The role of morphogenetic cell death during abnormal limb-bud outgrowth in mice heterozygous for the dominant mutation *Hemimelia-extra toe (Hm*)

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SUMMARY

A dominant mutation in the mouse, *Hemimelia-extra toe (Hm*)*, induces congenital limb malformations in heterozygotes. Typical expression includes axial shortening of the radius, tibia and talus ('hemimelia'), with supernumerary metacarpals, metatarsals, and digits ('polydactyly'). Pathogenesis was investigated during developmental stages 16 through 22 (11th through 15th days of gestation). Full expression was apparent during stage 20 when the limb pattern was comprised of pre-cartilaginous anlagen. Formation of a pre-axial protrusion on the autopod during stage 17 or 18 was the earliest gross abnormality, and foreshadowed the development of supernumerary digits. Microscopically, there was an alteration in the pattern of physiologic cellular degeneration (PCD) programmed to occur within the zeugopod and autopod. The 'opaque patch' (mesodermal necrotic zone normally occurring between tibial and fibular anlagen) was overextended pre-axially causing resorption of the tibial pre-cartilage. Additionally, PCD normally occurring within the basal cell layer of the apical ectodermal ridge (AER) and the 'foyer primaire préaxial' was not expressed in the mutant autopod. This occurred in association with outgrowth of the protrusion. The pre-axial portion of the AER remained in an abnormally thickened, viable, proliferative state, and did not undergo scheduled degeneration. This may have been the basis for prolonged induction of pre-axial outgrowth. Paucity of mesenchymal cell filopodial processes extended along the basal lamina, as well as a rarefaction of the filamentous material normally associated with the mesodermal face of the basal lamina, was detected at the pre-axial AER-mesenchymal interface on stage 18. A potential involvement of epithelial-mesenchymal interactions in the induction of epithelial PCD is discussed.

INTRODUCTION

Normal skeletal pattern in the limb can be disrupted by genomic mutations, but the precise mechanisms for pathogenesis are not understood (Hinchliffe & Ede, 1967; Johnson, 1969; Cairns, 1977; Rooze, 1977). Abnormal limb pattern may also be induced in genetically normal embryos by the administration of various anti-proliferative agents during a ‘critical phase’ of morphogenesis

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The pattern of abnormalities induced by such agents is stage-dependent, and susceptibility is lost once the pre-cartilage pattern emerges. Hence, the 'critical phase' encompasses the sequence of events from initial limb-bud outgrowth to pre-cartilage condensation. In genetic mutants which exhibit malformations of limb pattern, cellular abnormalities have invariably been found during this same period of development. Pertinent examples include the polydactylous talpid² (Cairns, 1977) and talpid³ (Hinchliffe & Ede, 1967) chick mutants, as well as Dominant hemimelia (Rooze, 1977) in the mouse. A genetic alteration in the pattern of cell death normally programmed to occur during the 'critical phase' of morphogenesis has been found in the limb buds of these mutants.

Another dominant mutation in the mouse, Hemimelia-extra toe (Hm²), induces specific pattern abnormalities localized to the limbs of heterozygotes. In some respects, these limb malformations are similar to those found in the talpids, Dominant Hemimelia, teratogenesis induced by anti-proliferative agents, and some human conditions (Freund, 1936). This report describes the malformations exhibited by Hm²-heterozygotes and some morphological features associated with pathogenesis during the 'critical phase' of pattern formation.

MATERIALS AND METHODS

Animals

Hemimelia-extra toe arose in the B10.D2-New/SnJ mouse strain in 1968 at the Jackson Laboratory (Bar Harbor, Maine). The colony maintained for our studies was started from seven mice received from Dr K. S. Brown, N.I.D.R., N.I.H. (Bethesda, Maryland). In our laboratory the colony was not strictly inbred, and did not produce skeletal malformations other than those associated with Hemimelia-extra toe. All animals were maintained on Purina Laboratory Chow ad libitum and kept in the light from 6 a.m. to 6 p.m. A group of seven females were caged with one male from 9 a.m. to 1 p.m. or from 6 p.m. to 9 a.m., and the presence of a vaginal plug immediately afterwards was regarded as the sign of successful mating, with this day designated as the first day of gestation. One of the parents was always heterozygous for Hemimelia-extra toe. Some pregnant mothers were killed by cervical dislocation on the eleventh to fifteenth days of gestation; others were allowed to deliver to obtain neonates. Further staging of gestational age during the embryonic period was adapted from the system described by Theiler (1972) which is based upon observations of developing hybrid mouse embryos at one-half-day intervals (Table 1). Embryonic stages 16 to 20 (11th–13th gestational days) were assessed by determining the number of somite pairs and other external morphological features. Fetal stage 22 (15th day) was assessed by crown–rump length and degree of interdigitation.
Limb development in Hm x/+ mice

Table 1. Stage designation of midgestation mouse embryos

<table>
<thead>
<tr>
<th>Day of gestation</th>
<th>Stage*</th>
<th>Somite no.†</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-0</td>
<td>14</td>
<td>13-20</td>
</tr>
<tr>
<td>10-5</td>
<td>15</td>
<td>21-29</td>
</tr>
<tr>
<td>11-0</td>
<td>16</td>
<td>30-34</td>
</tr>
<tr>
<td>11-5</td>
<td>17</td>
<td>35-39</td>
</tr>
<tr>
<td>12-0</td>
<td>18</td>
<td>40-44</td>
</tr>
<tr>
<td>12-5</td>
<td>19</td>
<td>45-49</td>
</tr>
<tr>
<td>13-0</td>
<td>20</td>
<td>50-</td>
</tr>
</tbody>
</table>

* Theiler (1972) designated these stages in hybrid mice where they corresponded to days 9-12 of gestation (plug day = day 0).
† The most caudal somite related to the post-axial border of the hindlimb bud was taken as somite 28.

Nomenclature

The original designation for Hemimelia-extra toe was Hx (see recent report by Kalter, 1980). However, because there is evidence for allelism on chromosome V between Hemimelia-extra toe and Hammer-toe (based upon the failure of these traits to cross over in over 700 matings), new symbols have been proposed by Dr K. S. Brown (personal communication) and will be adhered to for this study: Hammer-toe (Hm x) and Hemimelia-extra toe (Hm x). Thus, the normal phenotype bears a +/+ genotype, and the mutant phenotype bears a Hm x/+ genotype.

Whole-mount preparations of intact skeletons

Whole normal and mutant neonates were fixed overnight in 95% ethanol and processed for staining of their osseous skeleton by the rapid Alizarin red S dye method (Kochhar, 1973). Whole stage-22 foetuses were fixed overnight in 95% ethanol and processed for staining of cartilage/bone by the combined alcian blue/Alizarin red S technique of Inouye (1976) with the modification that, after staining, foetuses were not macerated with potassium hydroxide but instead were dehydrated in ethanol and cleared with methyl salicylate.

Light and transmission electron microscopy

Embryo or foetuses were dissected free of their extraembryonic membranes in ice-cold Tyrode’s saline and staged. Limbs from conceptuses of stages 16 to 22 were excised, fixed in cacodylated-buffered Karnovsky’s fixative (Karnovsky, 1965), postfixed in 1% osmium tetroxide in cacodylate buffer, dehydrated in ethanol, cleared in propylene oxide, then embedded in Araldite 502 (Electron Microscopy Sciences). One micron (μm) or serial 3 μm sections were cut in the anteroposterior plane, stained at 65 °C for four minutes with basic Azure II (Matheson, Coleman, and Bell), 0.25% in 0.5% sodium borate, and examined with a Nikon Biophot microscope. For electron microscopy, thin sections (silver)
Table 2. Monohybrid inheritance of the Hm* allele*

<table>
<thead>
<tr>
<th>Parental genotype</th>
<th>No. of successful pregnancies</th>
<th>No. of offspring</th>
<th>Average litter size†</th>
<th>Mutant (%)</th>
<th>Normal (%)</th>
<th>Implantation sites‡ resorbed (%)</th>
<th>P§</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ × +/+</td>
<td>26</td>
<td>183</td>
<td>7.0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>Hm*+/+ × +/+</td>
<td>107</td>
<td>661</td>
<td>6.2</td>
<td>47</td>
<td>53</td>
<td>9.7</td>
<td>0.02-0.05</td>
</tr>
<tr>
<td>Hm*+/+ × Hm*/+</td>
<td>43</td>
<td>265</td>
<td>6.2</td>
<td>35</td>
<td>65</td>
<td>5.3</td>
<td>0.70-0.50</td>
</tr>
</tbody>
</table>

* Eleventh gestational day to first day post-partum.
† Resorbed implantation sites excluded.
‡ During eleventh to fifteenth gestational day.
§ P, the probability that deviation from the expected ratio occurred by chance alone, was determined by chi-square analysis with a level of significance of P < 0.05. The expected ratios were based on simple autosomal dominant, homozygous lethal inheritance of the Hm* allele.

were cut on an LKB-Huxley ultramicrotome and examined with an RCA-3G electron microscope.

**Autoradiography**

Stage-19 embryos, freed of Reichert’s membrane and parietal yolk sac, were grown in whole embryo culture by techniques described elsewhere (Kochhar, 1975). After a five-hour incubation in the presence of [methyl-3H]thymidine (2 μCi/ml, New England Nuclear), embryos were freed of the visceral yolk sac and amnion, then rinsed several times in ice-cold Tyrode’s saline containing 100 μg/ml unlabelled thymidine. Limb buds were excised, fixed for microscopy as described above, and serially sectioned at 3 μm. Unstained sections were coated in the dark with a 50% aqueous dilution of nuclear track emulsion NTB-2 (Kodak) at 40 °C, air dried, refrigerated under desiccant for four weeks in light-tight boxes, developed for four minutes in D-19 photographic developer (Kodak), fixed with Kodak Rapid Fixer, washed, and then stained lightly for two minutes with Azure II.

**Assessment of cellular degeneration**

Dead or dying cells appeared under light microscopy as extravascular, heterogeneous, and densely azurophilic granules. Their ultrastructural features correlated well with descriptions of cellular degeneration provided by Kerr, Wyllie & Currie (1972); Schweichel & Merker (1973); and Kochhar et al. (1978). Zones of cellular degeneration were further confirmed, at the gross level, by incubating living embryos (dissected free of their extraembryonic membranes) at 37 °C for 30 min in Ringer’s saline solution containing the supravital dye Nile Blue A (1:200000, Allied Chemical), followed by a rinse with Ringer’s saline (without Nile Blue A) at 4 °C. Similar methods have been used successfully by Saunders, Gasseling & Saunders (1962), and Scott et al. (1977, 1980) for assess-
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...ment of normal and abnormal patterns of cell death during embryonic limb development.

RESULTS

Inheritance of the Hm\(^x\) allele

Monohybrid ratios of normal and mutant phenotypes, average litter size, and the percentage of implantation sites found resorbed are summarized in Table 2. In 107 matings between a normal and a mutant parent, no litters were composed entirely of mutants. In matings where either one or both parents were mutants, resorptions were more frequent and the average litter size was 14% less than in those matings where both parents were normal. The F1 monohybrid ratios obtained from a total of 176 matings were compared for goodness of fit (Chi-square test) to those expected if Hemimelia-extra toe was indeed an autosomal dominant, homozygous embryolethal mutation. \(P\), the probability that observed ratios deviated from the expected by chance alone, did not exist below the level of significance \((P \leq 0.05)\) in any of the three mating combinations, but was very low for normal-mutant matings \((0.05 < P < 0.02)\).

Gross morphology of the neonatal osseous skeleton

Alizarin red S-stained preparations of neonatal osseous skeletons, along with inspection of adults, demonstrated a range of gene penetration that was generally, but not strictly, related to parentage (the more penetrant in the parent, the more penetrant in the offspring). Hindlimbs in any individual were always more severely affected than the forelimbs, and commonly one side was more affected than the other. The heterozygote syndrome of hindlimb malformations is presented in Fig. 1. Forelimbs were similarly malformed, but to a lesser degree. The most severe deformities, taken as complete gene penetration, included bilateral absence of the tibia and distal half of the radius, with polydactyly in all four paws. The fibula and ulna were normal in size but bowed or stumpy secondary to reduction of the companion element. Polydactyly included six to seven metatarsals or metacarpals with seven to eight digits per paw. Supernumerary elements were always located pre-axially, and were of normal morphology (though ossification of the middle phalanx was commonly delayed). The humerus, femur or limb girdles were never affected, and the only other skeletal malformation observed was an occasional shortened tail.

A continuum of mutant phenotypes occurred, presumably due to varying degrees of incomplete gene penetration. For convenience of description, three levels of severity were noted: intermediate, mild/intermediate, and mild (Fig. 1). Intermediate penetration included absence of the distal half of the tibia, absence or reduction of the talus, with pre-axial polydactyly in all four paws. Mild/intermediate penetration included absence or reduction of the talus with pre-axial polydactyly in all paws. Mild penetration included absence of part or all of the talus, metacarpals and metatarsals I, II, III, and the proximal phalanx of...
Fig. 1. Neonatal hindlimbs stained with Alizarin red S then cleared to demonstrate ossified elements. Whole mounts, 19.5 ×. (A) +/+ phenotype. Pelvic girdle, pg; femur, F; tibia, T; fibula, f; talus, t; calcaneus, c; metatarsals, m; phalanges, p.
(B–D) Hm²/+ phenotype. Arrow denotes 'hemimelia'. (B) Complete penetration; (C) incomplete (intermediate) penetration; (D) incomplete (mild) penetration.
Fig. 2. Hindlimbs from +/- (A, C) and Hm+/+ (B, D) conceptuses. Whole mounts, 36 x. (A, B) Combined alcian blue/Alizarin red S staining on stage 22 of gestation. Distal shortening of the tibial cartilage (arrow) with supernumerary digits occurred in the mutant. (C, D) Stage 20 of gestation. A definitive protrusion (p) was present pre-axially on the autopod of mutant hindlimb buds.
Fig. 3. Morphology of hindlimb buds from stage-18 (40-somite) +/+ (A, C) and Hm²/+ (B, D) embryos. (A, B) Histological section of the distal, pre-axial quarter. Cellular degeneration (arrows) deep to the marginal vein (mv) was more extensive in normal mesenchyme, but mitotic figures (M) were more abundant in mutant mesenchyme. The most pre-axial segment of apical ectodermal ridge (aer) was thicker and less necrotic in the mutant. 3 μm sections, Azure II stain, 520×. (C, D) Osmicated hindlimb buds. The distal, pre-axial contour was altered in the mutant. White circles mark the necrotic zone of (A) and the mitotic zone of (B) in +/+ and Hm²/+ limbs, respectively. Whole mounts, 77×.
pre-axial digits. In mild penetration, pre-axial polydactyly occurred in the hindpaws only, but pre-axial ectrodactyly occurred in the forepaws. Several neonates that exhibited mild penetration died shortly after birth. When autopsied, they had no milk in their stomach, an air-expanded gastrointestinal tract, and a cleft of the hard palate.

Staining of whole stage-22 foetuses by the combined alcian blue/Alizarin red S method demonstrated the full syndrome of malformations (Fig. 2). All affected cartilage rudiments, when examined histologically, demonstrated normal organization (not shown).

**Morphology of stage-17 and -18 limb buds**

Litters were examined during the embryonic period characterized by early outgrowth of the undifferentiated limb bud (stage 16) to stage 22. The earliest gross phenotypic abnormalities occurred in limbs during stage 17 or 18. A precise age that mutant siblings were first distinguished varied between litters, but generally occurred around the 38-somite age if the carrier parent was severely affected. By the 44-somite age, all litters examined yielded the expected proportion of mutant phenotypes. One distinguishing feature was an excess of tissue located preaxially on the autopod giving mutant limb buds an altered contour (Fig. 3). In no litter was this change found before the 38-somite age, and no embryo had abnormally shaped forelimbs unless it also had affected hindlimbs. The microscopic features associated with altered contour were investigated in serial sections of hindlimb buds from 40-somite embryos. Abnormalities were found in the apical ectodermal ridge (AER) and underlying subridge mesenchyme, and were localized to the pre-axial portion of the autopod.

In normal hindlimbs, the AER was a stratified epithelium generally comprising four nuclear layers: an outer periderm of squamous cells, an innermost basal layer of columnar cells oriented perpendicular to the basement membrane, and two intermediate layers. Within the AER capping the pre-axial half of the normal limb bud, a band of cells appeared to be undergoing degeneration (Fig. 3). Ultrastructurally, many of the basal cells contained large, osmiophilic inclusions indicative of autophagic vacuoles within their cytoplasm; however, these cells did not exhibit signs of nuclear disorganization, cytoplasmic condensation, organelle disruption, or cell fragmentation (Fig. 4). The pre-axial mesenchyme underlying this segment of the AER was composed of cytologically homogeneous, undifferentiated cells which extended filopodial processes along the basal lamina. Some filamentous extracellular material was present in association with the mesodermal face of the basal lamina (Fig. 4). Additionally, a zone of physiologic cell death occurred within the mesenchyme deep to the marginal vein (Fig. 3).

Several abnormalities were found at the pre-axial ectodermal–mesenchymal region of comparably aged mutant hindlimbs. The AER was thicker than normal by one nuclear layer, and there was no sign of cellular degeneration in
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the basal layer. The pre-axial necrotic zone deep to the marginal vein was absent or greatly reduced, and an abundance of mitotic figures occurred at the comparable location (Fig. 3). Ultrastructurally, the sublaminer mesenchymal cells did not extend filopodial processes along the basal lamina, and there was less filamentous extracellular material associated with the mesodermal face of the basal lamina than in normal limbs (Fig. 4).

Hindlimb buds from several embryos were examined, at the 36-somite age, prior to overt manifestation of the mutant phenotype. No cellular degeneration was found within the pre-axial AER or mesenchyme in any embryo, either by light microscopy or by supravital staining with Nile Blue A.

**Morphology of stage-19 and -20 limb buds**

Hindlimb buds were examined on stages 19 and 20 to identify the early features associated with tibial agenesis and the emergence of supernumerary digits. Mesenchymal cell condensations for the stylopod (femur) and zeugopod (tibia, fibula) occurred during stages 18 and 19. A central block of cells within the zeugopodal condensation of all limb buds had become necrotic by stage 19. Resorption of these cells resulted in separation of tibial (preaxial) and fibular (postaxial) precartilage rudiments by stage 20. Concurrent with overt expression of this cell death in the mutant, a large percentage of presumptive tibial chondroblasts as well as cells within the adjoining myogenic region became necrotic (Fig. 5). On stage 20, the distal portion of the pre-cartilaginous tibia was absent in the mutant.

Continued limb outgrowth led to the formation of a definitive protrusion on the mutant autopod (Fig. 2). Within the AER capping this protrusion, the basal layer in comparison to normal was thicker, devoid of cellular degeneration, and exhibited a higher incorporation of \( [3\text{H}] \)thymidine (assessed by autoradiography following \textit{in vitro} exposure). The distribution of \( [3\text{H}] \)thymidine incorporated into the mesenchyme of this protrusion did not differ from normal (Fig. 6), and no cellular degeneration occurred here either. Our regional analysis of cellular degeneration is summarized in Fig. 7 for normal and mutant hindlimb buds.

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**Fig. 4.** Electron micrographs of the pre-axial ectodermal–mesenchymal interface from stage 18 (40-somite) +/+ (A) and \( \text{Hm}^x/+ \) (B) hindlimb buds. 7200 × . (A) Basal cells (c) of the AER exhibited osmiophilic vacuoles indicative of autophagic vacuoles (av). Mesenchymal processes (mp) extended along the basal lamina (b), the mesodermal (m) face of which was coated with fuzzy, filamentous extracellular material. (B) Basal cells did not exhibit any overt signs of autophagy. Mesenchymal processes did not extend along the basal lamina, and filamentous extracellular material was rarefied.
Fig. 5. Longitudinal sections (3 μm) of the pre-cartilaginous tibia (within the arrows) from stage-19 (46-somite) +/+ (A) and Hm+/+ (B) embryos. Azure II stain, 660×. (A) Prechondrogenic (pc) zones were distinguished from adjacent premyogenic (pm) regions by their less rounded nuclei and orientation perpendicular to the long axis of the rudiment. The ‘opaque patch’ (op) was a zone of cellular degeneration normally occurring between tibial and fibular rudiments. Several mitotic figures (asterisk) were present at the distal limit of the rudiment. Central artery, CA. (B) Cellular degeneration of the ‘opaque patch’ extended throughout the tibia, as well as the adjacent premyogenic region.
Fig. 6. Hindlimb buds from stage-19 $+/+$ (A, C) and $Hm^x+/+$ (B, D) embryos grown in embryo culture for 5 h in the presence of $[^3]H$thymidine (2 $\mu$Ci/ml); 3 $\mu$m sections, Azure-II stain. (A, B) Autoradiograms through the AER capping the pre-axial protrusion in the mutant (B), and a comparable segment of the normal (A). The normal AER was thinned compared to its appearance on stage 18, and exhibited cellular degeneration with few cells incorporating $[^3]H$thymidine into their nuclei. The mutant AER was thickened, especially the basal cell layer (BCL) which contained many cells incorporating $[^3]H$thymidine. Sub-ridge mesenchyme was more closely associated with the basement membrane in normal limbs (arrows). 2400 $\times$. (C, D) Autoradiograms through the pre-axial sub-ridge mesenchyme exhibited similar radiogranule distribution in mutant (D) and normal (C) limbs, but cellular degeneration (cd) was found in normal limbs only. 960 $\times$. 
Fig. 7. Composite diagrams representing observations of serially sectioned hindlimb buds from +/+ (A, B, C) and Hm²/+ (D, E, F) embryos. Cell condensations are represented as transverse lines; mesodermal degeneration as dots; AER degeneration is marked by squares or brackets. (A, D) Stage-18 (40-somite) of gestation. The most pre-axial segment of the AER in the mutant was thickened and free of degeneration (broad arrowhead). Marginal vein, mv; central artery, ca. (B, E) Stage 19 of gestation. (C, F) Stage 20 of gestation. Femur, F; tibia, T; fibula, f.

DISCUSSION

An autosomal dominant mutation in the mouse, Hemimelia-extra toe (Hm²), induces congenital limb malformations in heterozygotes. The homozygous condition, not investigated for this report, appears to be embryoletal during the eighth to ninth days of gestation (Dr K. S. Brown, personal communication). A 1:1 ratio of normal:mutant offspring was expected from matings between a normal and a mutant parent, but the actual ratio (1:0.82) deviated in favour of normal. Based upon statistical analysis, this deviation was not
significant and could be ascribed to random factors. However, these same
matings produced an abnormally high number of embryonic resorptions (inde­
dependent of whether it was the mother or the father which carried the mutant
allele), so the decrement in heterozygote number could also be accounted for
by an increased sporadic lethality.

All limb malformations were localized to pre-axial elements of the zeugopod
and autopod. Variation in gene penetration occurred perhaps because the mouse
strain was not inbred. There was always axial shortening of proximal skeletal
elements associated with the presence of distal supernumerary elements. Typical
expression included tibial and radial 'hemimelia', supernumerary metatarsals,
and polydactyly. In the mildest form the zeugopod was unaffected, but there
was an absence of some pre-axial metatarsals, with polydactyly or ectrodactyly.
Apparently, the regions along the longitudinal axis were affected depending
upon the penetrance of the mutant gene, with more proximal elements affected
in association with greater penetration. Since, during early limb outgrowth, the
longitudinal axis is specified in proximodistal fashion, increased penetration
implied that overt expression of the mutation occurred at an earlier develop­
mental age.

The earliest characteristics identified with the mutant phenotype appeared on
the eleventh to twelfth days of gestation. The precise age varied between
different litters, but generally occurred nearer the 38-somite age (stage 17) when
the gene was more penetrant in the heterozygous parent. The full syndrome of
malformations was apparent by the middle of the 13th gestational day (stage 20)
when the pre-cartilaginous limb pattern was complete. Because histodifferentia­
tion of affected elements proceeded normally, overt effects of the mutation may
have been confined to embryonic stages 17–20. As the shapes, sizes and scheduled
occurrence of mesenchymal condensations were initially unaltered, it is not
likely that the actual mechanisms underlying skeletal pattern formation were
disturbed. Rather, abnormal pattern was probably the result of disturbances in
other morphogenetic forces that normally shape the limb bud. One prominent
disturbance concerned the pattern of physiologic cellular degeneration (PCD)
programmed to occur at certain times in three locations within the zeugopod and
autopod: (1) the ‘opaque patch’ (a mesodermal necrotic zone occurring during
stages 18 and 19 between tibial and fibular pre-cartilage anlagen (Fell & Canti,
1934; Dawd & Hinchliffe, 1971)); (2) the ‘foyer primaire préaxial’ (fpp) (a
mesenchymal necrotic zone occurring during stages 17 to 20 within the autopod,
deep to the most pre-axial segment of the AER (Milaire, 1976)); and (3) cellular
degeneration within the most pre-axial segment of the AER itself (Scott et al.
1977, 1980).

In mutant limbs, expression of PCD in the opaque patch was accompanied by
necrosis within the tibial pre-cartilage. These two necrotic regions were con­
tinuous, so spatial overextension of the opaque patch is a possible cause for
resorption of the tibia. There are other instances of abnormal skeletogenesis in
which genetic or teratogenic alterations in the extent of normal PCD zones directly affect the size of pre-cartilage rudiments. Zwilling (1942, 1959) postulated an overextension in two systems: in the opaque patch as a cause of insulin-induced micromelia in chick embryos (Zwilling, 1959); and in the tail as a basis of dominant \textit{Rumplessness} in chicks (Zwilling, 1942). Furthermore, an absence of cell death in the opaque patch has been associated with fusion of tibial and fibular rudiments in the \textit{talpid$^a$} chick mutant (Hinchcliffe & Ede, 1967).

Preceding expression of PCD in the opaque patch, distinct abnormalities localized to the pre-axial epithelium and mesenchyme were evident in the autopod of mutant limb buds. The pre-axial AER remained in a thickened state and did not undergo scheduled degression during stages 18 to 20. This was associated with two abnormalities in its basal cell layer: (1) persistence in a rapidly proliferative state at least through stage 19 (assayed by autoradiography following a 5 h incorporation of [$^{3}$H]thymidine); and (2) failure to undergo cellular degeneration. The former may be related to the latter since withdrawal from the proliferative population commonly precedes morphogenetic cell death, as exemplified by epithelial autolysis during morphogenesis of the secondary palate in rats (Hudson & Shapiro, 1973; Pratt & Martin, 1975). The earliest signs of basal cell degeneration in normal limbs may have been indicative of either phagocytosis, autophagic remodelling of cellular organelles, or an early stage of lysosome-mediated autolysis. There was no evidence of the classic nuclear or cytoplasmic changes found during cell death (Kerr \textit{et al.} 1972; Schweichel \& Merker, 1973) at this time. Hence, a lysosome-mediated (type II) autophagy may provide the normal mechanism for programmed degression of the AER, but may or may not lead to autolysis in more advanced stages.

Delayed degression of the preaxial AER in mutant limbs would be expected to prolong its 'mesenchyme inductive' potential (Saunders, 1948; Zwilling, 1956\textit{a, b, c}), and possibly induce outgrowth of the pre-axial protrusion. The AER has been shown to exert a trophic influence on chick limb mesenchyme \textit{in vitro} maintaining it in a viable (Cairns, 1975), undifferentiated state of which rapid outgrowth is characteristic (Stark \& Searls, 1973; Globus \& Vethamany-Globus, 1976; Kosher, Savage \& Chan, 1979). Although mesenchymal proliferation is recognized as the primary motive force for outgrowth (Ede, Flint \& Teague, 1975; Summerbell \& Lewis, 1975), the proliferative profile of the mesenchyme within the protrusion was normal. Hence, outgrowth of the protrusion was not associated with an increased rate of proliferation. More likely, failure of PCD to occur at the fpp resulted in a larger population of proliferating cells. A large number of mitotic figures was present during stage 18 just prior to formation of the protrusion, at the expected location of the fpp. By stage 19 the mitotic profile was normal, indicating that a transient mitotic synchrony occurred within the region normally expected to undergo PCD. A delay in the onset of PCD pre-axially in the AER and mesenchyme has been described in the autopods of several other genetic mutants of the chick (\textit{talpid$^a$} (Cairns, 1977)}
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and talpid\(^3\) (Ede, 1971)) and mouse (Dominant Hemimelia (Rooze, 1977)), and in teratogenesis induced in rats by the anti-proliferative agents cytosine arabinoside, 5-fluorodeoxyuridine, and 6-mercaptopurine riboside (Scott et al. 1977, 1980). In these instances, a high incidence of pre-axial polydactyly was characteristic.

Other abnormalities occurred in the pre-axial mesenchyme of Hm\(x^+/+\) limbs, but their relationship to the altered PCD pattern is not clear. The paucity of mesenchymal cell filopodial processes extended along the basal lamina was similar to what has been described in the talpid\(^3\) chick mutant (Ede, 1971), in which aberrant cellular adhesive properties have been detected in limb mesenchyme (Ede, Flint, Wilby & Colquhoun, 1977). Also in Hm\(x^+/+\) limbs, the filamentous extracellular material associated with the mesodermal face of the basal lamina was less abundant than normal. This material, which is probably synthesized by the mesenchyme, may impart cellular adhesive properties to the basal lamina. Regional differences in basement membrane ultrastructure between digital and interdigital zones have been reported by Kelley (1973) for the early limb bud of man. In that study, a collagen-like fibrillar matrix was associated with the basement membrane at interdigital zones (where PCD occurred in the AER and mesenchyme) but was absent at the non-necrotic digital zones. In light of the proximity imposed between apical mesenchyme and the basal layer of the AER, there is a potential role for this matrix during induction of epithelial PCD. Direct correlation between the presence of collagenous stroma and morphogenetic cell death was reported by Ojeda & Hurle (1975) for the fusion of endocardial tubes during chick cardiogenesis, but a causal relationship was not resolved.

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REFERENCES


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