. Morphology of cell death in developing mouse limbs.

Semithin sections of developing limbs at days 10.5 (A), 11.5 (B), 12.5 (C, E-F), and 13.5 (D, G) of gestation. Sections were prepared as described in Materials and Methods, Chapter 2. Higher magnifications of: circle in C (E) shows area of condensation that will result in the formation of a digit; diamond area in C (F) shows interdigital cells maintaining minimal contact with each other; and the open triangle area in D (G) shows apoptotic bodies within an interdigital area as indicated by white arrows. Magnifications: A-D: 75X; E-F: 470X; G:434X.

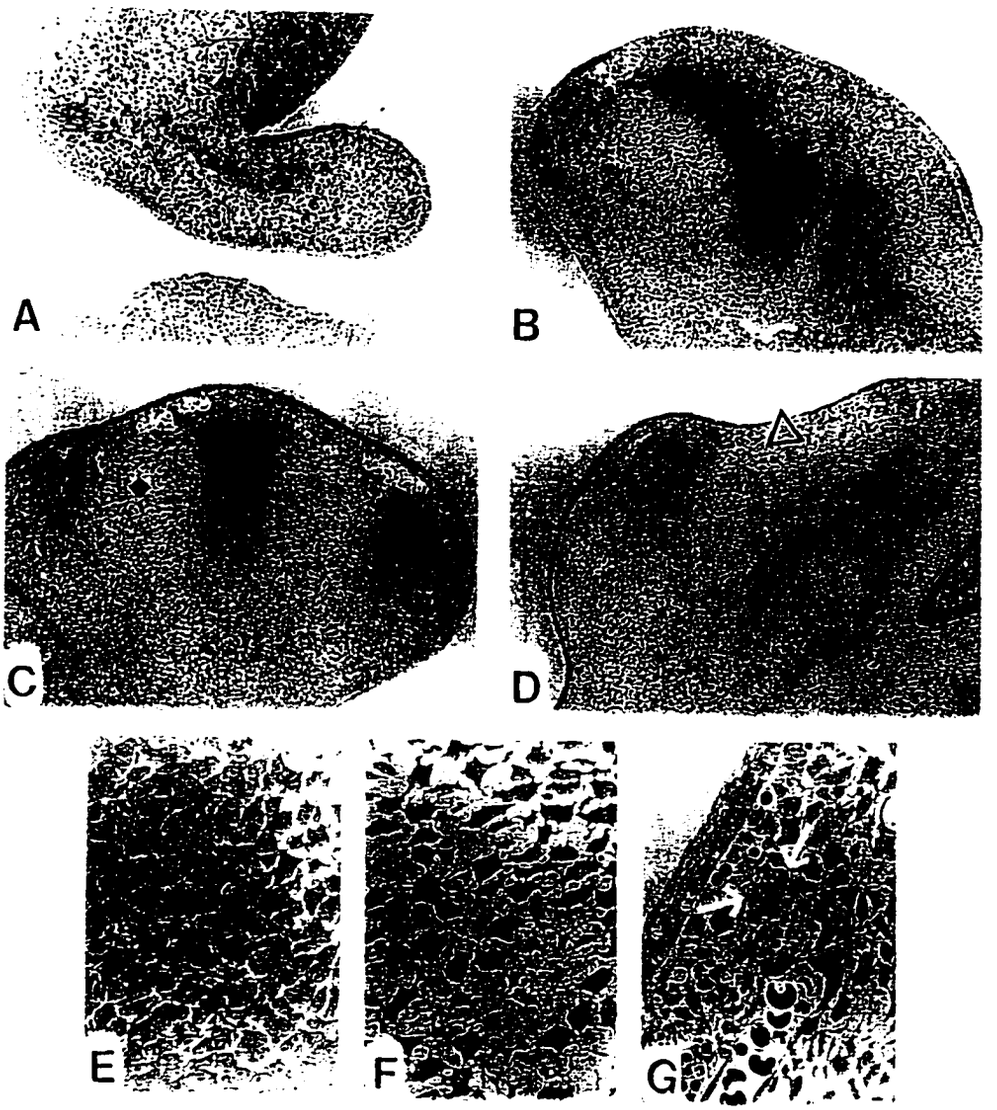


Figure 12. Localization of cell death in embryonic mouse limb by nile blue sulfate (NBS).

Embryos at days 12.5 (A), 14.5 (B), and 15.5 (C) of gestation were stained in NBS for 30 min. Dead cells were identified as dark circular bodies. In day 12.5 limbs, dead cells are seen in the anterior and posterior marginal zones (A, arrows). In day 14.5 limbs cell death is seen in the areas mentioned above as well in the interdigital regions (B, arrows). Minimal cell death is observed to the marginal zones in day 15.5 limbs (C, arrows). Magnifications: A-C: dissecting microscope, 40X.

Figure 13. Localization of cell death by recognition of phagocytic cells, their lysosomal activity, and DNA fragmentation in day 12.5 and 14.5 embryonic mouse limbs.

Day 12.5 (A, D, G) and 14.5 (B-C, E-F, H-I) embryonic limbs were removed and processed as described in Materials and Methods, Chapter 2. Anti-F4/80 antibody recognizes the macrophage surface antigen on phagocytic cells surrounding apoptotic bodies in the anterior marginal zone of the day 12.5 limb (A, arrows) and in the interdigital zone of the day 14.5 limb (B-C, arrows). Serial sections display acid phosphatase activity of the phagocytic cells of day 12.5 (D) and 14.5 (E-F) limbs. Day 12.5 (G) and 14.5 (H-I) limbs also show DNA fragmentation in condensed nuclei (arrowheads). C, F, and I are magnified views of B, E, and H, respectively. Magnifications: A, D, G: 100X; B, E, H: 25X; C, F, I: 1000X.

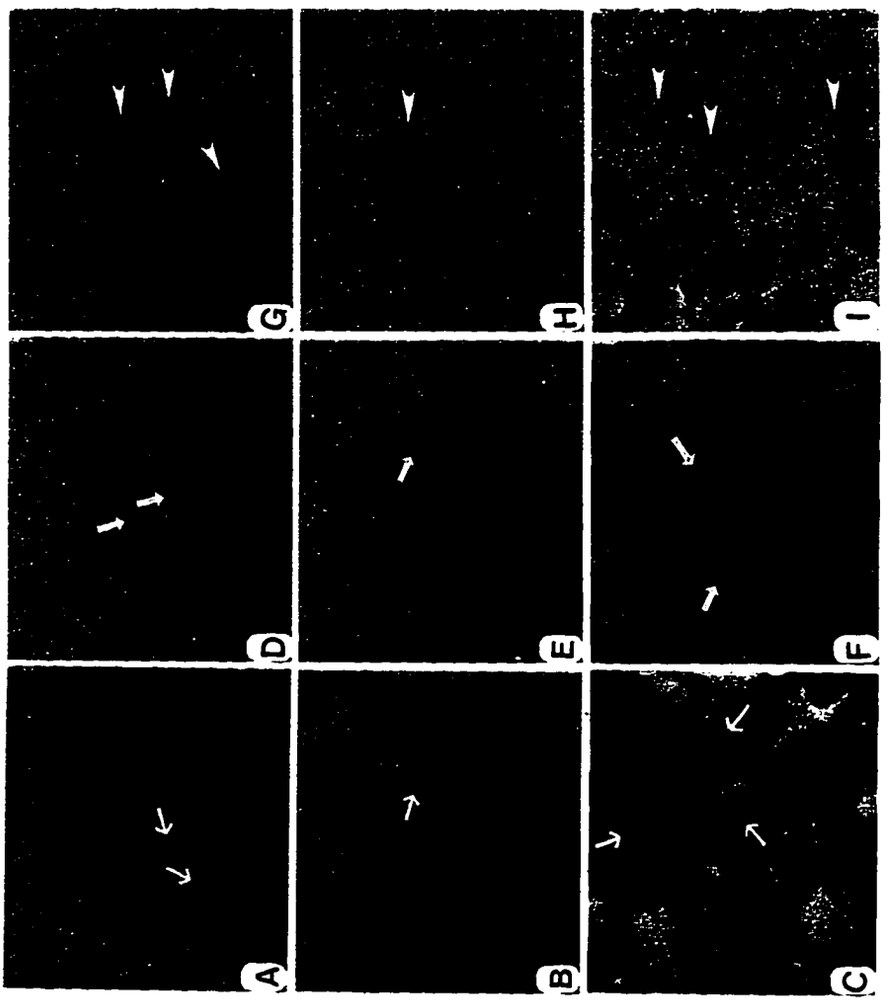


Figure 14. Profile of DNA isolated from day 14.5 interdigital regions.

DNA was isolated according to Materials and Methods (Chapter 2) from interdigital regions of fore (FL) and hind (HL) limbs of day 14.5 embryos. (A): Left= Total homogenate treated with proteinase K and RNAse A before electrophoresis; Right= DNA purified by phenol extraction. (B and C): DNA labeled by end-labeling technique; B= 3 hour exposure; C= Overnight exposure. Involuting thymocytes (Th) were used as positive controls for methodology of DNA fragmentation. Although the ladder pattern was detected in Th by all three techniques, ladders were not detected in FL or HL.

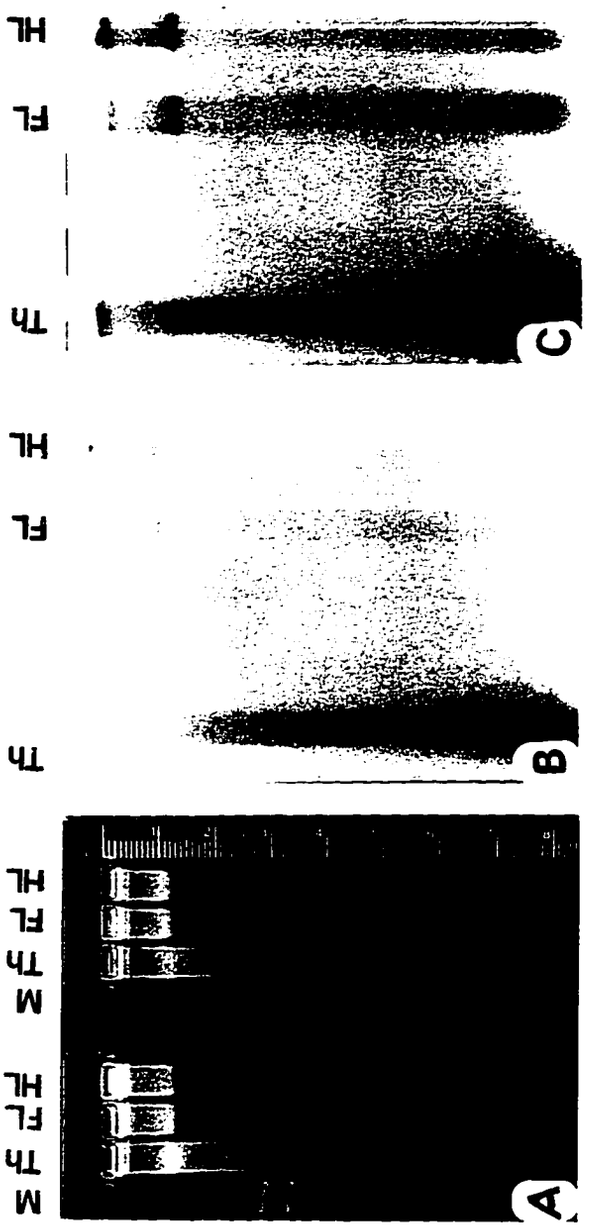


Figure 15. Suppression of cell death in the Hammertoe mutant limb measured by NBS.

Day 14.5 Hammertoe (Hm) limbs were examined for cell death by NBS. The wildtype (+/+) day 14.5 limb (A) displays cell death in the anterior and posterior marginal zones (open arrows) as well as in the interdigital zones (solid arrows). In contrast, the mutant Hm limb (B) shows dead cells in the anterior and posterior marginal zones (open arrows) and between digits 1 and 2 (solid arrow). Cell death is suppressed in the interdigital regions between digits 2 and 5. Magnifications: dissecting microscope A-B: 50X.

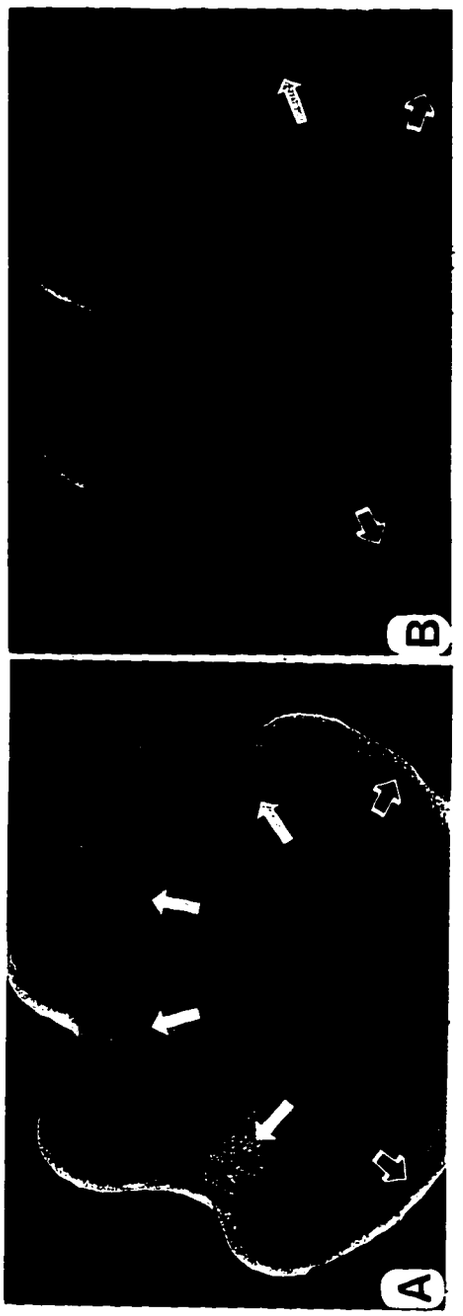


Figure 16. Suppression of cell death in the Hammertoe mutant limb detected by morphology, phagocytic staining, and DNA fragmentation.

Normal and mutant embryos at gestational day 14.5 were prepared as described in Materials and Methods, Chapter 2. Semithin sections display apoptotic bodies engulfed by phagocytic cells (D, arrows) in the interdigital region of the normal limb. Cell death was not detected in a similar section of the interdigital region of the Hm mutant limb (A). F4/80 staining indicating the presence of phagocytic cells was not detected in interdigital region of the Hm limb (B), but was found in phagocytic cells surrounding dead cell fragments in the posterior marginal zone of the Hm limb (E, arrows). Serial sections were stained for DNA fragmentation by *in situ* end labeling, and no fragmentation was observed in the interdigital region of the Hm limb (C), whereas nuclear staining was detected in the posterior marginal zone of the Hm limb (F, arrows). Stars indicate interdigital space and open arrowheads point to the ectoderm of the developing limb. Magnifications: A and D: 250X; B-C, E-F: 400X.

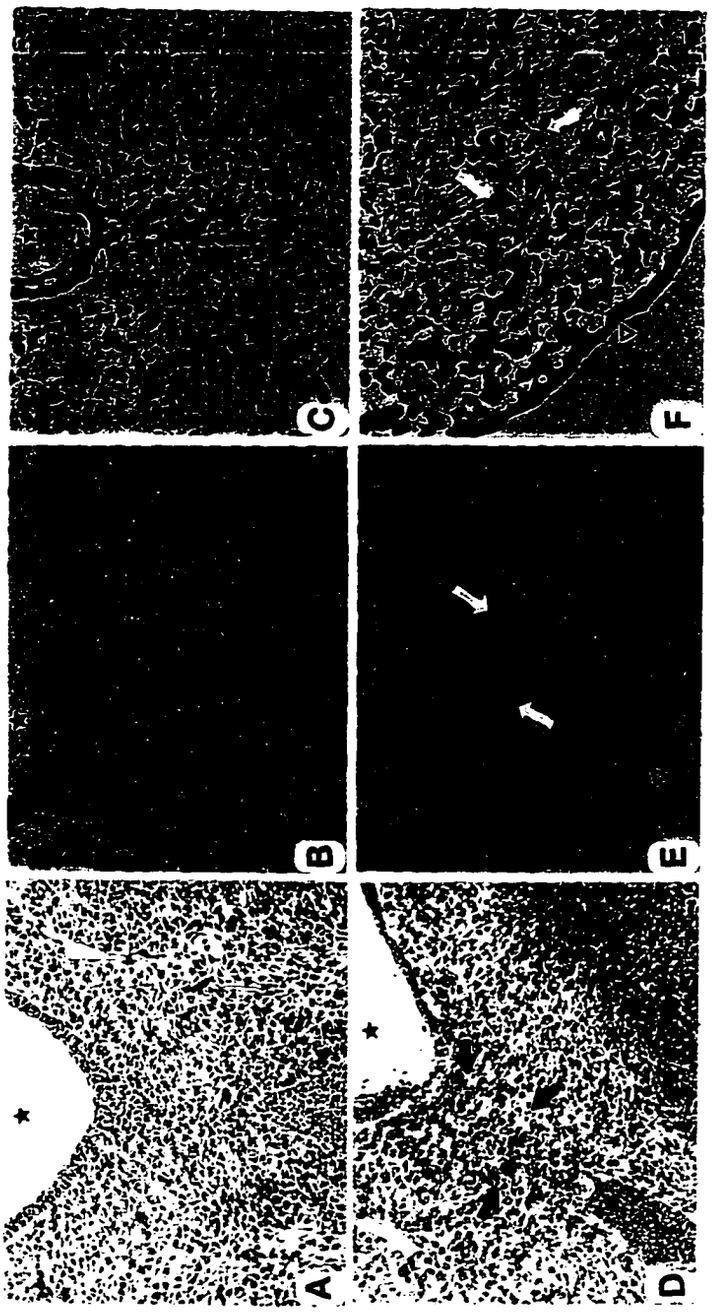


Figure 17. RA enhances apoptotic cell death in the normal developing limb.

Pregnant females were gavaged with 200 mg/Kg body weight of all-trans-RA at day 14 of gestation and embryos were recovered at day 14.5 of gestation and processed as described in Materials and Methods, Chapter 2. Nile blue sulphate stained limbs display cell death in the anterior and posterior marginal zones and the interdigital areas (A, thin arrows). Limbs treated with RA (B) display higher levels of cell death in these areas as indicated by thick arrows. RA-treated day 14.5 embryonic hindlimbs were recovered and serial sections of the interdigital region were analyzed for the presence of cell death as described in Materials and Methods. Hematoxylin stained section of the interdigital area of the RA-treated limb (C) shows apoptotic bodies (black arrows). F4/80 stains phagocytic cells in RA-treated limbs (D-E, white arroows) surrounding apoptotic bodies (E, black arrows). Acid phosphatase activity is indicated by the red staining in RA-treated (F, arrows) limbs. DNA fragmentation by *in situ* end labeling stains nuclei brown of RA-treated (G, arrowheads) limbs. Note the intensity of the stain by each of the markers in RA-treated limbs. Stars indicate interdigital space. Magnifications: dissecting microscope A-B: 30X; compound microscope: C, E: 1000X; D, F-G: 400X.

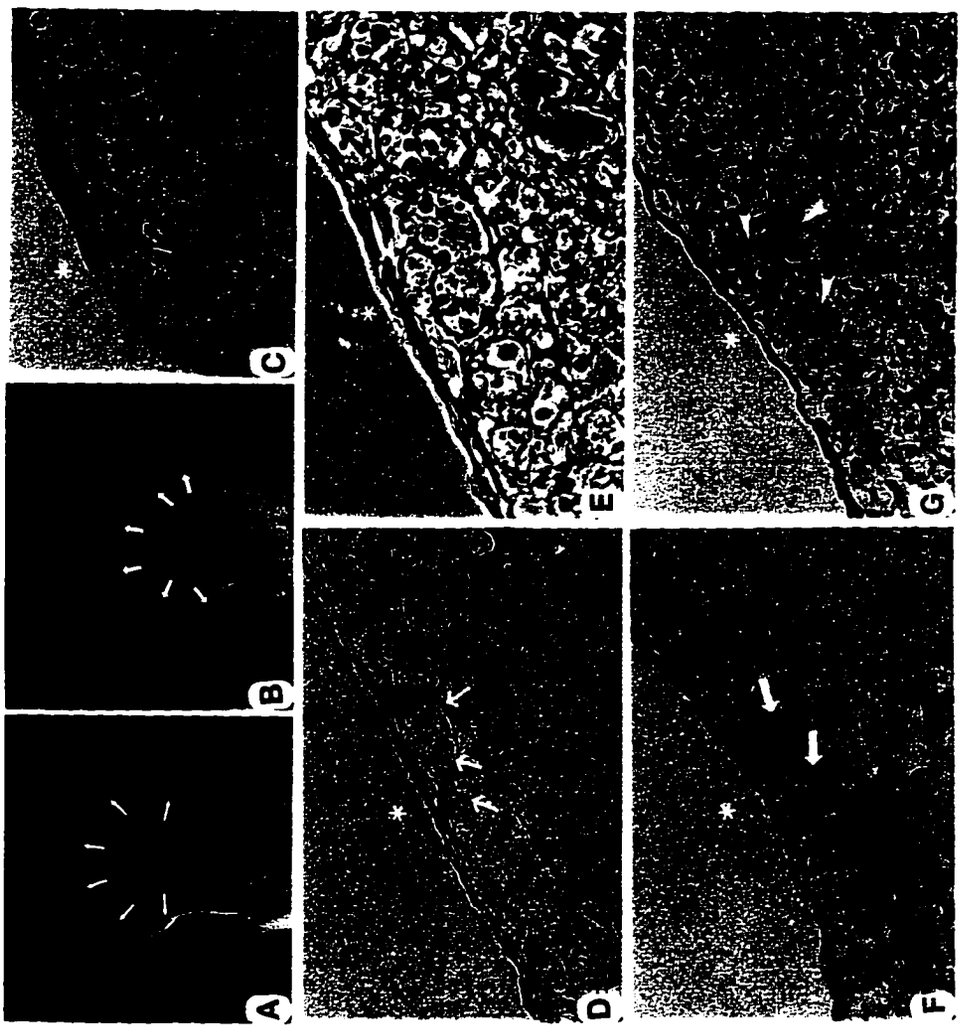


Figure 18. RA induces apoptotic cell death in the mutant Hammertoe limb.

Day 14.5 control (A) and RA-treated (B-C) Hammertoe mutant hindlimbs were examined for cell death by NBS. Compared to control hindlimbs (A), RA enhances cell death in the marginal zones and between digits 1 and 2 (B-C, arrowheads). In addition, it induces cell death between digits 2 and 5 (B-C, arrows). Similarly, induction of apoptotic cell death in the interdigital region of the Hm mutant limb is detected by F4/80 staining for phagocytic cells (D, arrows), acid phosphatase activity (E, arrows), and DNA fragmentation (F, arrowheads). Magnifications: dissecting microscope A-B: 30X; C: 40X. compound microscope: D-F: 400X.

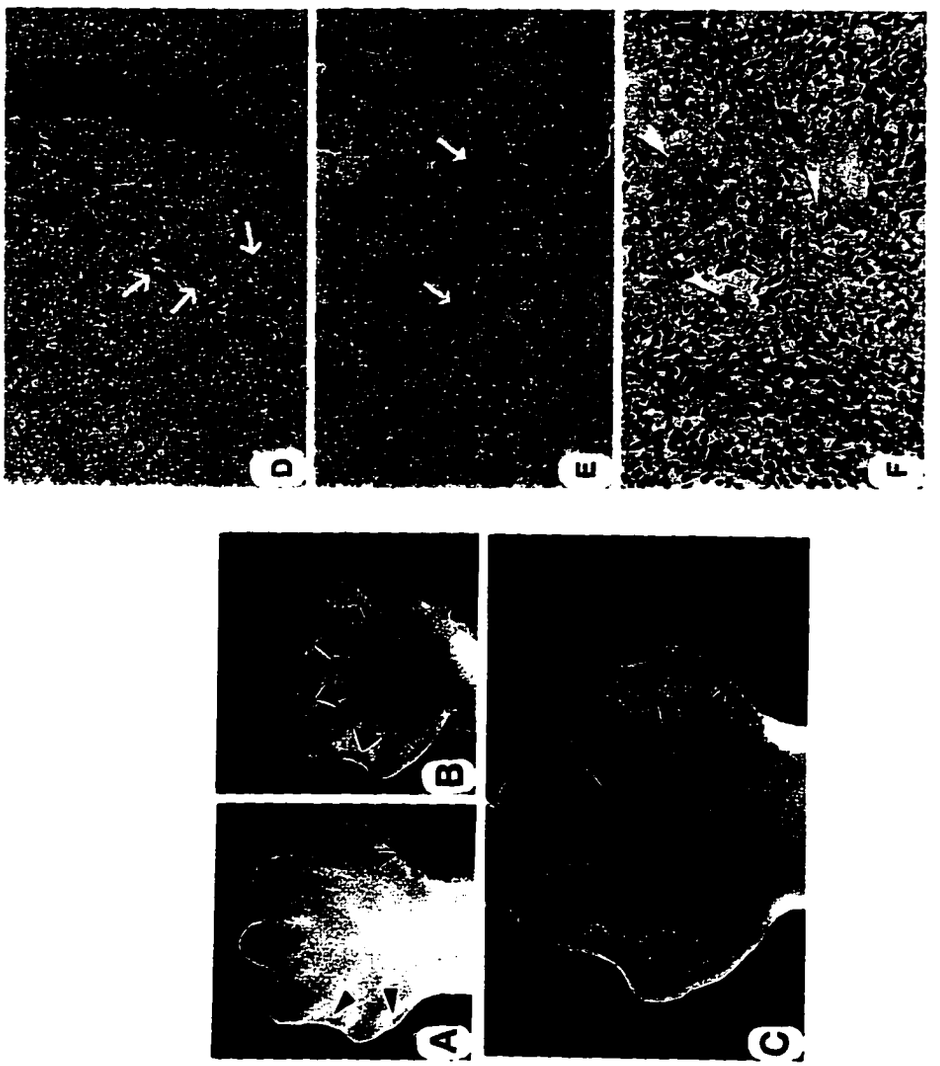


Figure 19. Tissue transglutaminase protein expression in normal, Hm mutant, and RA-treated limbs.

Day 14.5 limbs were processed as described in Materials and Methods. Chapter 3. Sections were stained with anti-tissue transglutaminase (tTG) and expression was detected in the apoptotic bodies of the normal developing limb (A-C, E arrows). B and C are higher magnifications of the area indicated by arrow "1" in A. Low levels of tTG were also detected in the membrane surrounding normal cells giving rise to cartilage (D, area indicated by arrow "2" in A). Compared to the normal limb (E, area indicated by arrow "3" in A), the RA treated limb (F, arrows) shows higher levels of tTG expression correlating with the increased number of apoptotic bodies. tTG expression was not detected in the interdigital zones of the Hm mutant limb (G) but was detected in interdigital dead cells of the RA-treated Hm limb (H, arrows). Bent arrows indicate expression of tTG in the blood cells of the developing limb. Magnifications: A: 5X, B, E-H: 40X, C-D: 1000X.

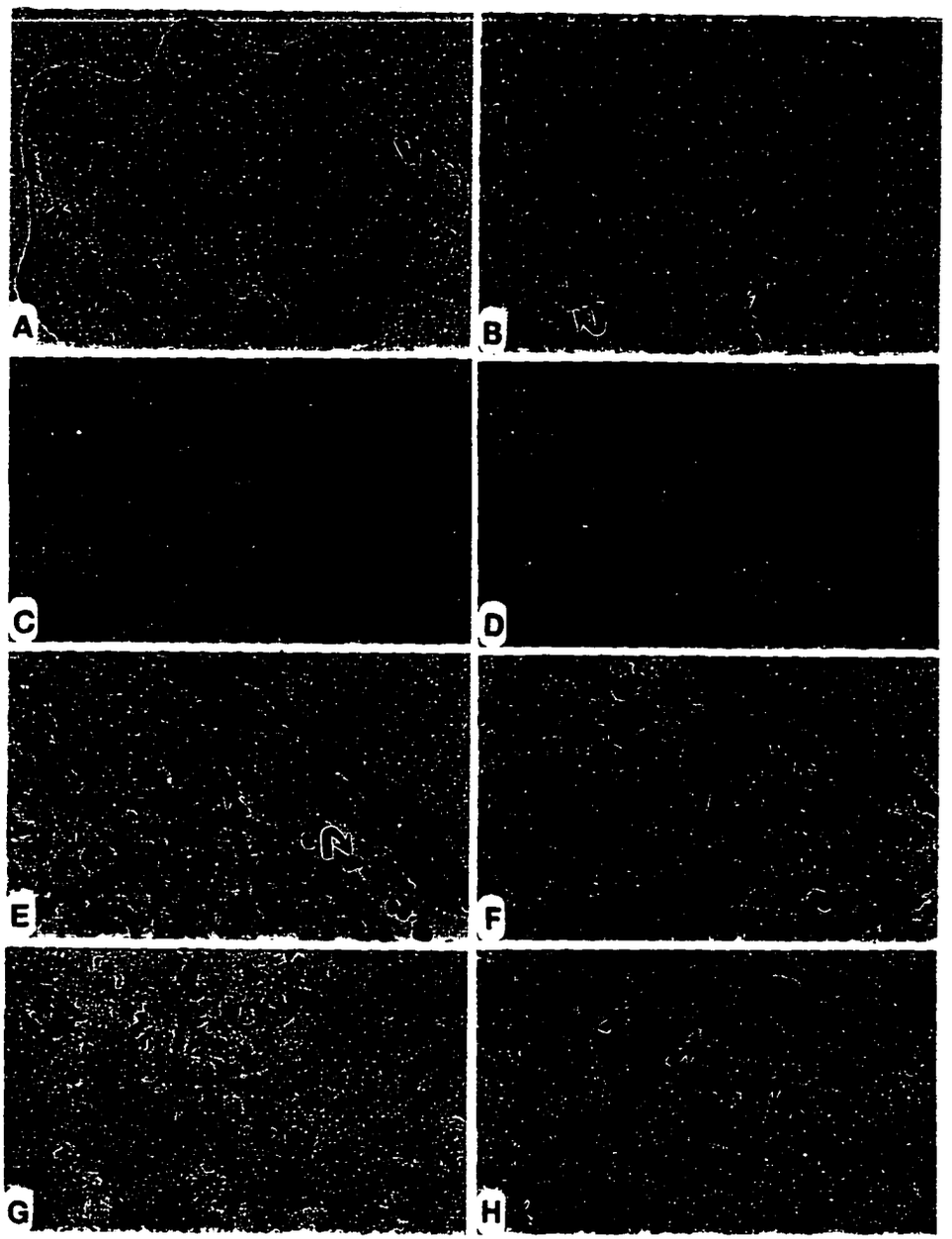


Figure 20. Localization of bcl-2 expression in normal and mutant limbs.

Day 14.5 normal (A, C-D) and mutant (B) limbs were processed as described in Materials and Methods, Chapter 3. Sections of normal limb indicate expression of bcl-2 mRNA in the digital zones of the developing limb (A, C-D). Expression is not detected in the interdigital areas or in the joints between the digits. Similarly, bcl-2 transcript is observed in the digital rays of the Hm mutant limb as well (B). C and D are dark field and bright field magnifications, respectively, of the area indicated by the white arrow in A. Magnigications: A-B: 100X; C-D: 400X.

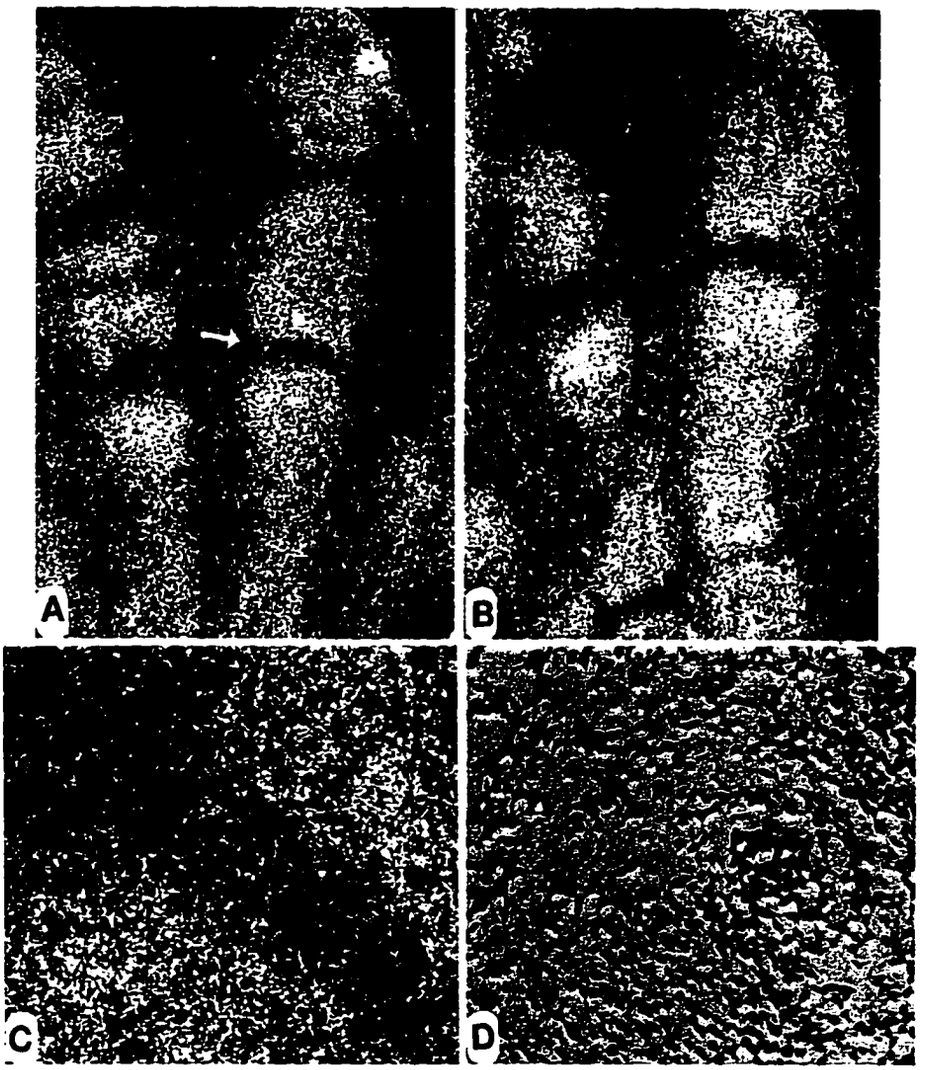


Figure 21. Localization of *Msx-1* expression in normal and mutant limbs.

Day 14.5 normal (A, C-E) and mutant (B) limbs were processed as described in Materials and Methods, Chapter 3. Sections of normal limbs (A, C-E) display *Msx-1* expression in the interdigital area of the developing limb. C and D are dark field and bright field magnifications, respectively, of the interdigital area in A. Higher magnification (E) shows that Msx-1 expression is not specifically associated with apoptotic bodies (black arrows) but is highly expressed in normal looking cells (E, white arrows). The suppression of cell death in the interdigital regions of the Hm mutant limb (B) does not effect the interdigital expression of Msx-1 that is similar to the normal limb. Magnifications: A-B: 100X; C-D: 400X; E: 1000X.

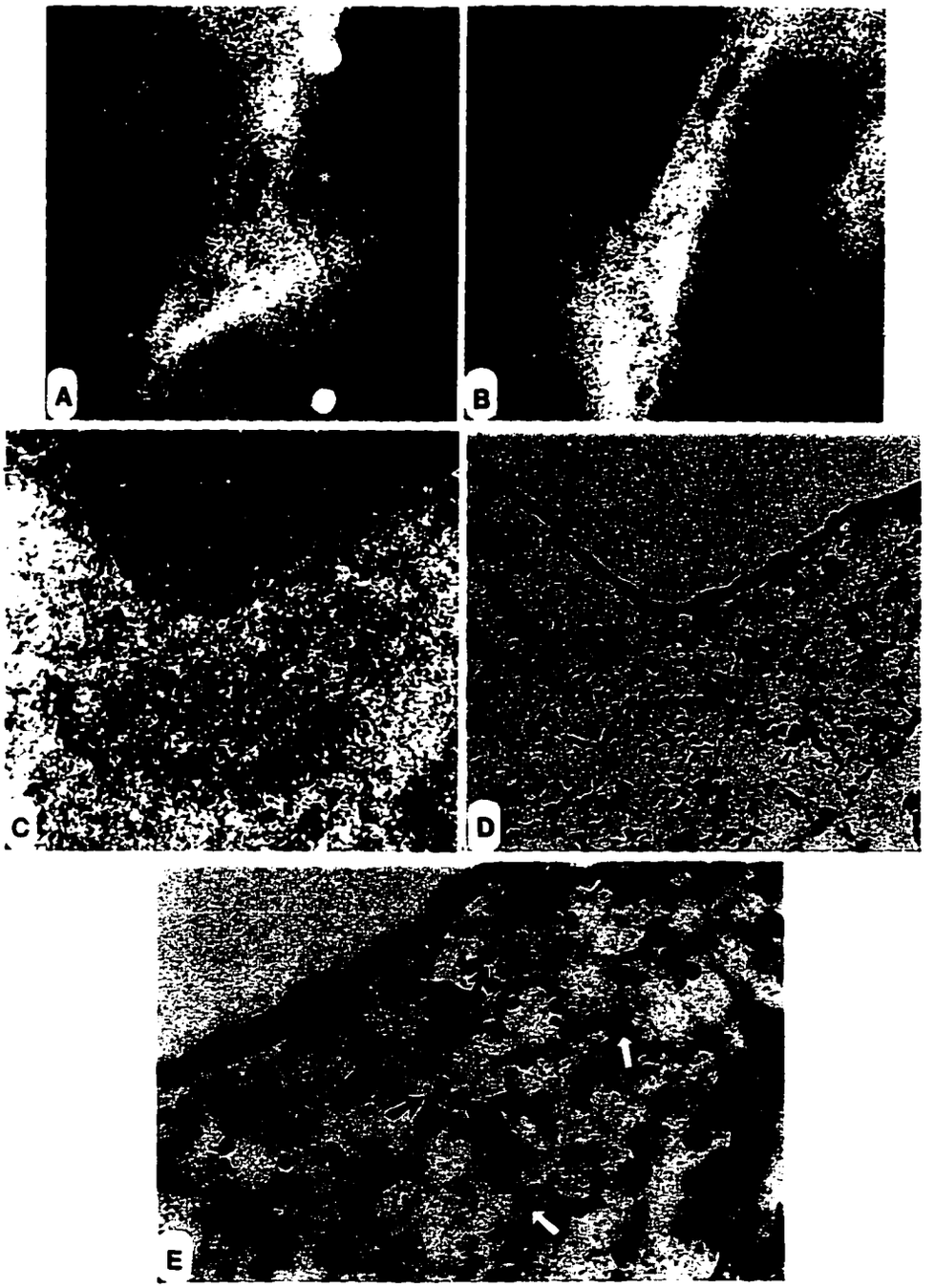


Figure 22. Localization of *Msx-2* expression in normal and mutant limbs.

Day 14.5 normal (A, C-E) and mutant (B) limbs were processed as described in Materials and Methods, Chapter 3. Sections of normal limbs (A, C-E) display *Msx-2* expression in the interdigital area of the developing limb. C and D are dark field and bright field magnifications, respectively, of the interdigital area in A. Higher magnification (E) shows that Msx-2 expression is not specifically associated with apoptotic bodies (black arrows) but is highly expressed in normal looking cells (E, white arrows). The suppression of cell death in the interdigital regions of the Hm mutant limb (B) does not effect the interdigital expression of Msx-2 that is similar to the normal limb. Magnifications: A-B: 100X; C-D: 400X; E: 1000X.

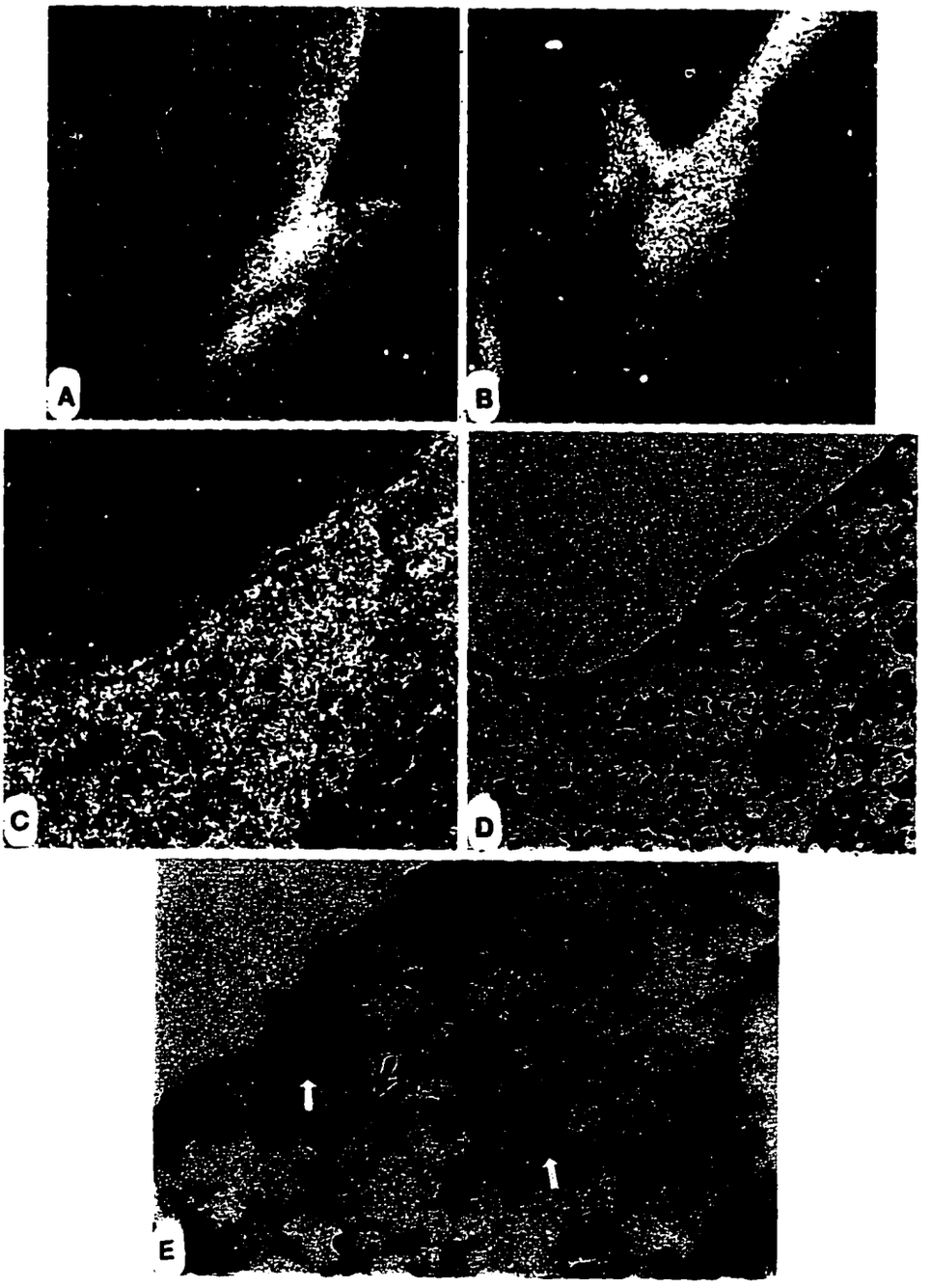


Figure 23. Localization of Msx-1 and Msx-2 in day 12.5 normal and mutant limbs.

Day 12.5 normal (A, C) and mutant (B, D) limbs were processed as described in Materials and Methods, Chapter 3. Sections of normal limbs (A, C) display Msx-1 (A) and Msx-2 (C) mRNA in the surrounding extoderm, subjacent mesenchyme, and interdigital regions of the developing limb. Sections of Hm mutant limbs (B, D) show similar expression patterns of Msx-1 (B) and Msx-2 (D) to that of the normal limb. Magnifications: A-D: 100X.

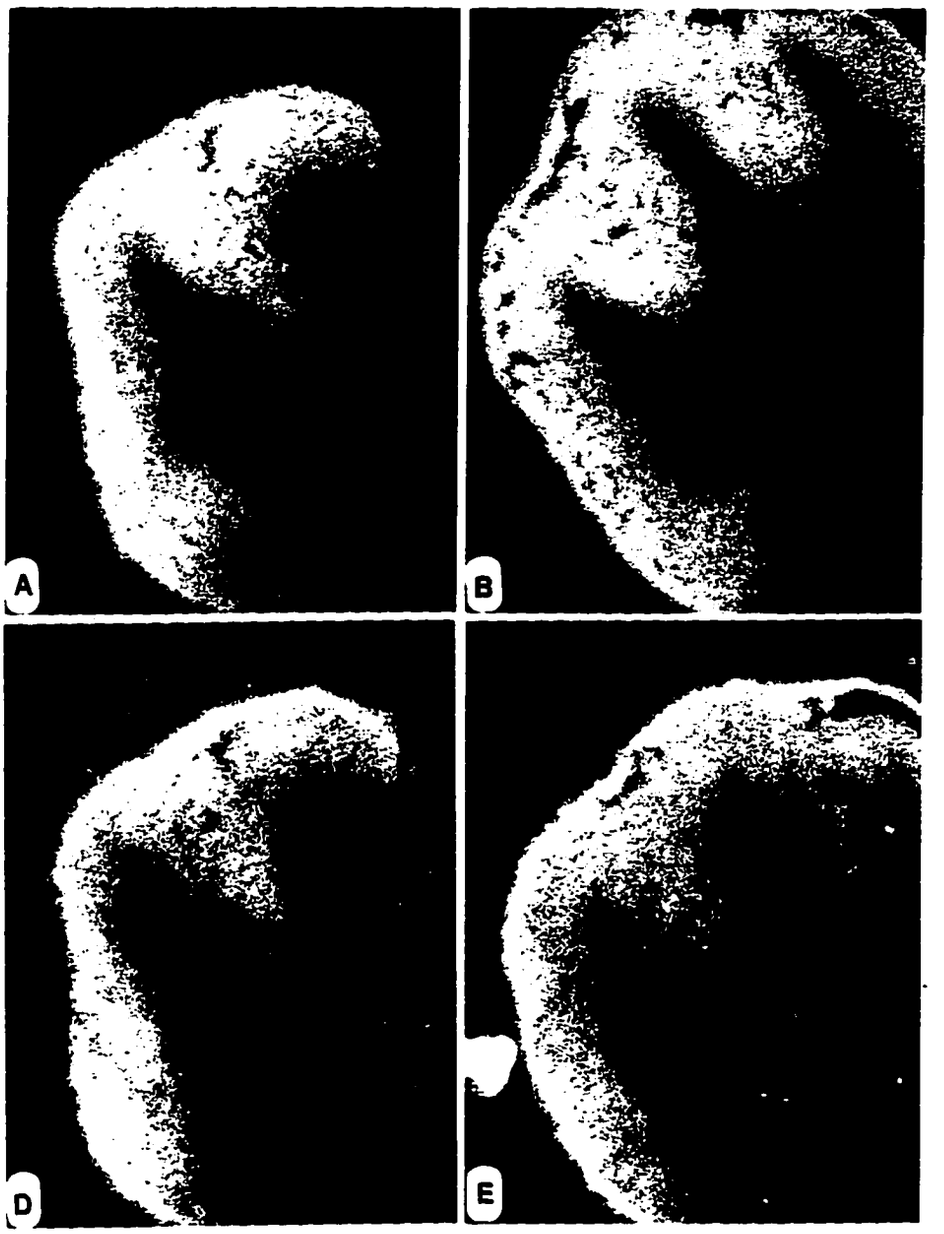


Figure 24. Localization of Msx-1 and Msx-2 in RA-treated day 14.5 normal and mutant limbs.

Day 14.5 normal (A), RA-treated normal (B,D) and RA-treated Hammertoe mutant (C,E) hindlimbs were processed and examined as described in Materials and Methods, Chapter 3. RA-treated normal (B) and RA-treated Hm mutant (C) limbs display a similar expression pattern of Msx-1 in the interdigital regions to that of the control normal limb (A). RA also does not alter the expression pattern of Msx-2 in normal (D) or mutant (E) limbs. Magnifications: A: 50X; B-E: 100X.

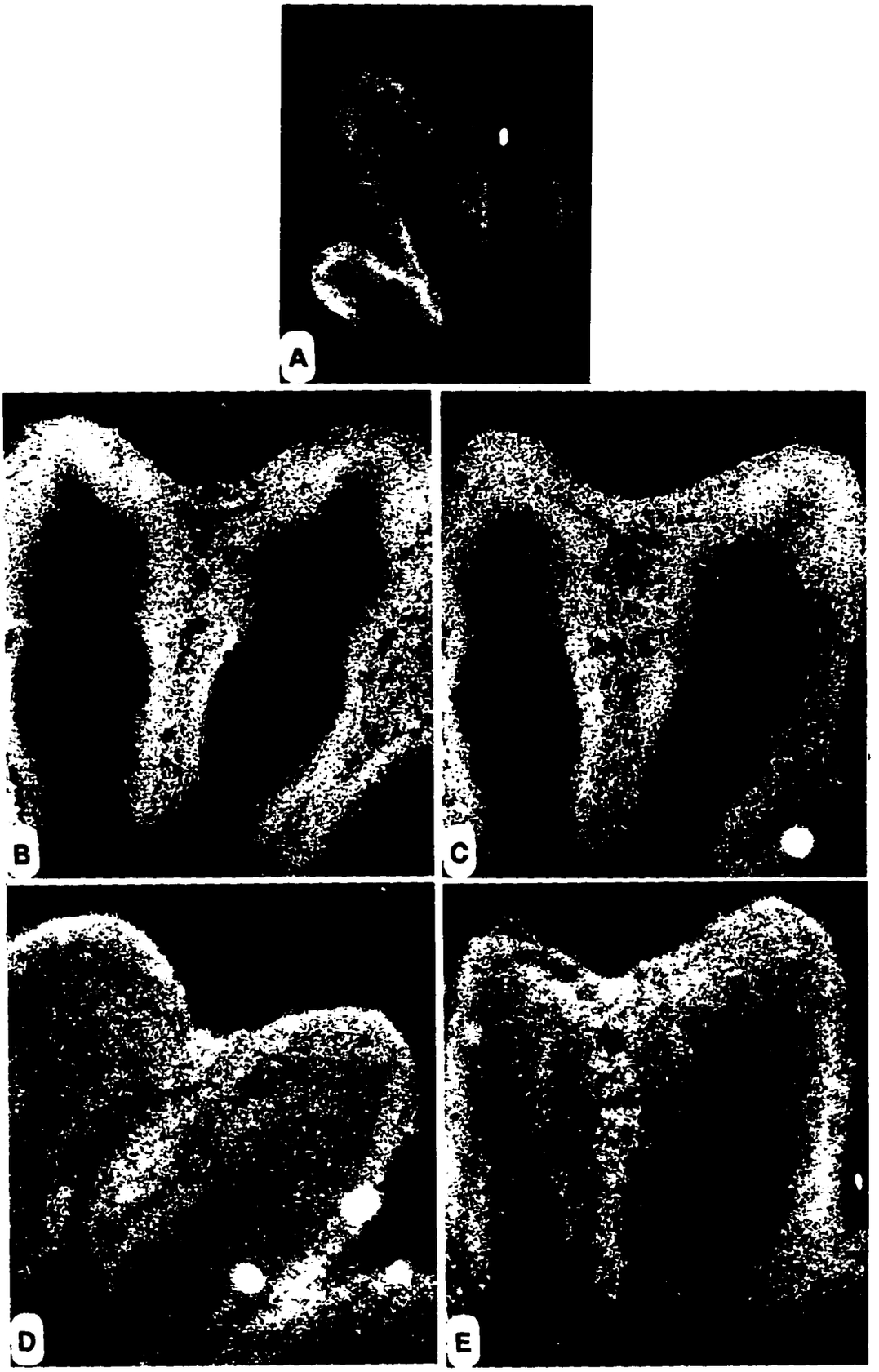


Figure 25. Localization of Cdk5 protein expression is associated with apoptotic cell death in developing and adult tissues.

Limbs, embryos, and adult ovaries were processed as described in Materials and Methods, Chapter 3. Cdk5 localization with Cdk5 antibody was detected in the interdigital cells of the day 14.5 limb (A, white arrows), the mesenchymal cells and retinal epithelium of the day 12.5 eye (arrows), the trigeminal ganglion of the day 14.5 embryo (C, arrows), and atretic follicles of the adult ovary (D, solid arrows). Expression of Cdk5 was not detected in the developing follicles of the ovary (D, open arrows). Magnifications: A-C: 100X; D: 400X.

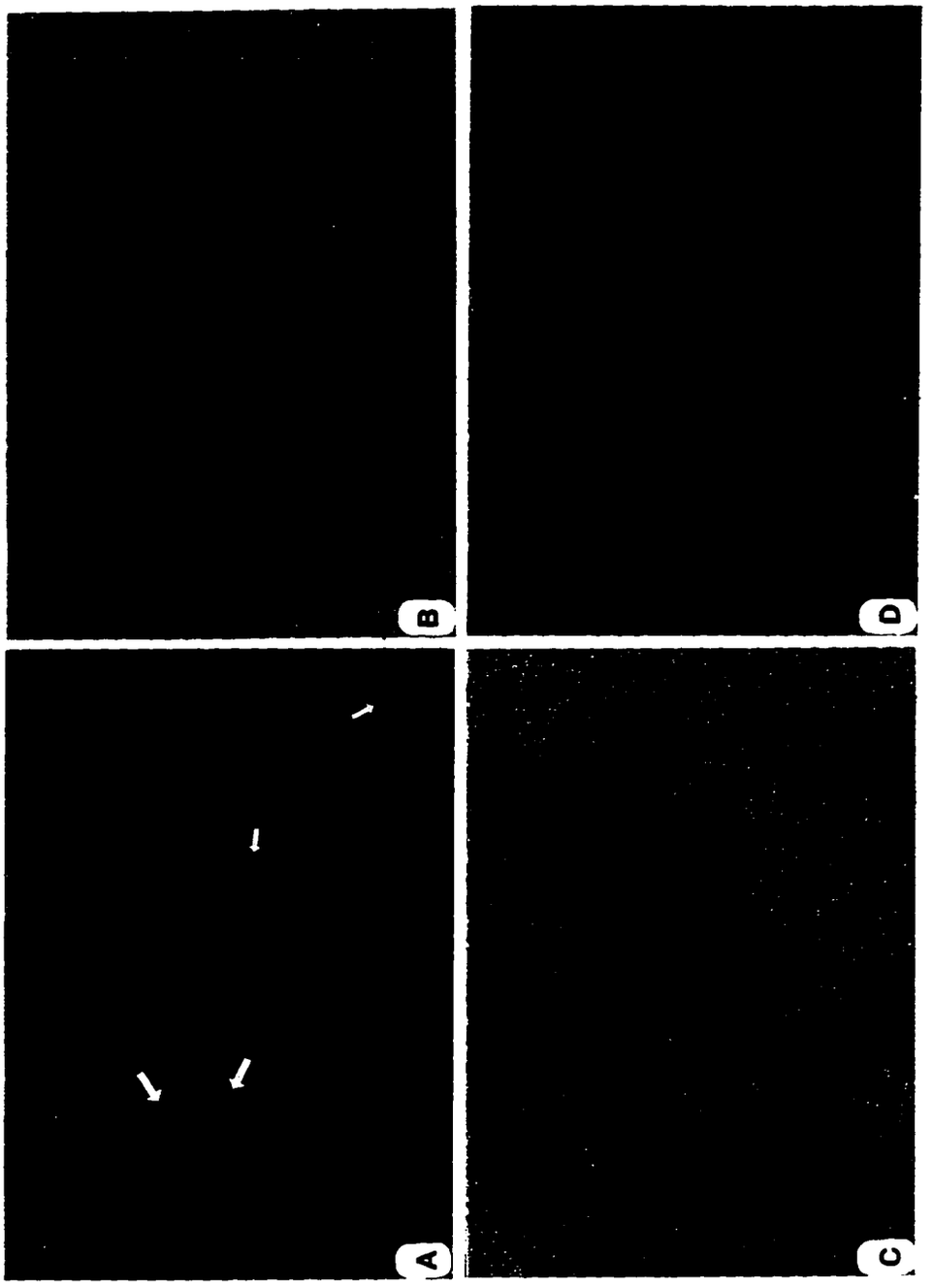


Figure 26. Association of Cdk5 to apoptotic cell death in the developing limb is not a general characteristic of Cdks.

Day 14.5 limbs were processed as described in Materials and Methods, Chapter 3. Section of day 14.5 limb displays abundant levels of Cdk5 expression in the interdigital apoptotic cells (A, arrows). Expression is not detected in serial sections incubated with Cdk1 (B) or Cdk2 (C) antibodies. Magnifications: A-C: 100X.

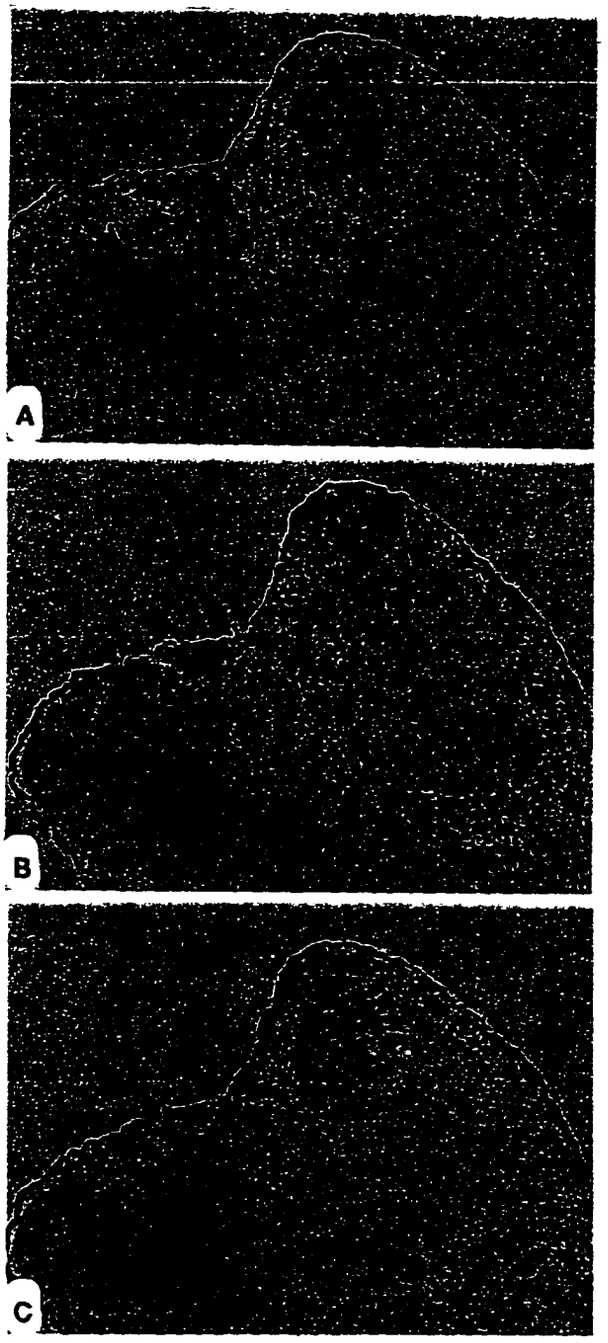


Figure 27. Histone H1 kinase activity in immunoprecipitates of Cdk5 in day 12.5 and 14.5 developing limbs.

Tissue lysates were processed from day 12.5 and 14.5 limbs and immunoprecipitation was performed with anti-Cdk5 antibody as described in Materials and Methods, Chapter 3. Incorporated radioactivity into labeled Histone H1 substrate displays increased levels of Cdk5 activity in the day 14.5 limb compared to the day 12.5 limb.

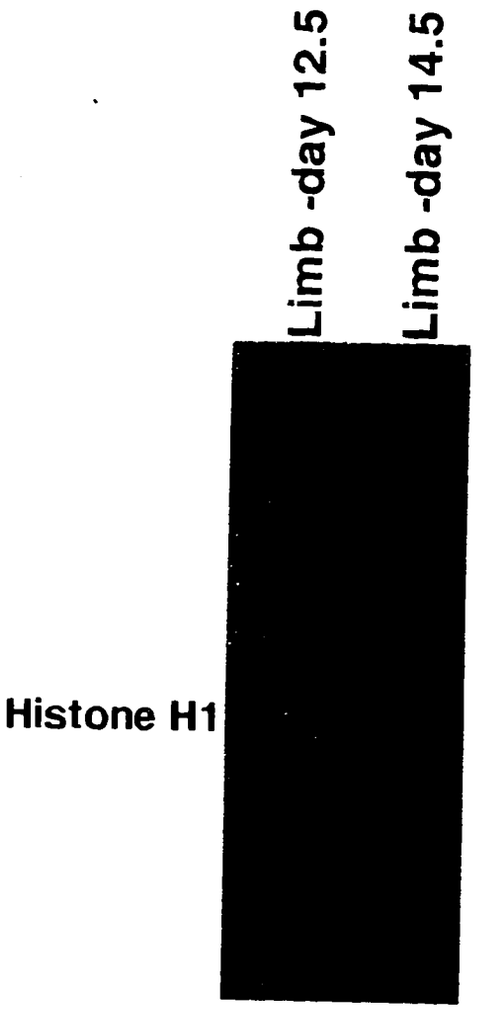


Figure 28. Cdk5 expression in normal, mutant, and RA-treated limbs.

Day 14.5 limbs were processed as described in Materials and Methods, Chapter 3. Compared to expression in apoptotic cells of the normal day 14.5 limb (A), Cdk5 protein is detected at high levels in the RA-treated limb (B, arrows). Few cells staining for Cdk5 antibody are detected in the cell death defective interdigital regions of the Hm mutant limb (C, arrow). In contrast, RA induces Cdk5 expression in correlation with induction of cell death in the Hm mutant limb (D, arrows). Magnifications: A-B: 400X; C-D: 100X.

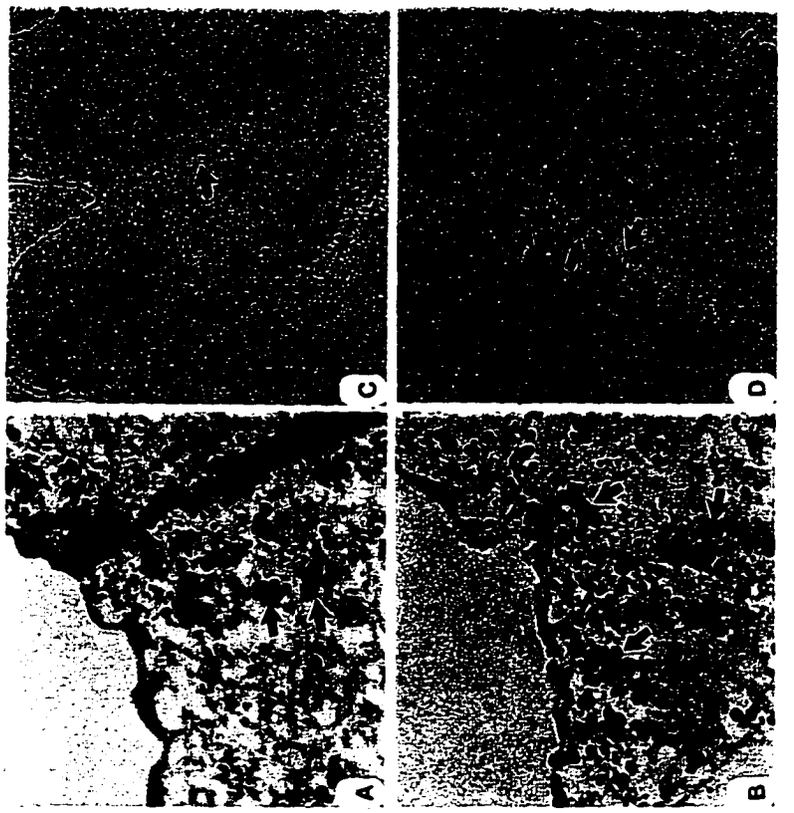


Figure 29. The effect of RA treatment on day 14 of gestation on the adult normal and mutant phenotype.

Normal (A-B), homozygous Hm mutant (C-D), and heterozygous Hm mutant (E-F) control (A, C and E) and RA-treated (B, D and F) hindlimbs were examined. Compared to the control normal limb (A), the RA-treated normal displays a slight curvature at the distal end of the digits (B). However, the effect of RA on mutant limbs is more dramatic. Compared to the control homozygous mutant limb (C), there is distal separation between digits 2 and 5, and as such more extension of the RA-treated mutant limb (D). In the RA-treated heterozygous mutant limb (F), the digits are well defined and the flexure is almost absent compared to the control heterozygous limb (E). Magnifications: dissecting microscope: A-F: 30X.

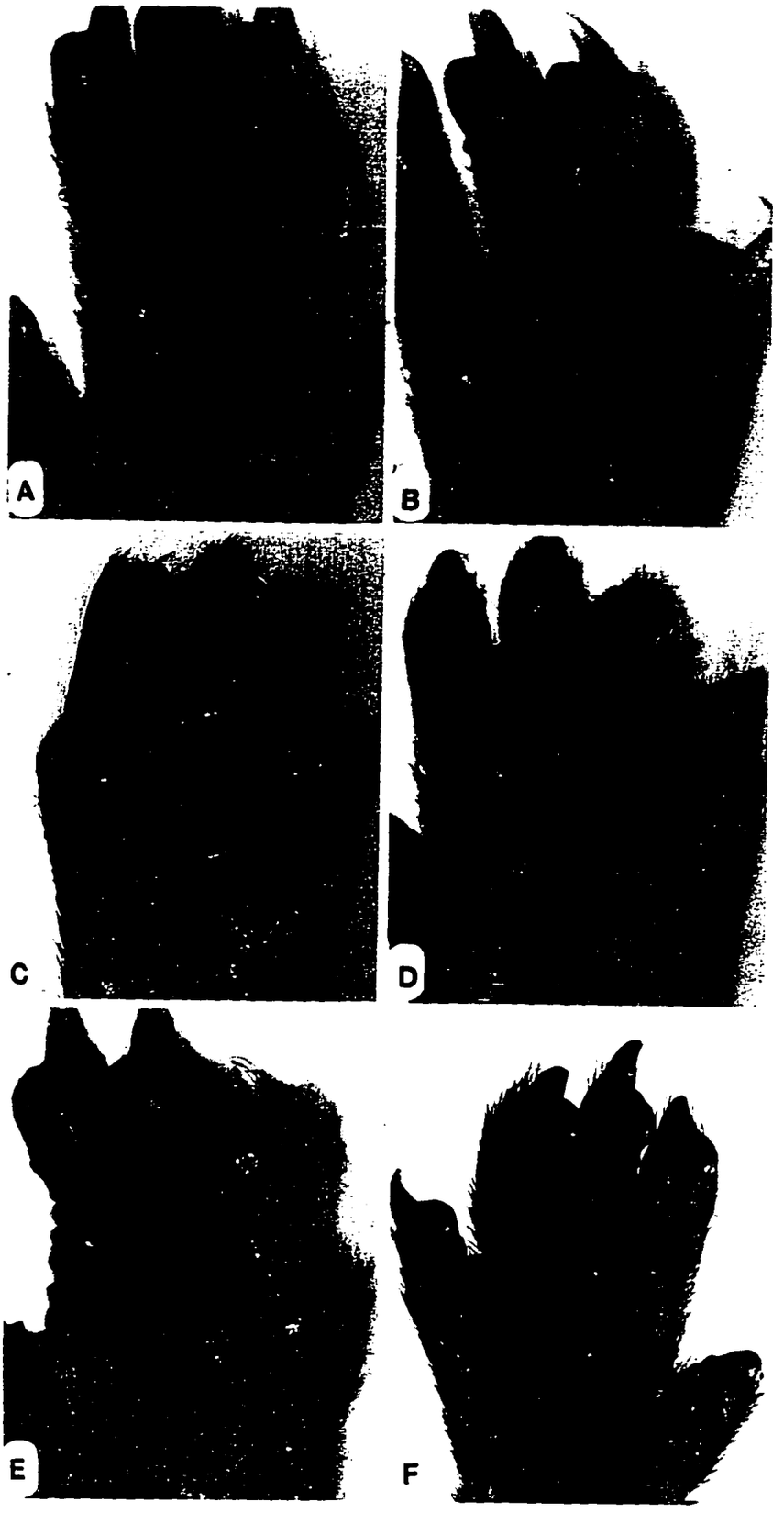


Figure 30. The effect of RA on different gestational days on adult normal and mutant phenotype.

Normal (A, C, E) and homozygous Hm mutant (B, D, F) were treated with RA on days 12 (A-B), 13 (C-D), and 15 (E-F) of gestation and examined one (A-B) or several (C-F) days after birth. RA did not seem to have a gross effect on normal (A, C, E) or mutant limbs (B, D, F) at these gestational stages as normal limbs still showed extended digit formation and mutant limbs continued to display syndactyly and flexure. Magnifications: dissecting microscope: A-F: 30X.

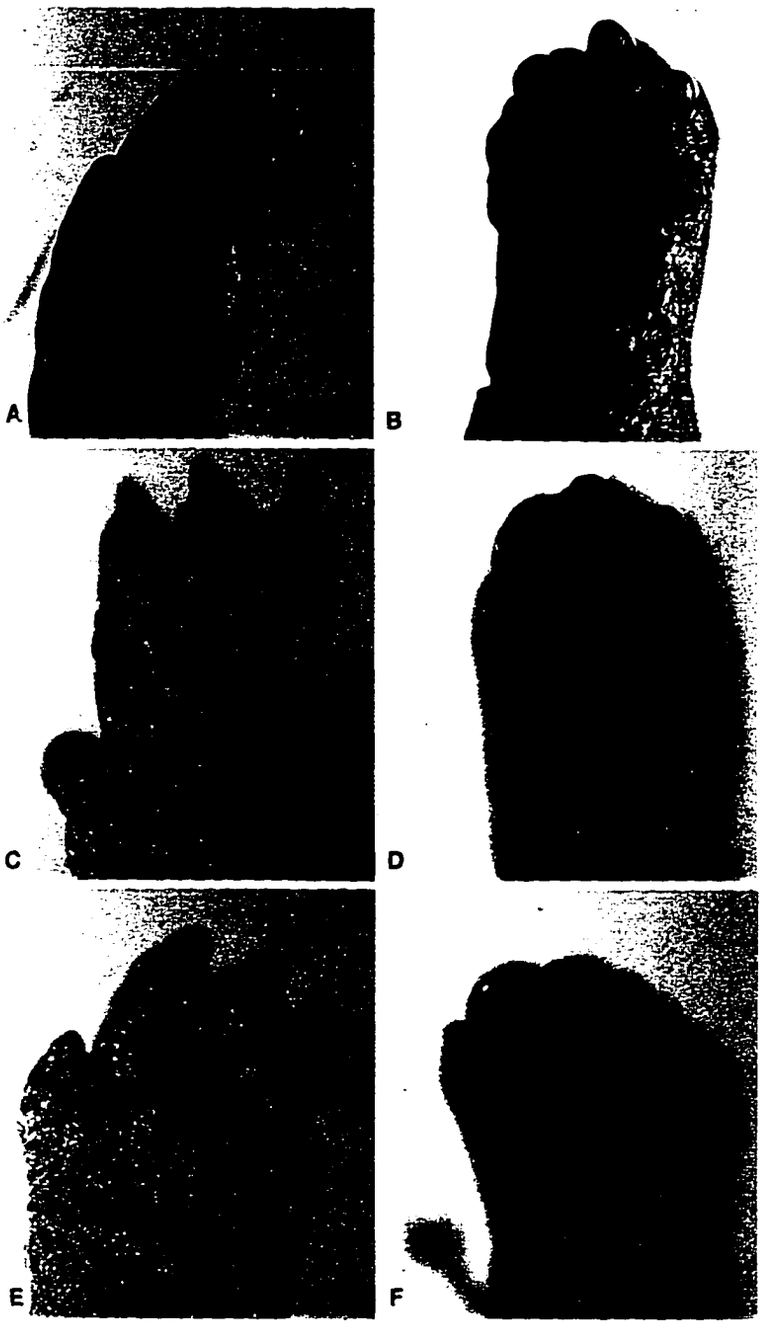


Figure 31. The effect of RA on the mutant phenotype is dose dependent.

Normal (A) and homozygous Hm mutant (B) limbs treated with 400 mg/Kg body weight of RA at day 14 of gestation. Although the normal limb does not display any gross effect with the double dosage of RA (A), the Hm mutant limb shows more extended and independent digits (B). Magnifications: A-B: dissecting microscope 30X.

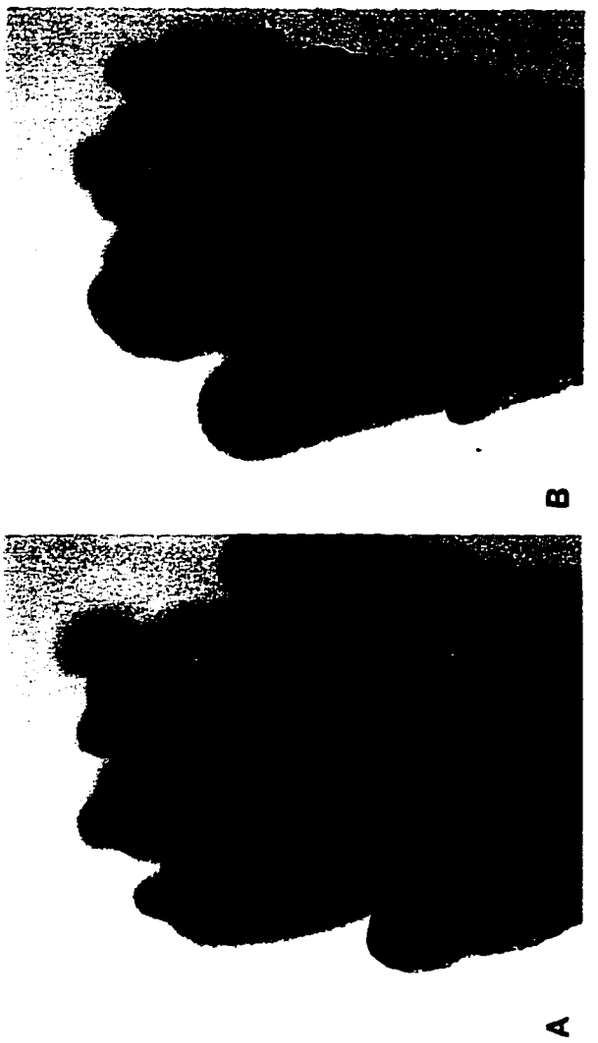


Figure 32. RA alters the Hammertoe mutant phenotype by affecting soft tissue only.

Mutant control (A) and RA-treated (B) two week old hindlimbs (ventral view) were compared to normal limbs (C-ventral view). In contrast to the extended digits of the wildtype limb, the Hm/Hm limb displays strong flexion and soft tissue syndactyly. This effect is improved with increased separation and reduced flexion of the RA-treated Hm/Hm hindlimb (B). The limb skeleton of mutant (D), RA-treated mutant (E), and normal (F) limbs (dorsal view) were stained for bone and cartilage. Besides the flexure in the Hm/Hm limb, no differences are detected in the bones or cartilage of the different limbs. Magnifications: dissecting microscope A-C: 30X; D-F: 15X.

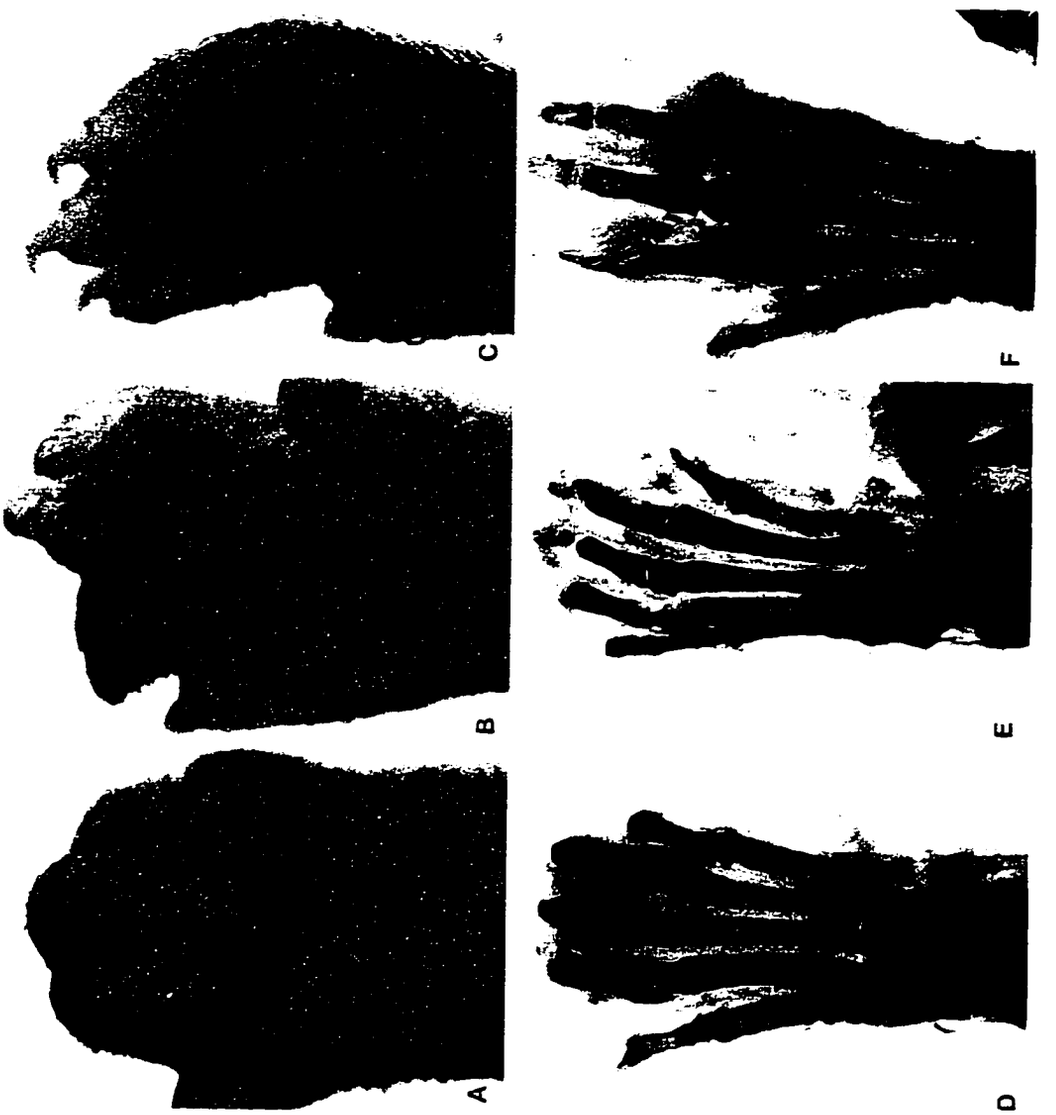
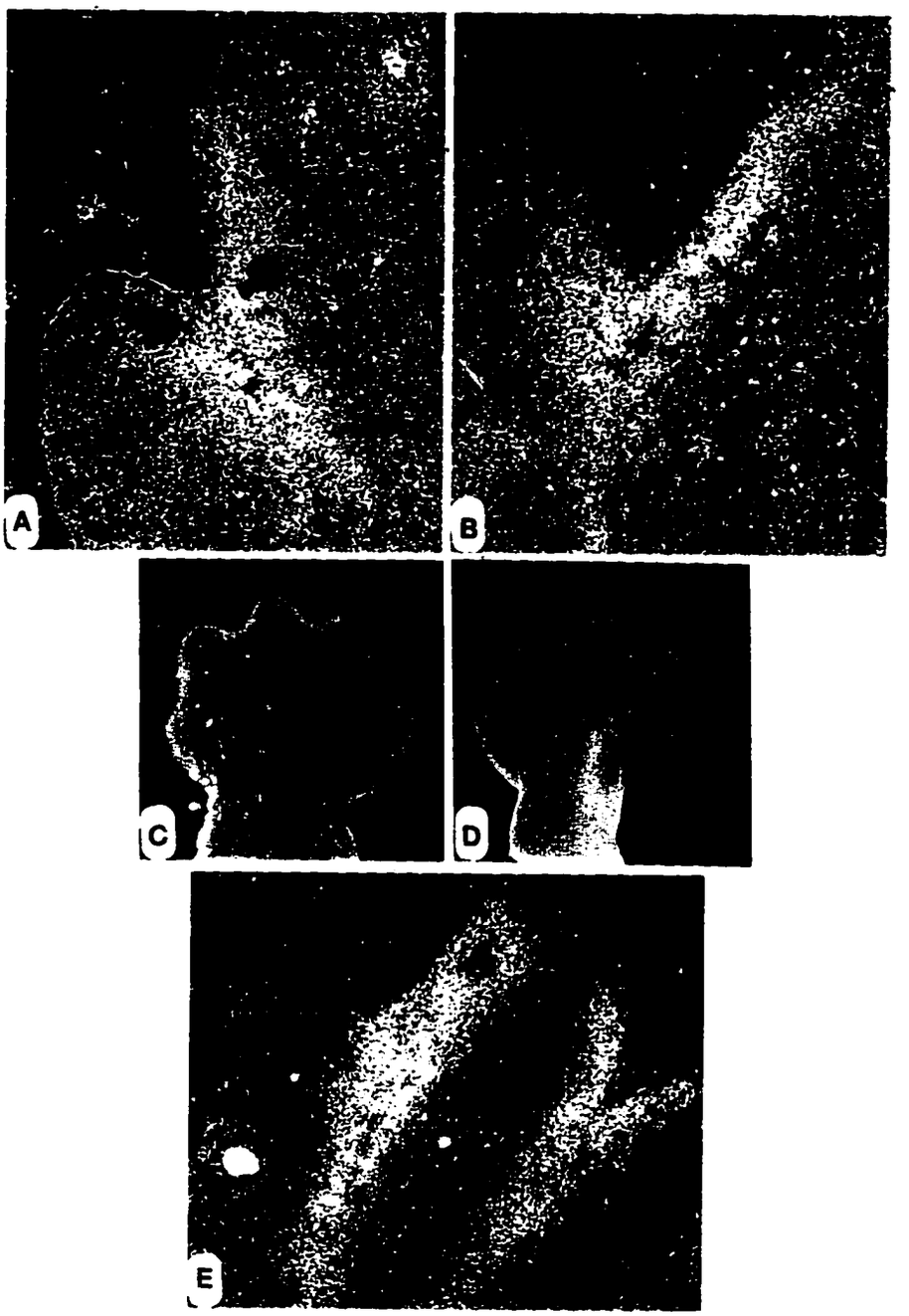


Figure 33. The pattern of *RARβ* and *RAREhsplacZ* are not altered in the Hammertoe mutant.

Gestational day 14.5 normal (A,C) and mutant (B,D) limbs were treated and hybridized (A-B) or stained with X-gal (C-D) as described in Materials and Methods. Chapter 4. The expression of *RARβ* is found interdigitally in the normal limb (A) and mutant limb (B). Transgene expression is also detected in the interdigits of normal (C) and mutant (D) limbs. No differences were observed in the pattern of expression in *RARβ* or in RA levels in normal limbs after RA treatment as well (E). Magnification: A-B, E: 100X; C-D: dissecting microscope: 30X.



PART IV. REFERENCES

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