All-trans-retinoic acid-induced disturbance of forelimb digital apoptosis in mouse embryos: a preliminary scanning electron microscope (SEM) tudy

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SUMMARY

Retinoids have been widely discussed in recent years as being able to induce a spectrum of malformations that include limb defects and digit malformations, where perturbation of cell death regulation is an important teratogenic mechanism. The present study assesses the effect of all-trans-retinoic acid (tretinoin) on forelimb digit development in mouse embryos. Pregnant Balb/c mice received retinoic acid by gastric intubation as follows: Group 1 (n = 5) at 100 mg/kg on gestational days 11 and 12; Group 2 (n = 5) at 50 mg/kg on gestational days 10, 11 and 12. Control animals, Group 3 and 4 (n = 5/group) were treated on gestational days 10, 11 and 12 with corn oil vehicle (Group 3) or were non treated (Group 4) respectively. The mice were euthanized on gestational day 18.

Embryos exposed to retinoic acid (R.A.) develop micromelia and oligodactyly in combination with brachydactyly and syndactyly. Apoptosis was incomplete between the digits, and atypical lateral ectodermal ridges were found. The observations were most striking in the embryos of Group 2 treated for three days. No abnormalities were noted in the embryos of either control group.

Key words: Limb development – Retinoic acid – Teratogenesis

INTRODUCTION

During limb development, cells differentiate into the proper tissue such as cartilage, bone, muscle, connective tissue and epidermis. In addition, cells also adopt the correct position relative to other tissues or cells and to the three axes of the developing limb: anteroposterior, dorsoventral and proximodistal (Trelstad 1977; O'Rahilly and Müller, 1996; Frenz and Liu, 1997).

The cell interaction between mesenchymal cells of the paddle-shaped limb and ectoderm gives rise to the stratified epithelium that forms the apical ectodermal ridge (AER). This plays the key role of exerting an inductive influence on the underlying mesenchyme and is a non-specific simulator of limb outgrowth, promoting normal development and growth. The continuing influence of the mesenchyme is required for apical epidermal ridge integrity (Carlson, 1996). The mesoderm, in particular the progress zone, which is located beneath

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the AER, controls the proximo-distal axis (Zwilling, 1972; Saunders, 1977).

The zone of polarizing activity (ZPA) consisting of mesenchymal cells on the postaxial border of the limb bud partly controls pattern development along the anteroposterior axis.

Physiological "cell death", referred to as "apoptosis", has been long recognized as an important component of pattern formation and organogenesis in normal embryos (Glucksmann, 1951). Apoptosis plays an important role in the differentiation of organs and tissues. In limbs, apopotosis first appears in the region of the future axilla; later in the elbow region, and then between the digits (Carlson, 1996), as exemplified by the histogenesis and remodeling of cartilage and bone (Moore and Persaud, 1998).

Apoptosis is essential for normal development, as dramatically demonstrated in null mice deficient in critical cell death. Perturbation of the cell death regulation after exposure to teratogens induces abnormal development. The molecular mechanisms of teratogen-induced cell death, which is apoptotic in nature, is now partially elucidated and two major pathways have been demonstrated: the receptor-mediated and the mitochondrial apopototic pathways (Mirkes, 2002).

Different experimental protocols of retinoic acid administration to the mother have been developed in order to induce malformations and analyze the multiple mechanisms taking place during normal and abnormal development (Kochhar, 1985; Abbott et al., 1990; Emmanouil-Nikoloussi and Foroglou, 1992; Agnish and Kochhar, 1993; Emmanouil-Nikoloussi et al., 1993; Degitz et al., 1998; Emmanouil-Nikoloussi et al., 2000 a, b, c, d, e; Emmanouil-Nikoloussi et al., 2003).

Cell death is encountered during normal and teratogenic development, and its occurrence in embryos after retinoid administration to pregnant animals has aroused special attention (Schweichel and Merker, 1973; Kochhar, 1977; Sulik et al., 1988; Sulik and Alles, 1991; Livrea and Packer, 1993; Von Schroeder et al., 1994; Sulik et al., 1995; Frenz et al., 1996; Grant et al., 1997; Emmanouil-Nikoloussi et al., 1998; Moore and Persaud, 1998; Emmanouil-Nikoloussi et al., 2000 b).

The present study addresses the effect of retinoic acid, a powerful morphogen and teratogen, in limbs and in particular in digit development.

Materials and Methods

Female Balb/c mice, 8-10 weeks of age and weighing 20-25 g were housed in a controlled temperature environment (18-22^{o}C) with a 12 hour light/dark light cycle. Food and water were provided *ad libitum*. One male was caged with two females for a three-hour mating period. The presence of a vaginal plug signified day zero of gestation. Retinoic acid (R.A.), tretinoin, all-trans-retinoic acid, stored under low light conditions in brown glass vials was suspended in corn oil at two different doses - 100 mg/kg and 50 mg/kg body weight. Pregnant mice were divided into four groups (n = 5/group). The first three groups received retinoic acid suspended in corn oil, or corn oil alone via gastric intubation. The fourth group (n = 5) served as non-treated controls. The animal groups and treatment schedules were as follows:

Group 1: 100 mg/kg of body weight (b.w.) on gestational days 11 and 12;

Group 2: 50 mg/kg/b.w. on gestational days 10, 11 and 12;

Group 3: received corn oil vehicle only, on gestational days 10, 11 and 12;

Group 4: non-treated.

All pregnant mice were euthanized by a lethal dose of CO_2 early in the morning of gestational day 18.

The guide for the care and use of laboratory animals of the National Academy Press (1996) was taken into account. Embryos were excised, weighed, and examined under a stereomicroscope and photographed.

For scanning electron microscopy (SEM) examination, embryos were washed with saline and fixed in 10% neutral formalin. The forelimbs of the embryos were microdissected under a stereomicroscope. They were postfixed in neutral formalin for a further three days, dehydrated in a gradual series of alcohol at room temperature, and finally dried by the critical point method (Critical Point Dryer, Balzers CPD 020). The embryonic limbs were attached to stubs with a drop of Dotie (XC-1, 2 Jeol/SVC), coated with a layer of gold 10 to 20 nm thick, and observed under a Jeol ISM-820 Scanning Microscope at 10 to 15 kV with a 10-6 A beam current.

Results

The forelimbs of untreated mice embryos and of the animals treated with corn oil

appeared normal under stereomicroscopic and scanning electron microscopy (SEM) examination. The five digits were well developed and the four digital rays were present, such that each digit was already independent from the adjacent one at 18 days of gestation (Fig. 1). At the distal end, nail development had begun.

The embryos treated with all-trans-retinoic acid developed micromelia and oligodactyly in combination with brachydactyly (Figs. 2 and 3) and proximal or complete syndactyly (Figs. 2, 3 and 4) at 18 days of gestation.

The digits formed in embryos from dams that received 50 mg/kg/bw (Group 2) showed a pronounced brachydactyly (Figs. 2 and 3). Their distal free ends were independent from those of the adjacent digits and were short in length. Interdigital areas were partly formed. At the surface of the digits, and particularly between the digits (Fig. 3), some flattened epithelial cells were observed, which seemed to have partially lost their adhesiveness but preserved some junctions at the ends of their cell processes.

The embryos from the dams that received 100 mg/kg/bw (Group 1) developed moderately shortened digits and also severe oligodactyly, with complete syndactyly at the distal extremity (Fig. 4). Interdigital tissues were not formed, although a slight depression in the form of a groove was seen between the digits, representing a failure of regression or involution of the tissue between the digits. The nails were clearly distinguished from the surrounding tissue. Some flattened epithelial cells remained at the surface between the distal digit extremities. Those epithelial cells were arranged to form a microscopic webbing, referred to as "zygodactyly" when it is more developed and macroscopically visible (Carlson, 1996).

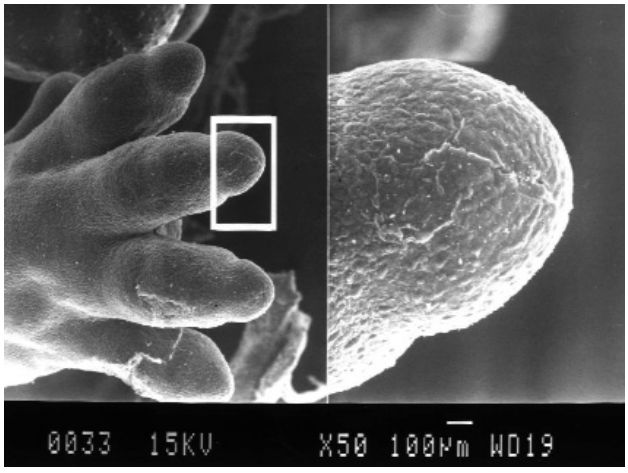

Fig. 1. Forelimb of untreated mouse embryo aged 18 days and observed by Scanning Electron Microscopy (SEM). Note the five well developed digits and the four digital rays separating the normally developed five digits at 18 days of gestation. At the distal end, nail development has begun. x 50 (left) and x 300 (right).

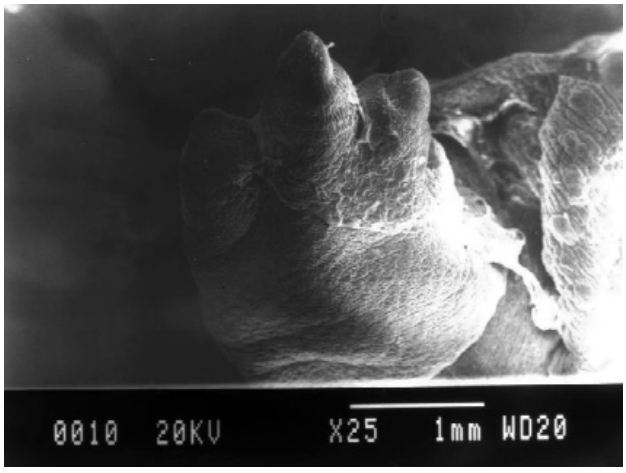

Fig. 2. Forelimb from a mouse embryo 18 days old from dams that received 50 mg/kg/bw (Group 2). Micromelia and oligodactyly in combination with brachydactyly. Note the pronounced brachydactyly and syndactyly with short adjacent digits. x 25.

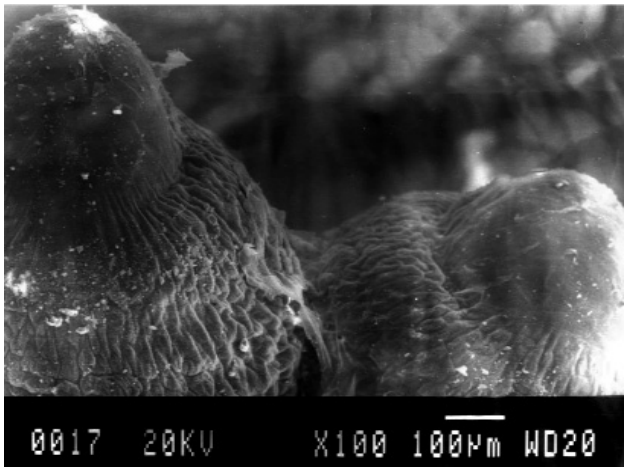

Fig. 3. Forelimb from a mouse embryo 18 days old from dams that received 50 mg/kg/bw (Group 2). Note some flattened epithelial cells between the digits. x 100.

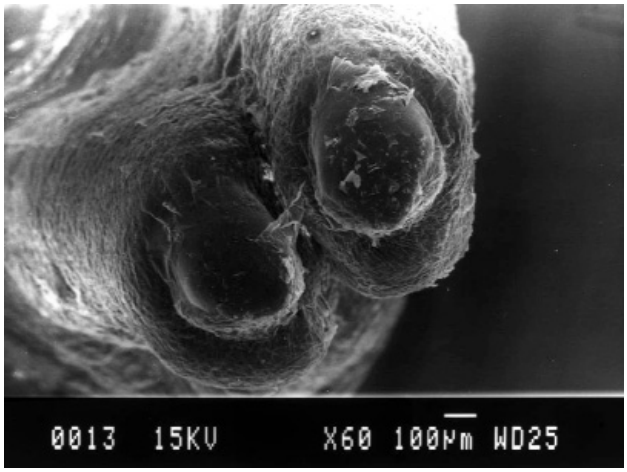

Fig. 4. Forelimb from a mouse embryo 18 days old from dams that received 100 mg/kg/bw (Group 1). Note the moderately shortened digits and severe oligodactyly and complete syndactyly up to the distal extremity. x 60.

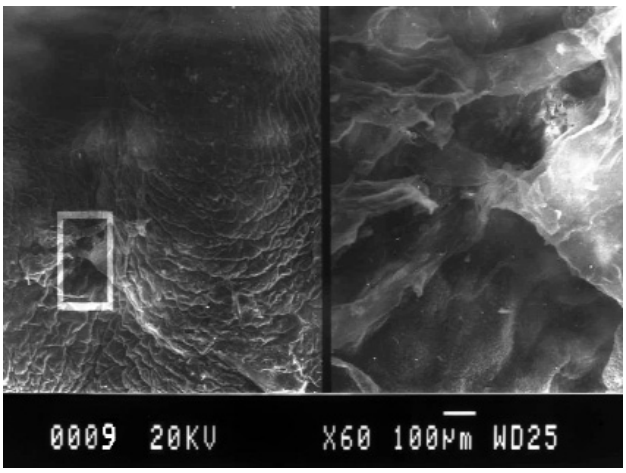

Fig. 5. Upper forelimb surface from a mouse embryo 18 days old from dams that received 100 mg/kg/bw (Group 1). Interdigital area. Note the flattened cells that remain attached to both adjacent digits by desmosome-like structures and that appear as bridges joining the cells between the digits. x 60 (left) and x 360 (right).

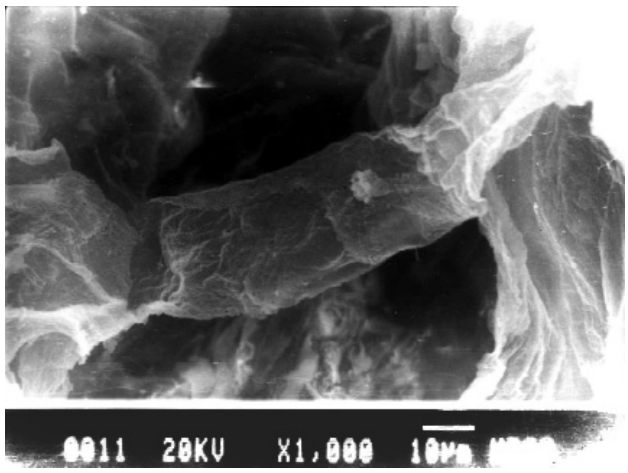

Fig. 6. Upper forelimb surface from a mouse embryo 18 days old from dams that received 100 mg/kg/bw (Group 1). Interdigital area. Note some of the flattered cells which show bubblings having the same morphological aspect as apoptotic bodies considered to be the first step of apoptosis. x 1000.

Between the digits, flattened cells remained attached to both adjacent digits by desmosome-like structures (thick arrow) and appeared as bridges joining them (Fig. 5). Moreover, some of the cells showed bubbling (fine arrows), having the same morphological appearance as apoptotic bodies considered to be the first step of apoptosis (Fig. 6). The dose of 100 mg/kg of body weight (b.w.) was administered for only two days, since additional exposure resulted in death of the dam. Days 11 and 12 were chosen since they correspond to the critical period of limb development (Moore and Persaud, 1998).

Discussion

Retinoic acid is one of the most important morphogens; deprivation or an excess results in a marked teratogenic effect (Lammer et al., 1985; Lammer et al., 1987; Lammer, 1988; Schulman et al., 1988; Adams, 1993; Agnish and Kochhar, 1993; Koren, 1993; Dolk et al., 1999; Nau, 2001; Tzimas and Nau, 2001).

Retinoic acid administered to rats during gestation induces severe alterations to craniofacial development in embryos, as shown in SEM studies (Emmanouil-Nikoloussi et al., 1998) and in extensive histological studies (Emmanouil-Nikoloussi et al., 2000 a, b, c, d, e; Emmanouil-Nikoloussi et al., 2003; Gunston et al., 2005).

Retinoid-induced teratogenicity has been shown to be associated with gene expression, and has provided a clearer understanding of the molecular basis of limb morphogenesis (Duboule, 1991; Means and Gudas, 1995; Lockshin et al., 1998).

The present study establishes that exposure of mouse embryos to all-trans-retinoic acid at different times of gestation affects limb development through interference with the action of R.A. on the developing limb. The changes seen were syndactyly and a persistence of interdigital webbing, brachydactyly and oligodactyly.

Over the past twenty years, the role of R.A. in the developing limb has been partly elucidated. R.A. controls most of the steps involved in limb development. Hox genes, whose expression of which is managed by R.A., regulate the position of the limb along the cranio-caudal axis in the flank regions of the embryo. When the cranio-caudal limb position has been determined, and once limb outgrowth has been initiated by FGF-8, the AER (apical ectodermal ridge) is formed and acts as a specific stimulator of outgrowth. AER activity has an important inductive influence on the adjacent mesenchyme, particularly on the progress zone, which remains an undifferentiated population of rapidly proliferating cells. FGF-2 and FGF-4 can substitute for AER in promoting outgrowth (Niswander et al., 1993) and maintain the progress zone active.

R.A. activates Meis 1 and 2 gene expression, an important determinant for promoting the proximalization of developing limbs, whereas FGF is now considered to be the main important factor responsible for AER activity and a promoter of distalization through inhi-

bition of R.A. production and signaling (Mercader et al., 2000).

The combined patterns of Hox A and Hox D expression are responsible for the differences observed in fore- and hindlimb structures. During the initial steps of development, Hox genes are expressed in the mesenchyme underlying the AER (Dolle et al., 1989) and their expression is controlled by R.A. It has been suggested that flank tissue with polarizing activity is very efficient at converting retinol to R.A. before limb buds appear and long before sonic hedgehog genes (Shh) are expressed (Helms et al., 1996). The zone of polarizing activity (ZPA) expresses sonic hedgehog genes (Shh), a secreted factor that plays a dominant role in patterning along the anteroposterior axis (Lu et al., 1999). Retinoic acid could first induce ZPA formation and then initiate Shh expression, which could then activate the BMP-2 genes. BMPs, members of the transforming growth factor β-like family, are important morphogens, and their gradient of distribution plays a role in the specification of cell position in the limb bud and is believed to assign a distinct identity to the cells and to control their further specification (Yang et al., 1997). The genes activated by R.A. or by ZPA graft in a host limb bud are Hoxd-11,-12,-13, FGF-4 and BMP-2, (Lu et al., 1999), establishing the fundamental R.A. control of activity in limb development. More recently, the expression of Twist gene (cTwist) has been studied during limb patterning in chicks. Sonic hedgehog (Shh) genes are able to maintain cTwist expression in the presence of AER secreting FGFs. The twist gene has also been reported to be regulated by AER, FGFs, R.A. (retinoic acid) and sonic hedgehog (Shh) genes (Tavares et al., 2001).

Both normal and teratogenic levels of retinoids' effects are mediated by retinoid receptors, retinoid-X-receptors (RXRs) and retinoic acid receptors (RARs) (Means and Gudas, 1995). All-trans-retinoic acid is a potent transcriptional activator of both RARs and RXRs (Winter and Tickle, 1993). Normal gene expression requires adequate RARs and RXRs receptors transducing the retinoid signal while altered gene expression with various secondary morphological defects are observed in RAR and RXR mutants (Lu et al., 1999). RAR and RXR occupancy is considered to be a regulator of homeobox gene expression (Means and Gudas, 1995), and binding sites for RARs and RXRs have been found in many Hox-genes (Hoxa-1, Hoxb-1, Hoxd-4 and Hoxd-11) controlling early embryonic development (Lu et al., 1999). In later limb bud development, RA regulates chondroblastic differentiation and controls appositional and longitudinal growth (Underhill et al., 2001).

Retinoid-binding proteins are expressed in the early embryonic period; notably cellular retinoic acid-binding proteins (CRABP I and CRABP II). These have been reported to vary as a gradient across the anterior/posterior axis and have been proposed as possible amplifiers of the retinoic acid signal (Maden et al., 1988; Perez-Castro et al., 1989; Miyagawa-Tomita et al., 1992).

Retinoic acid also affects programmed cell death, which is a very important process for normal limb pattern differentiation (Moore and Persaud, 1998). The mesenchyme, which is located adjacent to the apical ectodermal ridges, consists of undifferentiated rapidly proliferating cells, which die due to cellular apoptosis. The mesenchymal tissue present in the embryonic plate condenses to form the digital rays of the hand and the foot. The intervals between the digital rays consist of loose mesenchyme, which is quickly broken down, while the intermediate regions of mesenchyme form notches between the developing digital rays.

The link between retinoids and normal or abnormal limb development has been clearly established *in vitro* (Zakeri, 1993) and *in vivo* (Zakeri and Ahuja, 1994). The latter experiments were performed in mutant Hammertoe (Hm) mice; in this mutant the pattern of cell death is specifically altered in the interdigital region of the limb. The use of retinoic acid induces apoptosis between the digits and provides a specific correction to the mutation. In cultured limbs of Swiss-Webster mice, retinoic acid induces the process of digit separation and cell apoptosis in the interdigital area at E12 (E: embryonic day 12), although this process normally begins at E 12.5 *in vivo*, and cultured E12 limbs without retinoic acid do not show any induction of the separation process (Stewart et al., 2000).

GHox-8 (Coelho et al., 1992) and GHox-7 (Coelho et al., 1993) are expressed during normal development in the mesoderm of the posterior necrotic zone at the mid –proximal posterior edge of the limb bud, and in the mesoderm of the proximal anterior non-chon-

drogenic periphery of the chick limb bud, including the anterior necrotic zone.

Using retinoid acid-coated bead implants, Coelho et al. (1993) demonstrated that retinoids diminish GHox-7 expression and programmed cell death near the implants. The effects of bead retinoic implants (Tickle et al., 1985; Coelho et al., 1993) and of exogenous retinoic administration are quite different; the first reduces programmed cell death and the latter enhances apoptosis, with severe limb reduction defects (Kochhar, 1973; Zakeri, 1993).

The process of apoptosis responsible for tissue breakdown in the interdigital regions is mainly mediated by signaling molecules referred to as bone morphogenetic proteins (BMPs). These factors interfering with normal cellular events could account for the resulting syndactyly and webbing or fusion of the fingers or toes. The disappearance of the digit interzone during the early stages of limb development during GD 10-14 in mice, verified by Alizarin dye staining on GD 15, supports the concept of apoptosis as a necessary adjunct for normal morphogenesis (Zou and Niswander, 1996; Moore and Persaud, 1998).

Rosen and Chernoff (2002), examining the pathogenesis of the antineoplastic drug 5-Aza-2'-Deoxycytidine (dAZA) as a causative factor of limb anomalies, reported that the limb anomalies produced by the drug were consistent with a general insult to the limb mesenchyme during proximal-distal outgrowth of the limb. They also reported that the expression of scleraxis, a marker of early chondrogenesis, was reduced 12 hr after dAZA exposure, a time coincident with maximal cell death in long bone anlages, and acute cell death and reduced cell proliferation caused by the cytotoxic drug were also observed in the subridge limb bud mesenchyme.

When Affi-Gel beads, loaded with recombinant BMP-4 protein, were transplanted into the interdigital tissues of a day 12.5 mouse hindlimb, interdigital space cell apoptosis was precociously induced. In vitro, BMP-4 induces precocious interdigital tissue cell death when a flanking digit is left attached to the interdigital tissue. In contrast, when no digit is left with the interdigital tissue, cartilage is produced instead of cell death. The effects of BMP-4 protein effects on interdigital tissues could be dependent on the modulating influence of the digits (Tang et al., 2000).

When excessive doses of retinoic acid are given to a pregnant animal, as observed in our experiments, variations in the amount of apoptotic cells in the interdigital space result in the formation of digits markedly different from those expected in a morphologically normal hand and foot (Lockshin et al., 1998). Indeed, all-trans-retinoic acid may cause massive cell death with oligodactyly and brachydactyly (Figs. 2 and 4) and inappropriate cell death with syndactyly (Fig. 4). The drug affected distal limb development in a time- and dose-dependent manner, ranging from non detectable to markedly malformed digits.

Before the period of digit individualization, FGF-8 expression was coincident with the AER, but when cell death was first detected in the interdigits, FGF-8 expression became restricted to the tip of the growing digits; FGF-8 could be one of the factors responsible for differential digit-interdigit growth, and might also act as a survival factor on interdigital tissue. Moreover, FGF-8 did not overlap with the expression of RAR-beta, BMP-2, BMP-4, BMP-7, MSX-1 and MSX-2, which is generally considered to be involved in the activation of interdigital cell death (Salas-Vidal et al., 2001).

Our study suggests that exposure of mouse embryos to all-trans-retinoic acid at different times of gestation affects different mechanisms of outgrowth promotion, homeotic gene expression, balance between proximalization and distalization, and programmed cell death. Failure in apoptosis could result in syndactyly and a persistence of interdigital webbing, while massive apoptosis would lead to brachydactyly and oligodactyly.

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