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Programmed cell death in the developing limb

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ABSTRACT The sculpturing of shape in the developing limb together with the regression of the tail in anuran tadpoles constitute, perhaps, the most paradigmatic processes of programmed cell death. The study of these model systems has been of fundamental importance to support the idea that cell death is a physiological behavior of cells in multicellular organisms. Furthermore, different experimental approaches, including comparative analyses of the pattern of cell death in different avian species (i.e. chick interdigits *versus* duck interdigital webs) and in chick mutants with different limb phenotypes, provided the first evidence for the occurrence of a genetic program underlying the control of cell death. Two well known research groups in the field of limb development, the USA group headed first by John Saunders and next by John Fallon and the group of Donald Ede and Richard Hinchliffe in the U.K. provided a remarkable contribution to this topic. In spite of the historical importance of the developing limb in establishing the concept of programmed cell death, this model system of tissue regression has been largely neglected in recent studies devoted to the analysis of the molecular control of self-induced cell death (apoptosis). However, a considerable amount of information concerning this topic has been obtained in the last few years. Here we will review current information on the control of limb programmed cell death in an attempt to stimulate further molecular studies of this process of tissue regression.

KEY WORDS: *apoptosis, tissue regression, BMP, FGF, retinoic acid*

Patterns of Apoptosis in the Developing Limb

In the developing limb, major areas of programmed cell death occur in the undifferentiated mesoderm in association with the establishment of the prechondrogenic condensations of the skeleton and in the ectoderm of the AER. In addition cell death is also observed during the formation of the joints (Mitrovic, 1977; Mori *et al.,* 1995), in the establishment of the axon pathways (Tosney *et al.,* 1988) and during the remodeling of the vascular pattern (Hurle *et al.,* 1985; Feinberg, 1991). It must be mentioned that the areas of cell death in the limb have been formerly termed necrotic areas but they occur by apoptosis (Garcia-Martinez *et al.,* 1993). This contradiction is explained because the classical studies on limb programmed cell death have been performed prior to the introduction in the literature of the term apoptosis.

The areas of mesodermal cell death are related with the establishment of the shape and skeletal pattern of the limb and exhibit significant differences between species. A remarkable feature is that mesodermal cell death is a characteristic feature of amniota. In amphibians, limb develops without programmed mesodermal cell death (Cameron and Fallon, 1977). The biological significance of this difference between amniota and anamniota embryos remains to be explained.

In the early avian limb there are two areas of cell death, the Anterior Necrotic Zone (ANZ; Fig. 1C), and the Posterior Necrotic Zone (PNZ; Fig.1B), which have been related with the reduced number of digits in birds (three digits in the wing and four in the leg). These areas are absent in polidactylous avian mutants (Hinchliffe and Ede, 1967). The absence of wings in the *wingless* chick mutant is correlated with a dramatic increase in ANZ (Hinchliffe and Ede, 1973). In mammals ANZ and PNZ similar to those of the avians are not present (Milaire and Rooze, 1983).

The formation of independent rudiments for the zeugopodial bones (tibia-fibula; ulna-radius) is accompanied both in mammals (Alles and Sulik, 1989) and in birds (Dawd and Hinchliffe, 1971) by an area of cell death in the central mesenchyme of the limb bud which has been termed the Opaque Patch (OP; Fig.1A). The absence of this area of cell death in the *talpid3* chick mutant correlates with fusion of the skeletal pieces of the zeugopod (Hinchliffe and Thorogood, 1974)

Abbreviations used in this paper: AER, apical ectodermal ridge; ANZ, anterior necrotic zone; BMP, bone morphogenetic protein; INZ, interdigital necrotic zone; OP, opaque patch; PCD, programmed cell death; PNZ, posterior necrotic zone.

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The formation of free digits in all amniota vertebrates is accompanied by massive apoptosis of the interdigital mesoderm. These areas of cell death have been termed the Interdigital Necrotic Zones (INZs; Fig. 1D,F), and serve the function of sculpturing the shape of the digits (see reviews by Saunders 1966 and Hurle *et al.,* 1996). In species with free digits, such as the chick, quail, lizard, mouse or human, apoptosis extends through all the interdigital space. In species with webbed digits, such as the duck or turtle, apoptosis is limited to the distal part of the interdigit. In species with autopods of singular morphology, such as the digits with lateral membranous lobulations present in the moorhen (*Gallinula chloropus*) and in the coot (*Fulika atra*), or the splited autopod (zygodactyly) present in Chamaleons, the pattern of INZ correlates closely with their specific phenotypes.

The correlation between INZ and the phenotype of the digits is also observed in syndactylous mutant species (Hinchliffe and Thorogood, 1974) and in experimental limbs treated with drugs which inhibit cell death (see review by Hurle *et al.,* 1996). Interestingly, the inhibition of INZ is often followed by the presence of digit fusions or even by the formation of an ectopic digit (Gañan *et al.,* 1996). This finding indicates that the interdigital mesoderm has the potential to form digits.

In addition to mesodermal cell death, apoptosis is also an important feature of the ectoderm of the AER (Fig. 1E). In the chick limb bud ectodermal apoptosis appears to exert the function of controlling the extension of that structure (Todt and Fallon, 1986). In mammalians, apoptosis in the AER is more prominent than in birds and forms well-defined foci of cell death (Milaire and Roze, 1983). The inhibition or delay of this ectodermal cell death causes an enlargement of the AER followed by an increase in the amount of subridge mesenchyme which results in the induction of polydatyly (Naruse and Kameyama, 1982, 1986). In serpentiform reptiles with rudimentary limbs the regression of the limb primordium is mediated by massive cell death in the AER (see Raynaud, 1990).

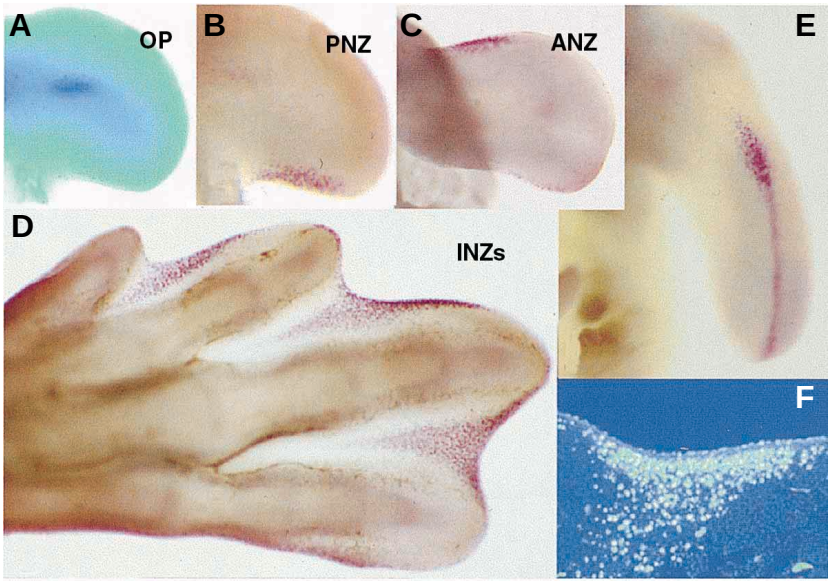

Fig. 1. The areas of cell death in the developing chick limb bud. (A) *Wing bud showing the OP vital stained with nile blue. (B-E) Neutral red vital staining showing the PNZ* **(B)**, *ANZ* **(C)** *INZs* **(D)** *and cell death in the AER* **(E). (F)** *Longitudinal section of the second interdigital space of a leg bud of the same stage to that showed in (D), illustrating the appearance of INZ after TUNEL labeling.*

BMPs are the Triggering Signal for Limb Programmed Cell Death

Evidence obtained mainly with the chick embryo indicates that programmed cell death in the limb bud shares many control mechanisms with those regulating proliferation and differentiation. BMPs have been identified as the triggering apoptotic signal for both the ectoderm of the AER (Gañan *et al.,* 1998) and the mesodermal cells (Gañan *et al.,* 1996; Zou and Niswander, 1996; Yokouchi *et al.,* 1996; Macias *et al.,* 1997). According to their pattern of expression BMP-2, BMP-4 and BMP-7 are the most likely physiological signals triggering apoptosis in the limb bud. However, these BMPs are also involved in the control of limb patterning (Pizette *et al.,* 2001) and in the regulation of chondrogenic differentiation (Macias *et al.,* 1997). In fact, local treatments with any of the above mentioned BMPs induce intense growth and differentiation in the prechondrogenic mesenchyme and massive apoptosis in the undifferentiated mesoderm (Macias *et al.,* 1997). The chondrogenic effect of BMPs appears to be mediated by the type Ib receptor (Yi *et al.,* 2000). However, the receptor implicated in the control of apoptosis remains to be identified. Inhibition of apoptosis have been obtained in over-expression experiments using dominant negative type IB and type IA BMP receptors (Zou and Niswander, 1996; Yokouchi *et al.,* 1996). However, since, these receptors are not expressed in the areas of programmed cell death at levels detectable by *in situ* hybridization the most likely explanation for these results is that the phenotype was secondary to depletion of BMPs which bind to the overexpressed receptors. Furthermore, interdigital induction of the type IB BMP receptor gene by application of TGFβ-1-beads is followed by inhibition of apoptosis and formation of an ectopic digit (Merino *et al.,* 1998).

The intracellular pathway activated by BMPs during apoptosis also remains unknown. However, there is evidence suggesting that the apoptotic effect of BMPs in the limb bud and in other developing model systems is mediated by the activation of cytoplasmic kinase TAK-1 rather than by the canonical intracellular pathway of BMPs involving phosphorylation of Smad proteins (Grotewold and Ruther, 2002).

In most embryonic models, BMP-signaling is additionally regulated by different types of BMP antagonists that play the function of modulating the intensity and/or the spatial distribution of the BMP signal. In the developing limb different BMP antagonists control the function of BMPs. One of these antagonists, Gremlin, is expressed in patterns that fit with a role in protecting the undifferentiated mesoderm from the apoptotic influence of BMPs (Merino *et al.,* 1999). It is remarkable, that Gremlin is expressed in the duck interdigital webs while in the chick interdigital expression of this BMP antagonist is down-regulated prior to the onset of INZ. Furthermore, interdigital implantation of beads bearing Gremlin in the chick leg bud inhibits INZ

and induces a membranous syndactyly similar to that found in the duck (Merino *et al.,* 1999). We have also observed that Bambi, a characteristic BMP antagonist acting at the level of the cell membrane, is expressed and regulated in the limb bud in the same fashion than cell death (unpublished observations)

Regulation of Programmed Cell Death by FGF-, TGFβs, and RA-Signaling

It is now clear, that the regulation of limb programmed cell death by BMPs is closely integrated with other signaling pathways implicated in the control of outgrowth and differentiation of the limb bud.

In the limb bud, FGF signaling is currently considered as responsible for outgrowth. However, experiments of gain-of- and loss-of-function have demonstrated that FGFs cooperate with BMPs in the control of mesodermal apoptosis (Montero *et al.,* 2001). When FGF-signaling is blocked by local application of FGF inhibitors, BMPs are not sufficient to trigger apoptosis. Furthermore, we have provided evidence suggesting that the reduced pattern of interdigital apoptosis observed in the interdigital webs of the duck might be due to a decrease in FGF-signaling rather than caused by absence of BMPs. The fashion by which BMPs and FGFs cooperates in the control of apoptosis awaits clarification, but the expression of several genes potentially involved in apoptosis requires the integrity of both FGF- and BMP-signaling (Montero *et al.,* 2001 and see below).

TGFβ2 has been implicated in the formation of digits. This factor is expressed in the developing digital both in birds and mice. Furthermore interdigital implantation in chick embryos of beads incubated either with TGFβ1, β2 or β3 proteins causes the formation of ectopic digits. However, Dünker *et al.* (2002) have recently observed that interdigital cell death is inhibited in *Tgfβ2(-/-)Tgfβ3(-/-)* double knockouts mice.

Retinoic acid signaling exerts major roles in limb patterning, including the control of apoptosis. In mouse, inhibition of interdigital cell death and subsequent syndactyly, has been reported in a variety of mutations of retinoic acid receptor genes (see Dupe *et al.,* 1999). Furthermore, the phenotype of the hammertoe mutant caused by defective apoptosis can be partially rescued by administration of retinoic acid to the pregnant females (Ahuja *et al.,* 1997). In the chick, we have observed that retinoic acid act in concert with BMPs to establish the interdigital regions (Rodriguez-Leon *et al.,* 1999). The function of RA-signaling consists of promoting the apoptotic effect of BMPs and at the same time inhibiting the chondrogenic effect of these factors. This may be of considerable importance for normal morphogenesis since in the developing autopod BMPs not only induce apoptosis but also promote a dramatic growth of the cartilage.

The Apoptosis Molecular Cascade

The molecular machinery responsible for apoptosis exhibits a high degree of conservation in the course of evolution. Four functional groups of genes have been identified in the regulation of apoptosis in the *C. elegans* (*Ced-3*, *Ced-4*, *Ced-9* and *Egl-1*). In vertebrates, these functional groups are conserved but each group includes many different genes. The homologous of Ced-3 in vertebrates is the large family of caspases, which are the direct effectors of the death program. The homologous of *Ced-4* is *Apaf-1* (*Apoptotic Protease-Activating Factor*) which is the prototype of a family of pro-apoptotic factors with the role of activating caspases. Ced-9 in *C.elegans* inhibits both Ced-3 and Ced-4. In vertebrates *Ced-9* is represented by the large *Bcl-2* gene family, which includes inhibitors of cell death and pro-apoptotic factors. Egl-1 in *C. elegans* promotes apoptosis by inhibiting Ced-9. The homologous of *Egl-1* in vertebrates include several members which repress the anti-apoptotic activity of *Bcl-2* (see review by Hurle and Merino, 2002).

During limb programmed cell death members of the different groups of apoptic regulators have been identified. As in other models of apoptosis, the final step of limb programmed cell death consists of the activation of caspases (Milligan *et al.,* 1995; Jacobson *et al.,* 1996; Mirkes *et al.,* 2001). Associated with the pathway of caspases are the pro-apoptotic factors *DIO-1* (*Death Inducer-Obliterator-1*; Garcia-Domingo *et al.,* 1999), *Gas1* and *Gas2* (*Growth Arrest Specific*; Lee *et al.,* 1999, Lee *et al.,* 2001). The involvement of *Apaf-1* in limb programmed cell death is supported by the occurrence of a reduced pattern of interdigital apoptosis and persistence of interdigital webs in mice mutant for this gene (Cecconi *et al.,* 1998). *Bax,* a proapototic member of the *Bcl-2* family, is expressed in the areas of cell death (Dupe *et al.,* 1999) and *Bax (-/-)Bak (-/-)* double knockout mice display persistence of interdigital webs (Lindsten *et al.,* 2000). In addition, several antiapoptotic members of this family, including *Bcl-2*, *Bcl-x* and *A1,* are expressed in the digital rays but not in the interdigital spaces of the mice autopod (Novack and Korsmeyer, 1994; Carrio *et al.,* 1996) while the interdigital regions prior to the onset of apoptosis express *Bag-1* which encodes an antiapoptotic protein which binds to Bcl-2 (Crocoll *et al.,* 2002). Another antiapoptotic factor, *Dad-1* (*Defender Against apoptotic cell Death*) has been implicated in the control of limb programmed cell death since heterozygous mutant mice for this gene display soft-tissue syndactyly (Nishii *et al.,* 1999).

Many additional genes are expressed and regulated in the limb bud in patterns that overlap with apoptosis. Some of these genes are associated with events accompanying the apoptotic process, including the arrest of cell proliferation (Tone *et al.,* 1988) or the lost of cell adhesion. In other cases their functional significance remains unknown.

The promyelocytic leukaemia zinc finger protein encoded by *Zfp145* gene appears to regulate cell death through a primary effect on the expression of BMPs (Barna *et al.,* 2000). Several genes implicated in the control of cell proliferation are involved in limb programmed cell death. *c-Fos* (Yano *et al.,* 1996) and *Cyclin-dependent kinase 5* (Zhang *et al.,* 1997) are expressed in the areas of interdigital cell death. *p53* has been implicated in the control of apoptosis in the mice limb buds exposed to apoptotic treatments (Moallem and Hales, 1998; Wang, 2001) and is expressed in the areas of cell death in mice (Dupe *et al.,* 1999). The gene for the FGF receptor 3 (*Fgfr3*), which mediates inhibition of cell proliferation is expressed in INZs and is regulated by treatments with FGFs and BMPs in the same fashion than apoptosis (Montero *et al.,* 2001). *Msx* genes (*Msx1* and *Msx2*), are homeobox-containing transcription factors, expressed in the undifferentiated mesoderm of the limb bud including the areas of programmed cell death (ANZ, PNZ and INZs, but not OP). In the duck limb, interdigital expression of *Msx* genes correlates with the reduced extension of INZ in the

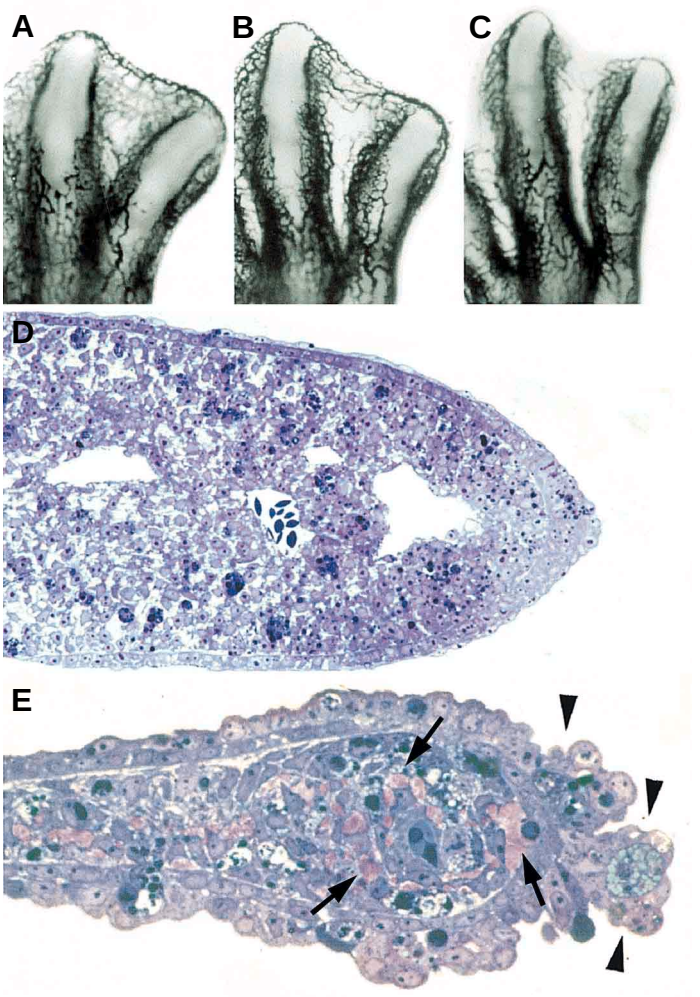

Fig. 2. Before the onset and during INZ. *(A-C) Limb buds microinjected with indian ink to show the progressive regression of blood vessels in the third interdigit of chick leg buds prior to the onset of INZ* **(A)**, *during INZ* **(B)** *and at the end of the degenerative process* **(C).** *(D,E) Longitudinal sections of the third interdigit during INZ* **(D)** *and at the end of the degenerative process* **(E)**. *Note in (E) that blood vessels are no longer present in the interdigit, the ectoderm is in the course of being detached into the amniotic sac (arrowheads) and the extracellular matrix appears in large clumps located between the degenerating mesodermal cells (arrows).*

interdigital webs. Similarly, *Msx* expression in ANZ and PNZ is inhibited in polydactylous mutants lacking these areas of cell death (Coelho *et al.,* 1993 and review by Chen and Zhao, 1998). The expression of *Msx* genes is positively regulated by FGFs, BMPs and RA, in parallel with the pattern of apoptosis induced by those factors. Furthermore over-expression of Msx-2 in the limb bud is followed by apoptosis (Ferrari *et al.,* 1998).

The implication of *Msx* genes in the control of limb programmed cell death appears to be related with the NF- kB family of transcription factors. It has been suggested that *c-rel*, a member of this family, is a positive regulator of apoptosis in the developing limb (Abbadie *et al.,* 1993). At the molecular level, NF-kß members appear to connect the FGF-signaling pathway with the expression of *Msx*-genes (Bushdid *et al.,* 2001).

Snail has been implicated in limb apoptosis by inhibiting cell adhesion (Montero *et al.,* 2001). *Snail* transcripts are detected in the limb mesoderm of ANZ, PNZ and INZ. In the duck, where interdigital cell death is reduced *Snail* expression is restricted to the areas of cell death. Moreover, induction of cell death is accompanied by the up-regulation of *Snail*.

Dickkopf-1 (Dkk-1) is a secreted protein that inhibits Wnt signaling. It is expressed in the limb bud in domains overlapping ANZ, PNZ and INZ. Furthermore it is regulated by FGFs and BMPs in the same fashion than apoptosis and its over-expression results in limb truncation (Grotewold and Rüther, 2002).

Other factors of potential importance in the control of apoptosis, expressed in the areas of limb programmed cell death include: the lysosomal membrane glycoprotein, LAMP-1 (Stewart *et al.,* 2000); tissue transglutaminase (Moallem and Hales, 1996; Dupe *et al.,* 1999); TNFa-like proteins (Wride *et al.,* 1994); insulin growth factor (van Kleffens *et al.,* 1998); polyamines (Gritli-Linde *et al.,* 2001); reactive oxygen species (Salas-Vidal *et al.,* 1998); the *Ft1* gene related to ubiquitin-conjugating enzymes (Lesche *et al.,* 1997) and *testosterone-repressed prostate message-2 gene*, (*TRPM-2*; Keino *et al.,* 1994).

Phagocytic Removal of Apoptotic Cells

A controversial question about the processes of programmed cell death concerns the origin of the phagocytic cells responsible for eliminating the apoptotic corps. In classical studies this function was assigned to the neighboring healthy mesenchymal cells. However, Cuadros *et al.* (1993) found evidence for the involvement of circulating macrophages in this process. More recently, Wood *et al.* (2000) showed that both 'professional phagocytes' and local mesenchymal cells are able to remove the apoptotic cells. According to their findings the circulating macrophages are the main responsible for the elimination of apoptotic cells, but the neighboring mesenchymal cells are also able to act as stand-in phagocytes. Thus, in macrophageless mutant embryos the regression of interdigital tissue is only slightly retarded, indicating that the compensatory phagocytosis by local mesenchymal cells is efficient in removing all apoptotic cells. Surprisingly, none of the engulfment genes, characteristic of professional macrophages are up-regulated in the non-professional mesenchymal phagocytes suggesting that these cells activate a distinct molecular cascade for recognition, engulfment and digestion of apoptotic cells.

Interdigital Tissue Regression

An important feature, often forgotten, concerning the areas of interdigital cell death is that these areas constitute zones of full tissue regression (Hurle *et al.,* 1986). The interdigits prior to the onset of cell death is covered by ectoderm and consist of a core of mesodermal cells rich in blood vessels and a complex extracellular matrix scaffold. In the avian embryos, the degenerative process occurs only in the mesodermal cells, but in subsequent stages the extracellular matrix is totally disintegrated (Fig.2E; Hurle and Fernandez-Teran, 1983), the blood vessels regress (Fig. 2 A-C; Hurle *et al.,* 1985) and the ectodermal tissue become detached into the amniotic fluid (Fig. 2 D-E: Hurle and Fernandez-Teran, 1983). The molecular basis underlying this complex process of tissue regression awaits clarification but most evidence point to a major

role of the extracellular matrix. According to this interpretation syndactyly has been found both in mice lacking the laminin α5 chain (Miner *et al.,* 1998) and in mice null for *fibrillin 2* gene (Arteaga-Solis *et al.,* 2001). It is also likely that matrix metalloproteinases in coordination with their tissue inhibitors exert an important role in remodeling the interdigital matrix. *Stromelysin-3* is expressed in the developing limb bud and in other processes involving apoptosis (Lefebvre *et al.,* 1992; Dupe *et al.,* 1999) and tissue inhibitor of metalloproteinases-3 (*TIM-3*) is specifically expressed in the interdigital regions prior to cell death (Zeng *et al.,* 1998).

Other factor of potential importance for the regression of the interdigital tissue may be the involvement of *Slit2* gene in preventing the incorporation of axons into the interdigital regions (Yuan *et al.,* 1999).

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