Scanning Microscopy

Volume 9 | Number 2 Article 21

4-28-1995

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J. M. Hurle
Universidad de Cantabria

M. A. Ros
Universidad de Cantabria

V. Garcia-Martinez
Universidad de Extremadura

D. Macias
Universidad de Extremadura

Y. Ganan
Universidad de Extremadura

Scanning Microscopy, Vol. 9, No. 2, 1995 (Pages 519-534) 0891-7035/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

CELL DEATH IN THE EMBRYONIC DEVELOPING LIMB

J.M. Hurle*, M.A. Ros, V. Garcia-Martinez[1], D. Macias[1] and Y. Gañan[1]

Departamento de Anatomía y Biología Celular, Universidad de Cantabria, 39011 Santander, Spain
[1]Departamento de Ciencias Morfológicas, Universidad de Extremadura, Badajoz, Spain

(Received for publication January 23, 1995 and in revised form April 28, 1995)

Abstract

In amniote vertebrates, the development of form and structure of the limb bud is accompanied by precise patterns of massive mesodermal cell death with morphological features of apoptosis. These areas of cell death appear to eliminate undifferentiated cells which are required only for a limited time period of limb development. Predictable skeletal and morphological anomalies of the limb occur when the pattern of cell death is modified in mutant species or under experimental conditions. Most evidence points to the occurrence of local triggering mechanisms to account for the establishment of the areas of cell death and the subsequent activation of cell death genes. Modifications of the extracellular matrix and diminution in the contribution of growth factors by neighbouring tissues appear as the most likely potential candidates for triggering the cell death program. Information on the genetical basis of cell death in the developing limb is very scarce. Among the increasing number of cell death genes identified in other cell death systems, such as p-53 and the *ced-3*/ICE and *ced-9*/*bcl-2* gene families, only *bcl-2* has been studied in detail during limb development and yet, the information obtained is contradictory. *Bcl-2* is not expressed in the areas of cell death of the developing limb, but normal limbs develop in mice with disruption of the *bcl-2* gene. Obviously, the clarification of the role of the cell death genes constitute a major task in future studies of cell death in the developing limb.

Key Words: Apoptosis, death genes, endonuclease, lysosomes, extracellular matrix.

*Address for correspondence:
Juan M. Hurle
Departamento de Anatomía y Biología Celular,
Facultad de Medicina Polígono de Cazoña,
39011 Santander,
Spain

Telephone number: +34-42-201920
FAX number: +34-42-201903

Introduction

It is now widely recognized that cell death is a controlled behaviour of cells with a key role in growth, differentiation and tissue homeostasis. The idea that cell death constitutes a physiological cell behaviour characteristic of multicellular organisms has been advanced in the fifties and sixties on the basis of the observation of massive degenerative processes in the course of normal embryonic development and larval metamorphosis (Glücksmann, 1951; Saunders, 1966). Since then, the study of cell death has received increased attention; in the last few years, it became a fashionable topic of research due to its emergence as a subject of extraordinary biological interest. A distinction between such physiological or programmed cell death and cell death caused in response to noxious physical or chemical insults to the cells was soon proposed on the basis of the morphological characters of the dying cells. Kerr *et al.* (1972) proposed to restrict the term of necrosis for the dying processes resulting from cell injury and to use the new term "apoptosis" for physiological cell death. The initial morphological distinction between necrosis and apoptosis has been confirmed by biochemical and molecular studies and both processes are now considered as two distinct and unrelated entities (Buja *et al.*, 1993).

In the last few years, an impressive amount of information has been accumulated and has shown the existence of a suicide program in cells which can be activated or repressed by a variety of physiological and pathological stimuli. This suicide program involves an active self-destruction of the cell and constitutes the molecular basis of apoptosis. Necrosis, on the contrary, is a passive phenomenon consisting of the disruption of the cell integrity caused by external agents (Buja *et al.*, 1993). The suicide program appears to be very complex and includes genes accounting for the onset of cell death and genes protecting cells from entering the death program.

In an early theoretical article, Umansky (1982) proposed that in evolution, cell-death genes are activated at the early stages of the formation of multicellular

organisms, and play the role of eliminating damaged or abnormally functioning cells presenting a real or potential danger for the survival of the whole organism. This hypothesis has received considerable support with advances in the knowledge of the molecular basis of cell death. It has been found that the presence of damaged or single stranded DNA constitutes one of the mechanisms which activate the cell death program. Conversely, many viruses carry genes encoding proteins which block the cell death program-suggestive of an evolutionary specialization of these viruses to inactivate the possibility of an antiviral defensive strategy of the multicellular organisms based on destruction of the cells infected by the virus [see review by Vaux *et al.* (1994)].

With regard to such incorporation in eukaryote cells of a molecular machinery finely tuned to self-destroy the cells, it is worth mentioning the occurrence of comparable killing molecular mechanisms in prokaryote. Many plasmids contain DNA which encodes a long-lasting protein lethal for the host bacteria and also an unstable antidote protein. This molecular machinery ensures the permanence of the plasmid in the bacteria. If the plasmid is eliminated, the host bacteria are killed by the stable poison protein due to the interruption in the synthesis of its corresponding antidote protein [see, Bernard *et al.* (1993)].

In the course of evolution, the organisms would have found a number of uses for the cell death program. In fact, examples of the participation of cell death in physiologic processes are innumerable. As mentioned above, during embryonic development and larval metamorphosis, the formation of many parts of the body involves the elimination by cell death of large cell populations or even all the cellular components of an organ rudiment (Saunders, 1966; Lockshin, 1981; Hurle, 1988). In the same way, the cell turnover and differentiation of most, if not all, adult tissues involve the controlled elimination of cells by cell death. The elimination of lymphocytes during receptor repertoire selection, and the differentiation of the red blood cells or the atrophy of tissues linked to endocrine modifications are remarkable examples which illustrate the widespread participation of cell death in differentiation and tissue homeostasis (Strange *et al.*, 1992). However, the extraordinary biological interest of physiological cell death is due to a great extent to its transcendence in pathology and by the emerging possibility of manipulating the death program for therapeutic purposes.

Many congenital malformations are due to alterations in the normal pattern of cell death in the embryo (Hurle, 1988). Excessive survival of cells resulting from blockage of death genes or by overexpression of genes inhibiting cell death has been found to be linked with oncogenesis (Williams, 1991; Symonds *et al.*, 1994; Wright *et al.*, 1994). Other diseases, such as acquired-immune deficiency syndrome (AIDS), appear to be the result of triggering the normal program of cell death in specific target cell populations by external agents such as a virus (Meyaard *et al.*, 1992). In some cases, cell death is not the first target for the pathologic agent but it is induced as a secondary response to the initial alterations in the organ. This might be the case in the transition to heart failure in hypertrophic heart disease by chronic pressure overload (Bing, 1994).

With regard to therapeutics, it has been found that, in many cases, the efficiency of anticancer agents depends on their ability to induce cell death by apoptosis in the target tissue (Trauth *et al.*, 1989; Eastman, 1990; Fisher, 1994). There are also promising results in the utilization of death-genes via recombinant adenoviral vectors as anticancer therapeutic agents (Liu *et al.*, 1994).

In spite of the impressive amount of information collected in recent years, many important questions concerning the biological significance and the control of physiological cell death await clarification. Whether there is an universal genetic program for cell death, how the death program is triggered or inhibited in each particular cell type, and the unraveling of the cascade of events leading from the expression of the first death genes to cell disintegration are key topics for the understanding of cell death subjected to intense investigation at present. In this article, we will survey our current knowledge on the involvement of cell death during embryonic limb development. This topic, along with the elimination of the tail in amphibian anura larval metamorphosis, were probably the first to attract the attention of biologists to the study of programmed cell death. However, at present when molecular biology approaches are being extensively applied to the study of cell death, the embryonic limb cell death model has been largely neglected. In this review, we will pay particular attention to the above mentioned questions concerning the control and the mechanism of cell death.

Distribution of cell death in the developing limb

In vertebrates, the formation of each limb takes place from an initial primordium which grows on the lateral surface of the embryonic body. The initial limb bud is a very simple structure consisting of a core of mesenchymal cells covered by an ectodermal cap. The ectoderm covering the distal margin of the bud, termed the apical ectodermal ridge (AER), induces the proliferation of the subjacent mesenchyme and the bud undergoes progressive outgrowth. Concomitantly with this process, the mesenchymal core of the bud differentiates into the cartilaginous anlage of the limb skeleton (Fig. 1). This differentiation process follows a proximodistal sequence.

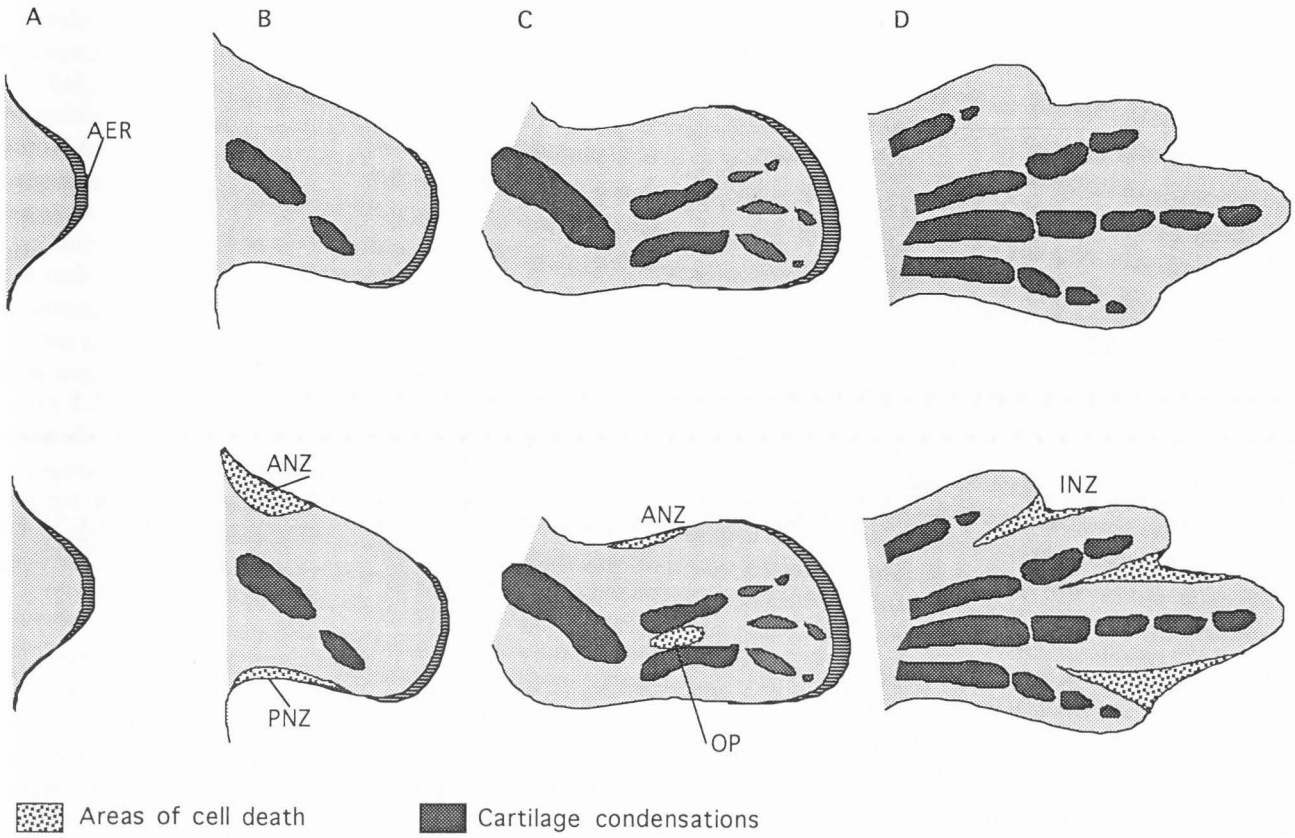

Figure 1. Schematic representation of the establishment of the skeletal condensations and the areas of cell death during chick limb development. No differences between wing and leg in the pattern of cell death have been represented. **Column A** represents stage 20 limb bud. **Column B** represents stage 23-24 limb bud, the humerus/femur and the initial ulna/tibia condensations appear laid down. **Column C**, represents stage 26-27 limb bud where the full zeugopodium (ulna-radius/tibia-fibula) and the initial condensations of the autopodium are illustrated. **Column D** represents a stage 32 autopodial segment of the limb showing the primordium of the digits. **Top row** only shows skeletal condensations while **bottom row** indicates both skeletal condensations and the adjacent areas of cell death. Abbreviations: **AER**, apical ectoderm ridge; **ANZ**, anterior necrotic zone; **PNZ**, posterior necrotic zone; **OP**, opaque patch; **INZ**, interdigital necrotic zones.

Initially, the most proximal segment of the bud forms a single central skeletal piece which corresponds to the femur/humerus. The primordia of the tibia-fibula/ulna-radius are formed next in the intermediate segment of the limb bud. The tarsa/carpa and the digital rays are the last and the most distal pieces to be formed. The muscles, blood vessels and connective tissue differentiate around the cartilaginous skeleton.

Concomitantly with the process of formation of the skeletal primordium of the limb, a number of well defined areas of cell death are identified (Fig. 1). The extent of cell death within each of these areas as well as their temporal and spatial pattern of distribution in the limb bud, is constant for each animal species. Differences between species in the pattern of distribution of the areas of cell death are significant but, as we shall discuss later, what is particularly interesting is that these differences can always be related with parallel differences in the morphology of the limb. An exception to this rule are the anamniote vertebrates. In these species, limb morphogenesis takes place without cell death (Cameron and Fallon, 1977).

In the chick limb bud, the first two areas of massive cell death by apoptosis to appear extend through the undifferentiated mesenchyme located respectively anterior and posterior to the central chondrogenic mesenchyme of the proximal segment of limb (see, review by Hinchliffe, 1982). These areas have been termed the anterior and the posterior necrotic zones (ANZ and PNZ; it should be mentioned that by the time these areas were first described, the term apoptosis had not been introduced in the literature). Next, a third area of cell death appears in the central mesenchyme of the limb bud delimited by the condensations of the two skeletal pieces of

Figure 2. Avian leg autopodium showing the pattern of interdigital cell death after vital staining with neutral red. Bar = 0.5 mm.

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the zeugopod (tibia-fibula/ulna-radius). This area was termed the opaque patch (OP). Later in development, a further set of areas of cell death is identified in the undifferentiated mesenchyme located between the developing digital rays. These areas of cell death have been termed the interdigital necrotic zones (INZ), again, using a terminology from prior to the adoption of the term of apoptosis.

In addition to these major areas of cell death, several other events of limb development appear to involve the participation of cell death [see Hurle *et al.* (1995)], but the extent of cell death in these events is much less prominent and experimental approaches to ascertain their control and significance are few.

Role of cell death in limb morphogenesis

The involvement of the areas of massive cell death in limb morphogenesis is now clearly established. For almost all the different areas of cell death, three different lines of observation and experimental approaches support the hypothesis of an important role in limb morphogenesis (Hinchliffe, 1982; Hurle, 1988; Hurle *et al.*, 1995). These include: (1) comparative studies in species with different limb morphology; (2) studies carried out in mutants with modifications of the normal pattern of cell death; and (3) analysis of the effects caused by the administration of drugs interfering with the normal pattern of cell death. The results obtained from these observations show that all the areas of mesodermal cell death which have been mentioned account for the removal of mesenchymal cells with skeletogenic potential which are no longer necessary once the limb anlage has attained some critical volumetric parameters and levels of tissue differentiation.

The areas of interdigital cell death (Fig. 2) illustrate that contention well. Interdigital necrotic areas are present in all the amniote vertebrates but their extension shows a wide range of variations depending of the morphology of the digits in each species. In species with free digits, such as the chick (Saunders and Fallon, 1967; Pautou, 1975), quail (Fallon and Cameron, 1977), lizard (Fallon and Cameron, 1977), mouse (Zakeri *et al.*, 1994) or human (Kelley, 1973), the areas of cell death extend through all the interdigital space. In species with webbed digits, such as the duck (Saunders and Fallon, 1967; Hurle and Colvee, 1982) or the turtle (Fallon and Cameron, 1977), interdigital cell death is limited to the distal part of the interdigit. In species with free digits, but having a membranous lobulation along the margins of the digits, such as, the moorhen (*Gallinula chloropus*) or the coot (*Fulika atra*), interdigital cell death is restricted to the central part of the interdigital tissue (Hurle and Climent, 1987). In syndactylous mutant species, interdigital cell death is inhibited (Hinchliffe and Thorogood, 1974; Van der Hoeven *et al.*, 1994; Zakeri *et al.*, 1994). Finally, when embryos are treated with drugs which inhibit cell death, the limbs exhibit membranous syndactyly (Toné *et al.*, 1983) accompanied in some cases by the presence of ectopic interdigital cartilages (Fernandez-Teran and Hurle, 1984).

Experimental analysis to ascertain the developmental potential of the interdigital mesenchyme prior to the onset of cell death provide conclusive evidence that the interdigital tissue contain all the information required to form a full digit (Hurle and Gañan, 1986, 1987; Gañan *et al.*, 1994). Several experimental manipulations, destined to isolate the interdigital spaces from the influence of the neighboring digits, abolish the death program leading to the development of ectopic digits (Hurle *et al.*, 1989; Hinchliffe and Holder, 1993; Lee *et al.*, 1993; 1994; Gañan *et al.*, 1994; Ros *et al*, 1994). Similar results are obtained from grafting into the flank of a host embryo of limb bud-like recombinants composed of a young limb ectodermal cap and a core of interdigital mesoderm (Ros, Fallon and Hurle, in preparation).

On the basis of all the information mentioned, it appears that prior to the appearance of the digital rays, all the mesenchymal tissue of the distal digit-forming segment of the limb bud has the potentiality to form digits. The establishment of each digit would cause a lateral antichondrogenic inhibitory effect in the adjacent interdigital mesenchyme followed by cell death (Gañan *et al.*, 1994). The intensity of interdigital cell death, which can be presumed to be dependent on the characters of the autopodium in each species, will result in the sculpturing of the shape of the digits. A comparable mechanism involving the formation of an initial excessive number of cells followed by its regulation by cell death is a common feature during the development of the nervous system (see, review by Oppenheim, 1991).

Control and molecular mechanisms of cell death in the embryonic limb

On the basis of actual knowledge of the molecular mechanism accounting for the control of cell death, three distinct hierarchic levels of death control should be distinguished. First, the cells appears to have a set of genes which by its balanced activation triggers the cellular machinery which accounts for the self-destruction of the cell. Here, we shall call these genes the "death-genes", though some of them have in fact an anti-death effect. Second, as a result of the activation of the appropriate death-genes, the cells committed to death initiate a cascade of cellular events which lead to their disintegration. We shall call here all these events the "death-machinery". Finally, the activation of the death-genes in cells according to appropriate temporal and spatial parameters is dependent on information that we will term here "patterning of cell death".

"Death genes" Although we are still far from having a clear picture of the genetic basis of cell death, there is evidence of the existence of inducer as well as repressor death-genes. The first discovery of genes directly related with cell death was obtained in the nematode *Caenorhabditis (C.) elegans* (Ellis and Horvitz, 1986). As described here for the vertebrate limb, during normal development of this worm, several cells undergo cell death according to precise temporal and spatial patterns. Mutations that inactivate either of two genes termed *ced-3* and *ced-4* (ced: cell death gene) result in the survival of almost all cells that normally die during development (Yuan and Horvitz, 1990). The significance of the *ced-4* gene product in the dying process is obscure and expression of this gene is not restricted to the dying cells (Yuan and Horvitz, 1992). In contrast, the study of *ced-3* is yielding very promising information for the understanding of the control of cell death.

The *ced-3* gene product appears to act as a cystein protease and shares functional and sequence similarity with the mammalian interleukin-1β (IL-1β)-converting enzyme (ICE) (Yuan *et al.*, 1993; Wilson *et al.*, 1994). The human ICE is a substrate specific protease involved in the production of IL-1β, a cytokine which mediates a wide range of biological responses including inflammation, wound healing, hematopoiesis, and growth of certain leukemias. Remarkably, it has been found that overexpression of ICE in rat fibroblasts causes cell death with morphological features of apoptosis (Miura *et al.*, 1993). Furthermore, microinjection of an expression vector containing an specific inhibitor of ICE (*crmA* gene), inhibits physiological cell death in cultured developing neurons (Gagliardini *et al.*, 1994). More recently, a death gene, *Ich-1* (also termed Nedd-2) belonging to the ICE/*ced-3* family, has been characterized in mammalian cells (Kumar *et al.*, 1994; Wang *et al.*, 1994). Interestingly, this gene produces two distinct proteins by alternative splicing: one of them induces programmed cell death while the other suppresses cell death (Wang *et al.*, 1994).

A second set of genes controlling cell death was identified after the isolation in *C. elegans* of a gene, termed *ced-9*, apparently a repressor of cell death (Hengartner *et al.*, 1992). Abnormal activation of *ced-9* prevents cell death in *C. elegans*. Conversely, a mutation that inactivates *ced-9* causes cells that normally live to undergo cell death. More recent studies indicate a more complex participation of *ced-9* in the control of cell death. Studies on *ced-9* (Hengartner and Horvitz, 1994), and especially, the identification in vertebrates of a set of homologous genes, are indicative of a double role of effector and repressor for cell death of the genes of this family (Boise *et al.*, 1993; Oltvai *et al.*, 1993). The first identified vertebrate gene homologous to *ced-9* was *bcl-2*. Activation of *bcl-2* inhibits cell death in many, but not all, models of cell death in which it was studied. Furthermore, *bcl-2* is able to prevent programmed cell death not only in vertebrate cells (Garcia *et al.*, 1992), but also in *C. elegans* (Vaux *et al.*, 1992). However, *in vivo bcl-2* appears to be associated with bax, a protein which is also homologous to *ced-9* and which accelerates apoptotic cell death (Oltvai *et al.*, 1993). It has been suggested that the ratio *bcl-2* to bax determines survival or death following an apoptotic stimulus. This interpretation fits with the identification of another gene homolog of *bcl-2* and *ced-9*, termed *bcl-x* (Boise *et al.*, 1993). As described above for *Ich-1*, *bcl-x* produces two distinct proteins by alternative splicing with opposite effects on cell death. One of the proteins is very similar in size and structure with the predicted protein of *bcl-2* and shares the same inhibiting effect on cell death. The other protein inhibits the ability of *bcl-2* to enhance cell survival. Thus, the family of *ced-9*/*bcl-2* appears as a second group of genes, highly conserved in evolution,

with a key role in the control of physiological cell death.

A third group of genes proposed to play a role in the control of cell death corresponds to genes controlling proliferation and oncogenesis in vertebrate cell lines. These include p53 (Debbas and White, 1993; Morgenbesser *et al.*, 1994), *c-rel* (Abbadie *et al.*, 1993), *c-myc* (Evan *et al.*, 1992), and *c-fos* (Buttyan *et al.*, 1988; Gonzalez-Martin *et al.*, 1992; Smeyne *et al.*, 1993). In all these cases, it has been found that, in addition to a well established role in the control of cell proliferation, their expression in some cell populations is accompanied by cell death. The fact that the function of some of these genes can be modified by viruses along with the occurrence of carcinogenic transformation in the cells after alteration of these genes confers special interest to the study of their role in the control of cell death. However, as for the other groups of death-genes their mechanism of action in the death process remains unknown and except for p53, their direct involvement in cell death has been questioned (Vaux and Weissman, 1993; Carrasco *et al.*, 1994; Hermeking and Eick, 1994). In this regard, it is important to mention that the action mechanism of p53 appears to be integrated in the *bcl-2*/bax complex described above (White, 1993; Chiou *et al.*, 1994; Miyashita *et al.*, 1994).

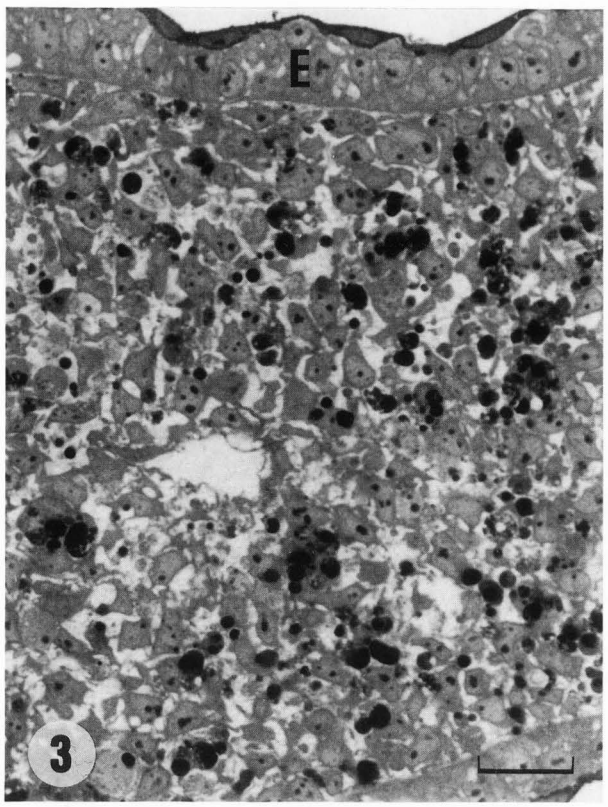
Unfortunately, information on the involvement of all the death genes mentioned in cell death during limb development is very scarce. The possible participation of the *ced-3*/ICE gene family in cell death during vertebrate limb development has not yet been analyzed, although ICE-deficient mice do not exhibit limb malformations (Li *et al.*, 1995). Similarly, there are only partial studies on the participation of *ced-9*/*bcl-2* gene family. During embryonic limb development, *bcl-2* protein expression was detected in the digital rays while it was absent in the interdigital tissue programmed to death (Novack and Korsmeyer, 1994). This observation fits with a role of *bcl-2* in the control of cell death processes in the developing limb. However, it should be mentioned that normal limbs develop in *bcl-2* gene-disrupted mice (Veis *et al.*, 1993). The existence of other genes in this family, such as *bcl-x*, may account for that discrepancy but again its possible expression in the areas of limb mesodermal cell death remains to be analyzed.

With regard to the involvement of the third group of death-genes in the limb model, neither p53 nor *c-fos* have been yet studied in the areas of cell death of the embryonic limb. *C-rel* expression in the limb bud has studied by *in situ* hybridization by Abbadie *et al.* (1993) who reported a specific labeling for *c-rel* in the dying cells of all areas of cell death of the chick embryonic limb. However, the expression of this gene in other cell populations not subjected to programmed cell death, along with the absence of its expression in some embryonic cell populations destined to die (Carrasco *et al.*, 1994), makes it difficult to propose a role for *c-rel* in embryonic cell death. In our laboratory, we have analyzed the distribution of *c-myc* expression by *in situ* hybridization in the chick developing limb bud (Ros, Delgado and Leon, in preparation) and found that none of the areas of cell death exhibited *c-myc* labeling, either prior to or during the dying processes. In fact, at advanced stages of limb development, the digital chondrogenic rays showed some labeling contrasting with the negative labeling of the dying interdigital tissue.

The recent identification of a mouse mutant (***Fused toes***), induced by the integration of the human *Ha-ras* gene characterized by inhibition of interdigital cell death (Van der Hoeven *et al.*, 1994), may provide, in the future, new information for the identification of death-genes involved in cell death during limb development. In this mutant, the inhibition of interdigital cell death is also accompanied by thymic hyperplasia and by a lower susceptibility of the thymocytes to undergo apoptosis after cortisone treatment. This coincidence is indicative of a common molecular mechanism for interdigital cell death and cell death associated with thymocyte maturation. However, neither *c-myc* nor *bcl-2* were found altered in the mutant mice (Van der Hoeven *et al.*, 1994).

"Death-machinery" In contrast with the recent advances in the identification of the genes controlling cell death, the mechanisms employed by the cells for self-destruction constitute an obscure topic of cell biology. Many basic questions are still unanswered. A first major unanswered question is whether the activation of the cellular machinery for programmed cell death is directed by the synthesis of one or several poisonous factors or if cell death is caused by an active interruption of mechanisms blocking a specific suicide machinery present in a latent state in all cells. A further unanswered key question is whether programmed cell death always involves the same cellular mechanisms or if the execution of the death program is dependent on the cell lineage or the degree of cell differentiation.

The term of apoptosis proposed by Kerr *et al.* (1972) assumed the occurrence of an unique mechanism of programmed cell death. This contention was based in the common morphological features of the cells undergoing cell death by a supposed intrinsic mechanism, in contrast with the very different morphology of the dying cells killed by exogenous injuries (necrosis). It should be mentioned, however, that programmed cell death in some models exhibit morphological features quite different from the typical apoptosis. This is the case in the degeneration of the larval intersegmental muscles of the lepidoptera insects after ecdysis (Lockshin and Beaulaton, 1975). In this model, the first cellular alteration consists of the activation of an intense autophagic



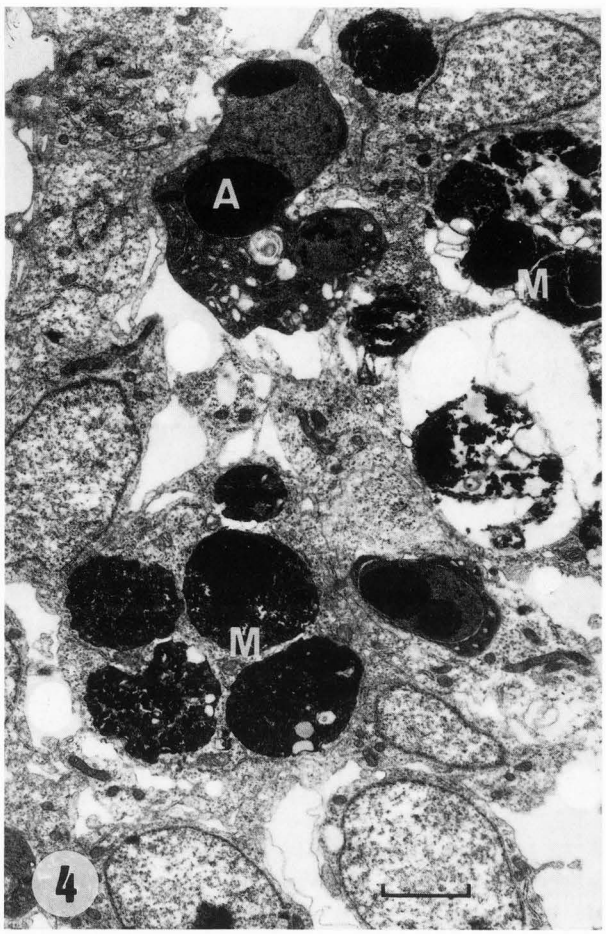


Figure 3. Semi-thin section of the interdigital space of a chick leg bud at the stage of maximum intensity of cell death (day 8 of incubation). Note the abundance of dark apoptotic cells. Ectoderm (**E**). Bar = 20 μm.

Figure 4. Transmission electron micrograph illustrating cell death in the interdigital space of the chick leg bud. An isolated dead cell with the typical morphology of apoptosis (**A**) and several dead cells engulfed by large macrophages (**M**) are clearly identifiable. Bar = 1 μm.

Figure 5. Scanning electron micrograph of the interdigital space illustrating the same stage of cell death shown in Figure 3. Note the abundance of small rounded cell fragments (arrowheads) resulting from the fragmentation of the dying cells. Arrow shows a cell fragment in course of engulfment by a macrophage (**M**). Ectoderm (**E**). Bar = 4 μm.

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process. Nuclear alterations in these dying cells appear relatively late when cytoplasmic and cell membrane alterations are very prominent (Lockshin and Beaulaton, 1975).

In all the areas of cell death of the limb bud and in all vertebrates where they are present, the morphology

of the dying cells correspond to typical apoptosis. It should be mentioned, however, that in the interdigital regions of cell death and at advanced stages of the degeneration process, cells with morphological features which are typical for necrosis, such as, disintegration of the cell membrane, are identifiable (Garcia-Martinez and Climent, 1985). Since blood vessels also degenerate in the areas of interdigital cell death (Hurle *et al.*, 1985), these necrotic cells could be the result of local anoxia or, alternatively, they may represent a secondary degeneration of unphagocytosed apoptotic cells.

Basically, early dying cells in the areas of cell death of the limb bud exhibit both nuclear and cytoplasmic alterations detectable by light (Fig. 3), transmission (Fig. 4) and scanning electron microscopy (Fig. 5; Hurle and Hinchliffe, 1978). In transmission electron microcopy, the nucleus shows the chromatin condensed and often segregated in a marginal region. The cytoplasm appears also condensed with moderate vacuolization of the organelles. A further precocious feature of the dying cells is the rounding of the cellular contour which is particularly evident under the scanning electron microscope (Hurle and Hinchliffe, 1978). These early changes are soon followed by cell and nuclear fragmentation giving rise to small apoptotic bodies which contain a small pycnotic nuclear spherule. A characteristic feature of the areas of cell death in the limb bud and observed also in other areas of cell death of the embryo, is that the cells are very rapidly engulfed by phagocytes (Garcia-Martinez *et al.*, 1993)

It has been proposed that apoptosis involves a primary perturbation in which activation or de novo synthesis of endonuclease(s) results in cleavage of DNA at linker region between nucleosomes to form fragments of double-stranded DNA (Wyllie, 1980; Compton, 1992). The characteristic nuclear condensation of the apoptotic cell would be the morphological parallel to this biochemical event. However, the intensification of studies focussed on ascertaining the role of endonuclease participation on apoptosis showed that nuclear condensation could be explained by other alterations preceding internucleosomal DNA fragmentation (see, Oberhammer *et al.*, 1994). Cohen *et al.* (1992) reported the occurrence of cell death by apoptosis in thymocytes in the absence of endonuclease participation. Also, in contrast with the hypothesis of internucleosomal DNA fragmentation, patterns of DNA cleavage in 30 or 50 Kbp fragments without or preceding internucleosomal fragmentation, have been reported during apoptosis in several cell types (Oberhammer *et al.*, 1993; Cohen *et al.*, 1994). In the areas of interdigital cell death, internucleosomal DNA fragmentation is only detected concomitantly with the appearance of cells with clear apoptotic features, but non-specific DNA fragmentation is also present at the same time (Fig. 6; Garcia-Martinez *et al.*, 1993; Zakeri *et al.*, 1993).

Activation of tissue transglutaminase has been also reported to be a precocious feature of dying cells (Fesus *et al.*, 1987; Piacentini *et al.*, 1991). According to these studies, transglutaminase activation would not be a primary factor in the death mechanism but it would play a key role in preventing cellular disintegration prior to engulfment of the dying cells. In the embryonic chick limb, an increase in tissue transglutaminase activity has been detected in mesodermal cell death induced by retinoic acid administration (Jiang and Kochhar, 1992), but there are no reports of its occurrence in the areas of programmed cell death.

Activation and liberation of the lysosomal enzymes was perhaps the first hypothesis proposed to explain the mechanism of self-destruction in programmed cell death (Weber, 1969). However, the introduction of the concept of apoptosis assumed that lysosomes do not participate in programmed cell death and the presence of secondary lysosomes in the areas of cell death was explained as a secondary phenomenon due to the phagocytic uptake of the apoptotic fragments. In the areas of cell death of the limb bud, secondary lysosomes are often observed in the mesenchymal cells prior to and during the establishment of the areas of cell death (Hurle and Hinchliffe, 1978). The interpretation of this observation in relation to the death process is difficult. One interpretation may be that irreversible commitment of cells to death is preceded by an initial autophagic process. Alternatively, the cells containing acid-phosphatase positive vacuoles may be hemopoietic macrophages destined to remove the dying cells (Cuadros *et al.*, 1992; Hopkinson-Wooley *et al.*, 1994; Rotello *et al.*, 1994).

In the absence of a coherent explanation of the mechanisms involved in the dying process in the areas of programmed cell death of the developing limb, there is now significant information obtained in this model which should be taken into account in future studies. Fernandez *et al.* (1994) have reported that the dying cells of the limb bud express a specific antigen which is also present in cells induced to die by necrosis. This study rises the possibility that necrosis and apoptosis share some common molecular processes. DNA, RNA and protein synthesis have been reported to decrease in the prospective dying cells of the limb (Pollak and Fallon, 1974; 1976; Toné *et al.*, 1988). It has also been found that prior to death the prospective dying cells enter an S-period of the cell cycle which could be critical for the commitment of cells to death (Toné *et al.*, 1988). A further characteristic of the areas of cell death of the developing limb is the expression of clusterin gene (TRPM-2; Buttyan *et al.*, 1989) which codes for a glycoprotein possibly involved in membrane remodelling

which is characteristic of some (not all) cell death processes but it is also involved in other biological events [see Rosenberg *et al.* (1993) and Wong *et al.* (1994)].

A final topic to be taken into account for the understanding of the dying machinery in the areas of cell death of the developing limb is the participation of macrophages. Recent studies, using a variety of specific labeling techniques, revealed that removal of the dying cells involve the participation of migrating macrophages (Cuadros *et al.*, 1992; Hopkinson-Wooley *et al.*, 1994; Rotello *et al.*, 1994). This participation of macrophages is a very precocious phenomenon since no temporal gap between the appearance of the first dying cells and the identification of macrophages can be established (Garcia-Martinez *et al.*, 1993). The importance of this feature comes from the observation in *C. elegans* (Ellis *et al.*, 1991) of a requirement for the presence of macrophages for the establishment of some programmed cell death processes.

"Patterning of the areas of cell death" The knowledge of how the genetic death program is triggered in each particular cell type constitutes a further key question for understanding the biology of cell death. Some death-triggering mechanisms may be shared by all cell populations. For example, cell death may be a common defensive phenomenon in response to modifications of the genome (secondary to virus infection or to oncogenic transformation) which are of potential danger for the survival of the whole living organism. However, a first glance at the variety of processes in which cell death is involved indicates that there must be triggering mechanisms specific for the cells in relation to their differentiation or with the character of their local environment.

The possibility of a circulating signal in the control of cell death in the limb bud, as occurs in hormone mediated cell death during larval metamorphosis or in the regression of components of the reproductory system can be discarded. One key property of the developing limb bud from the very early stages of its morphogenesis is its autonomy for self-differentiation when isolated from the embryonic body [see, Hinchliffe and Johnson (1980)]. Limb buds grafted to different regions of host embryos of different stages or to the chorio-allantoic membrane undergo normal development. Furthermore, a relatively normal development of the limb bud takes place in appropriate organ culture conditions. These facts limit the possibility of cell death control in the developing limb to local factors.

The introduction of molecular biology techniques to the study of embryonic development in the last few years has produced a considerable advance in the understanding of the molecular basis of the morphogenesis of the limb bud. It has been found that a precise spatial and temporal pattern of expression of homeobox-containing genes precedes the appearance of the skeletal primordia of the limb [see review by Izpisúa-Belmonte and Duboule (1992)]. Furthermore, evidence of a causal relationship between such pattern of homeobox gene expression and the morphogenesis of the different skeletal elements of the limb has been obtained in experiments inducing disruption or overexpression of such genes (Morgan *et al.*, 1992; Dollé *et al.*, 1993; Small and Potter, 1993). We have attempted to analyze whether the pattern of interdigital cell death may be also related with the pattern of homeobox gene expression (Ros *et al.*, 1994). Our observations revealed that experimental inhibition of interdigital cell death resulting in the formation of extra digits is not accompanied by modifications in the pattern of expression of the 5'-located Hoxd genes which are supposed to be involved in the patterning of the digital rays. The same study failed to record any precocious modification in the pattern of *msx*-1 and *msx*-2 gene expression, in spite of the fact that these two genes during normal development exhibit a domain of

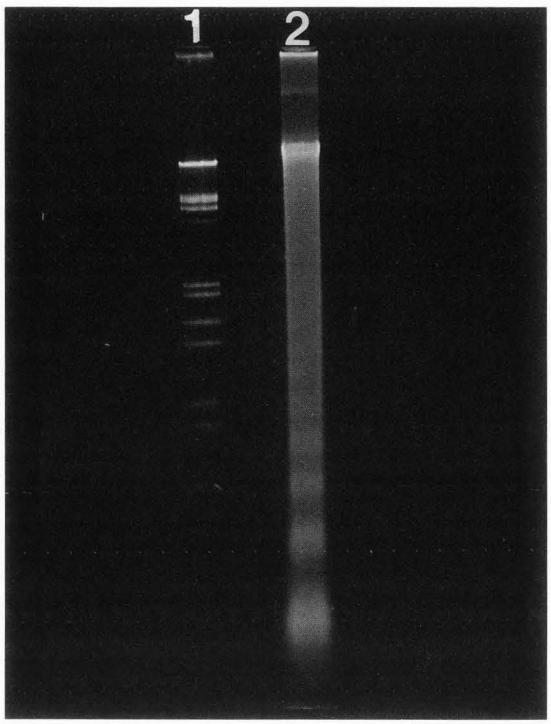

Figure 6. Agarose gel showing DNA fragmentation in extracts from the interdigital mesenchyme of day 8 of incubation (Lane 2; this is the same stage as that shown in Figures 3 and 5). Lane 1 is a digest of λ-phage DNA with EcoRI and HindIII. DNA fragments of 200 bp multiples are identifiable at this stage but a remarkable smear of degraded DNA is also detected but absent in stages lacking cell death.

expression relatively coincident with the zones of interdigital cell death. These observations point to other still unidentified genes in the patterning of the areas of interdigital cell death. In this regard, it should be mentioned that the interdigital spaces at the time of establishment of interdigital cell death exhibit a characteristic pattern of expression of genes related to retinoic acid including, RAR-β gene (retinoic acid receptor β) CRBP-I gene (cellular retinol binding protein) and CRABP II gene (cellular retinoic acid binding protein; Ruberte *et al.*, 1992). BMP-2A gene (bone morphogenetic protein 2A), another gene presumably involved in embryonic signalling processes, also exhibits a characteristic domain of expression in the interdigital spaces (Lyons *et al.*, 1990). The possible significance of this pattern of gene expression in the interdigits remains to be clarified.

One of the best known mechanism of death control in developing systems involves the local interaction between cells with the participation of growth factors. This is the case with cell death in the developing nervous system. During the development of most elements of the nervous system, the organ rudiments contain an initial excessive number of cells. When the neuronal connections become established only the neural cells which contact the appropriate target tissue survive. It has been clearly demonstrated that contact with the target provide the developing neurons with growth factors required for survival (see review by Oppenheim, 1991). In the early developing limb, growth factors also appear involved in the control of cell survival. As mentioned previously, the ectoderm of the distal margin of the limb bud termed the AER causes proliferation in the underlying mesenchyme. The function of the AER appears closely related with the production of a fibroblast growth factor (Fallon *et al.*, 1994; Niswander *et al.*, 1994). Removal of the AER in early stages of limb development is followed by intense apoptosis in the subjacent mesenchyme which is inhibited by local administration of fibroblast growth factor (Rowe *et al.*, 1982; Fallon *et al.*, 1994). Addition of fibroblast growth factor to organ cultures of prospective PNZ and OP regions of the chick limb bud, also rescues the cells from the death program (MacCabe *et al.*, 1991). In an early study, we failed to inhibit cell death in the interdigital spaces by local microinjection of several growth factors, including FGF, TGFβ1, TGFβ2 and EGF (Gañan *et al.*, 1993). However, during more recent experiments in our laboratory, implanting in the interdigits affigel blue beads soaked in FGF-2 or FGF-4 resulted in a delay of the onset of cell death followed by the appearance of syndactyly.

In some processes of programmed cell death, it has been found that the death program can be triggered by local tissue interactions involving modifications of the extracellular matrix (Lefebvre *et al.*, 1992; Meredith *et al.*, 1993; Frisch and Francis, 1994). There are several lines of evidence suggesting a role for extracellular matrix in the establishment of the areas of cell death in the developing limb. The segregation of the developing autopodium into alternated digital/interdigital regions is accompanied by the establishment of comparable digital/interdigital patterns of extracellular matrix involving a wide range of matrix molecules (Hurle *et al.*, 1994). Furthermore, experimental manipulations of the interdigit leading to inhibition of interdigital cell death and formation of extra digits (Hinchliffe and Horder, 1993; Gañan *et al.*, 1994) are accompanied by precocious changes in the pattern of distribution of several matrix molecules including fibronectin, tenascin and elastin (Hurle *et al.*, 1995, and unpublished observations). It has also been found that, in the embryonic mouse, the interdigital tissue exhibits temporally and spatially restricted domains of expression of genes encoding enzymes related with extracellular matrix remodeling (Carroll *et al.*, 1994).

The possible involvement of the extracellular matrix in the establishment of the areas of cell death in the developing limb is also supported by studies carried out in other fields of study of cell death. The cells of the OP, for example, undergo cell death when they are explanted under organ culture conditions, but not when they are disaggregated and cultured in monolayer conditions (MacCabe *et al.*, 1991). Cell death in the PNZ is inhibited by local ectoderm removal, which has an important effect on the organization of the underlaying extracellular matrix (Brewton and MacCabe, 1988).

Conclusions

In amniote, the development of form and structure of the limb bud is accompanied by precise patterns of massive mesodermal cell death. Most evidence suggests that cell death accounts for the removal of undifferentiated mesenchymal cells with skeletogenic potential which are no longer necessary once the limb anlage has attained some critical volumetric parameters and levels of tissue differentiation. Variations in the limb morphology and skeletal pattern among different vertebrate species are accompanied by parallel modifications in the pattern of cell death during embryogenesis of the limb. Abnormal increase in cell death during limb development results in the deletion or rudimentation of the limb skeleton while inhibition of cell death causes increased chondrogenesis eventually leading to the formation of extra skeletal pieces.

The morphology of the dying cells in all the studied species corresponds with that of apoptosis. Internucleosomal DNA fragmentation is only detected concomitantly with the appearance of cells with clear apoptotic features but non-specific DNA fragmentation

is also present at the same time.

Most evidence points to the occurrence of local triggering mechanisms accounting for the establishment of the areas of cell death which are followed by the activation of cell death genes. Modifications of the extracellular matrix secondary to the segregation of the limb mesoderm into skeletal and non-skeletal forming regions and diminution in the contribution of growth factors by neighbouring tissues appears as the most likely potential candidates for triggering the cell death program.

Information on the genetic basis of cell death in the developing limb is only at very preliminary stages. Among the increasing number of cell death genes identified in other cell death systems, such as p-53 and the *ced-3*/ICE and *ced-9*/*bcl-2* gene families, only *bcl-2* has been studied in detail during limb development and yet the information obtained is contradictory. *bcl-2* is not expressed in the areas of cell death of the developing limb, but normal limbs develop in mice with disruption of the *bcl-2* gene. Obviously, the clarification of the role of the cell death genes constitute a major task in future studies of cell death in the developing limb.

Acknowledgments

This work has been supported by a grant from the DIGICYT (PB 90-0340).

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Discussion with Reviewers

U. Heinzmann: Did you find differences in the *bcl-2* expression between fore- and hindlimbs during morphogenesis in chicken?

Authors: The expression of *bcl-2* during limb development have only been studied in the mouse. From the results obtained in the mouse it could be inferred that in the chick *bcl-2* would be expressed in the digital chondrogenic areas while its expression would be undetectable in the areas of cell death.

U. Heinzmann: With your biological system, you should be able to examine the occurrence of macrophages according to the pattern of cell death. Who triggers whom?

Authors: As far as we know, the only experimental approach to analyze the role of macrophages in the establishment of cell death in the developing limb has been done by Kieny and Sengel (1974). These authors inhibited the phagocytic process in the areas of interdigital cell death of the chick by the administration of cytochalasin B. After this treatment, phagocytosis is fully inhibited but cell death proceeds on schedule.

U. Heinzmann: What do you speculate is the role of RA and FGF in the programmed cell death of your biological system?

Authors: The effect of RA depends on the doses at which it is administered. At high doses, RA is an inductor of cell death. In this way, it is interesting to note that the addition of RA to the culture medium inhibits the syndactyly which often accompanies the development of the autopodium in organ culture conditions (Lussier *et al.*, 1993). Current experiments in our laboratory show that FGF may play a key role in the establishment of the areas of interdigital cell death. Implantation in the interdigital spaces of affigel blue beads soaked in a solution of FGF-2 or FGF-4 delays the onset of cell death and results in the formation of soft-tissue syndactyly. The importance of this observation comes from the fact that FGF appears to be responsible for the proximodistal outgrowth of the limb bud (Fallon *et al.*, 1994; Niswander *et al.*, 1994). According to these observations, cell death in the limb may be due to the cessation of the stimulus which maintain the outgrowth of the limb bud.

Reviewer III: Do any of the SEM images confirm, expand, or contradict the hypothesis that extracellular matrix is an important factor in determining the survival or death of the cells? This question comes to mind since not all cells in the interdigital region die.

Authors: It is difficult to analyze changes in the extracellular matrix by SEM. Using immunohistochemical procedures and confocal microscopy, we have observed very significant differences in the pattern of distribution of several extracellular matrix proteins between the digital rays and the interdigital regions (Hurle *et al.*, 1994).

Reviewer III: Can one find, by SEM or confocal images, macrophages in the vicinity of dying cells? Do these images suggest that macrophages differentiate *in situ*, that they migrate to the area, or that they kill the cells?

Authors: Both the number of macrophages and the amount of phagocytosed material present in each macrophage correlates precisely with the pattern of cell death in the limb. The occurrence of migration of macrophages to the areas of cell death has been demonstrated by several labeling procedures (Cuadros *et al.*, 1992; Hopkinson-Wooley *et al.*, 1994; Rotello *et al.*, 1994), however, many morphological evidences suggest that local mesenchymal cells are also able to remove by phagocytosis the dying cells as happens in other areas of cell death of the developing embryo (Hurle *et al.*, 1978). We do not think that macrophages kill the cells in the areas of cell death of the developing limb. As mentioned above, inhibition of phagocytosis by administration of cytochalasin B does not modify the dying program.

Reviewer III: Are you suggesting that ultimately all the embryonic cell death in vertebrates will be found to be induced by a lack of growth factors or an inappropriate matrix, as opposed to the situation in *Caenorhabditis elegans* where, according to Yuan and Horvitz (1990), the death of the cells is controlled by the cell itself and nor by its neighbors?

Authors: Most evidence, in the developing limb, indicates that the establishment of the areas of cell death does not differ from other embryonic processes. If this is true, one can expect that the expression of the death genes is controlled by signals produced in the course of the development including changes in the extracellular matrix, changes in cell adhesion etc., which obviously would be ultimately codified by one or several genes. The existence of genes in *C. elegans* which control cell death only in some specific cell populations (Ellis and Horvitz, 1991) points to this interpretation.

Reviewer III: To what extent does induced apoptosis resemble embryonic apoptosis? Is the morphology the same? How do the induction pathways relate to each other? Do the cell death mutants and experimental treatments suggest a greater vulnerability of cells in specific regions?

Authors: The morphology of apoptotic cells is very similar in all locations regardless of the mechanism triggering cell death. In the embryo, there are many critical regions and events which appear more susceptible to the action of teratogens and in fact very often these events involve the participation of cell death. A classic review by Menkes *et al.* (1970) provides a thorough survey of the involvement of cell death in teratogenesis.

Reviewer III: In liver, free apoptotic cells persist only a very short time before being phagocytosed. Do the arguments relating macrophages to cell death account for these dynamics?

Authors: In the limb bud the engulfment of dying cells by macrophages also appears to be very rapid, however, at the stages of maximum intensity of cell death there is a significant accumulation of isolated dying cells. Our interpretation is that macrophages at this stage are not able to remove all dying cells. It is at this stage when cells with morphological features of necrosis are detected in the interdigital space. This observation parallels the finding of Kieny and Sengel (1974, see above) using cytochalasin B to inhibit phagocytosis.

Reviewer III: If one can induce cell death experimentally, how important are cell death genes?

Authors: Cell death genes appear to constitute a cellular machinery able to self-destroy the cells when triggered by a variety of signals. This genetic machinery does not discard other mechanisms of killing cells, but in the later case, the morphology of the dying cells appears to correspond with "necrosis".

Reviewer III: What is the structure of the DNA or chromatin as the cells begins to undergo apoptosis? Is it attached to the nuclear membrane? Is the first step now considered to be large-size cleavage, alteration of the structure, or fragmentation by calcium-activated endonuclease? Does this mark the beginning of the irreversible stage?

Authors: At present, we have no response for your questions. Our research projects for the near future are focussed on these issues.

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