Molecular mechanisms underlying limb anomalies associated with cholesterol deficiency during gestation: implications of Hedgehog signaling

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Received January 28, 2003; Revised and Accepted March 14, 2003

Human disorders caused by inborn errors of cholesterol biosynthesis are characterized by dysmorphogenesis of multiple organs. This includes limb malformations that are observed at high frequency in some disorders, such as the Smith–Lemli–Opitz syndrome, indicating a pivotal role of cholesterol in limb morphogenesis. Recently, it has been demonstrated that cholesterol can modulate the activity of the Hedgehog proteins, that act as morphogens to regulate the precise patterning of many embryonic structures, among which the developing limbs. To provide insight in the functions of cholesterol during limb development and in the potential role of Hedgehog signaling in the genesis of limb defects, we developed an in vivo rat model of cholesterol deficiency. We show here that treatment with Triparanol, a distal inhibitor of cholesterol biosynthesis, induced patterning defects of the autopod at high frequency, including pre-axial syndactyly and post-axial polydactyly, thus reproducing limb anomalies frequently observed in humans. Using in situ hybridization, we show that these malformations originate from a modification of Sonic Hedgehog signaling in the limb bud at 13 days post-coitum, leading to a deficiency of the anterior part of the limb. This deficiency results in an imbalance of Indian Hedgehog expression in the forming cartilage, ultimately leading to reduced interdigital apoptosis and syndactyly. Our study thus unravels the molecular mechanisms underlying the genesis of limb defects associated with cholesterol deficiency in rodents, and most probably in humans.

INTRODUCTION

Cholesterol has long been recognized as a key regulator of eukaryotic membranes properties and as a precursor in the synthesis of steroid hormones, bile acids and lipoproteins. Recently it has been demonstrated that cholesterol has also the potential to modulate the activity of a class of cell-signaling proteins, the Hedgehog (Hh) proteins. This activity is particularly critical during embryogenesis, as these proteins act as key regulators of growth, patterning and morphogenesis of many embryonic structures (reviewed in 1).

To be activated the Hh proteins undergo an autoprocessing reaction that involves an internal cleavage which releases the amino-terminal peptide (Hh-N) that is responsible for all signaling activities (2–5). The cholesterol participates to this cleavage and at the end of the process a cholesterol molecule is covalently attached to the carboxyl terminus of Hh-N (= Hh-Np), causing a preferential association of the peptide with the cell membrane (6,7). In addition to its role in the genesis of an active signal, cholesterol is also important for Hh signal transduction (reviewed in 8–10). The Hh receptor, Patched (Ptc), contains a sterol sensing domain (SSD), a motif originally identified in proteins that function in cholesterol homeostasis (11), thus implying a role for cholesterol in mediating the receptor–ligand interaction that activates the signaling pathway. Recent discovery of two other genes in Drosophila suggests that diffusion of Hh protein from cell to cell and release of the protein Hh from the synthesizing cell are also influenced by cholesterol. The first one, tout-velu (tv), encodes an enzyme involved in proteoglycan biosynthesis, that is required for movement of cholesterol-modified, but not -unmodified, Hh-N (12,13). The second one, dispatched (disp), encodes a 12-transmembrane protein that contains an SSD (14). In the absence of disp, normal levels of Hh protein are...
produced and processed, but Hh-Np fails to be released from synthesizing cells, suggesting a role in intracellular trafficking of the cholesterol-modified form of Hh. Interestingly, disp is not required for signaling by unmodified Hh.

In view of these data it is reasonable to hypothesize that any alteration of sterol balance within embryonic tissues will result in alteration of Hh biogenesis, trafficking and/or signal transduction, leading to impaired range and/or activity of the signal. Indeed, deficit in cholesterol biosynthesis during gestation is associated with the occurrence of specific anomalies at birth, both in humans and animals, the most spectacular being of the holoprosencephalic realm. These anomalies have been proposed to result form altered activities of Hh proteins during early development (15,16). In agreement, we recently demonstrated that brain defects observed in a rat model of cholesterol deficiency are associated with abnormal signaling of the vertebrate Sonic Hedgehog (Shh) protein in the developing neural tube (17,18).

In addition to craniofacial and brain defects, limb malformations are also frequently associated with deficit in cholesterol biosynthesis in humans and mutant animals. The Smith–Lemli–Opitz syndrome (SLOS) is the better known human syndrome in which cholesterol biosynthesis is altered. It is caused by mutation in the 7-dehydrocholesterol reductase (7-DHCR), the last enzyme in the Kandutsch–Russell pathway of cholesterol biosynthesis. One of the most common anomalies of SLOS patients is the distinctive Y-shaped syndactyly of the second and third toes, which has been found in more than 80% of biochemically proven cases (19,20). Bilateral or unilateral postaxial polydactyly of the hands and, less common, of the feet, have been observed, particularly in the severely affected patients (21–24). Syndactyly and postaxial polydactyly have been reported at lower frequencies in other human syndromes displaying altered metabolism of cholesterol: the Conradi–Hünermann–Happle X-linked dominant disease (CDPX2), the Greenberg skeletal dysplasia and the Bare Patches (Bpa) mouse mutant (Table 1) (reviewed in 16). In addition to digit patterning defects, limb shortness and even dwarfism seem to be universally associated with deficiency in cholesterol biosynthesis during embryonic development (Table 1).

As two members of the vertebrate Hh family, Shh and Indian hedgehog (Ihh), play critical functions for the patterning and differentiation of limb structures, we speculated that limb defects generated by cholesterol deficiency and/or sterol imbalance during gestation could result from impaired Hh activity. A genetic mouse model of the SLOS has been produced recently (25). However, these mice develop only mild craniofacial anomalies and no limb defects, possibly due to the high sterolemia of this particular species and to the placental transfer of maternal cholesterol to the embryos. In contrast, due to its relatively low sterolemia and to its susceptibility to develop severe cholesterol deficiency under exposure to distal cholesterol biosynthesis inhibitors, the rat provides an excellent model system in which to study the impact of cholesterol deficiency (26). Indeed, this methodology has been successfully used to unravel the molecular mechanisms underlying the pathology of brain defects in a model of the SLOS (17,18). To investigate the relationship between cholesterol, Hh activities and limb development, we thus developed a rat model of cholesterol deficiency using the pharmacological inhibitor Triparanol, that acts at the level of the 24-dehydrocholesterol reductase (24-DHCR) (27). Pregnant rats were treated at stages critical for limb development, 9 and 10 days post-coitum (dpc). We report here that these treatments induce a high frequency of patterning defects of the limb. We show that these malformations originate from a modification of Shh signaling in the limb bud at 13 dpc, leading to a deficiency of the anterior part of the limb, through a process that involves increased cell death. This deficiency results in an imbalance of Ihh expression in the forming cartilage, ultimately leading to reduced interdigital apoptosis and syndactyly. Our study thus unravels the molecular mechanisms underlying the genesis of limb defects associated with cholesterol deficiency in rodents, and most probably in humans. It also shows that cholesterol is required for two specific activities of Shh in the developing limb, the long-range patterning and the regulation of the posterior necrotic zone, and that its role cannot be fulfilled by other sterols.

### RESULTS

**Triparanol exposure induces cholesterol deficiency in treated embryos**

The influence of the Triparanol treatment at 9 dpc on sterol levels was assayed in maternal serum and fetal tissues by gas chromatography–mass spectrometry (GC–MS) at different stages after exposure: 15, 17 and 19 dpc. Maternal cholesterol levels in control animals varied from one dam to another and from one stage to another, but was within the range of 60–80 mg/dl (Fig. 1A). Exposure to 200 mg/kg of Triparanol once at 9 dpc reduces cholesterol concentrations to values varying from 23 to 36% of control values (Fig. 1A). In parallel, cholesterol precursors that were in undetectable amounts in controls accumulate in the serum of treated dams. The two major precursors detected were desmosterol (cholesta-5,24-dien-3β-ol or 24-dehydrocholesterol) and zymosterol (cholesta-8,24-dien-3β-ol). In treated animals, desmosterol values were equal or higher than cholesterol values, while zymosterol values were ~20–40% of cholesterol values.

The sterolemia was more severely modified in fetal tissues. The total sterol values were decreased to about 15–20% of levels

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SLOS, Smith–Lemli–Opitz syndrome; CDPX2, the Conradi–Hünermann–Happle X-linked dominant disease; CHILD, Congenital Hemidysplasia with Ichthyosiform erythoderma and Limb Defects (for references, see 16).
measured in control fetuses (Fig. 1B). Precursors were present in detectable amount in control fetuses, mainly desmosterol was present in amounts that were 10–15% of cholesterol concentrations. Following treatment, desmosterol concentrations dramatically increased to values that were two to five times those of cholesterol. However, the most abundant precursor detected at 15 and 17 dpc was zymosterol, that accumulated at very high concentrations (Fig. 1B).

Triparanol treatment induces macroscopical limb defects

Cholesterol deficiency and precursors accumulation induced by Triparanol treatment at 9 dpc resulted in several morphological alterations of the limbs, that concerned principally the anterior half of the autopod of both forelimbs (FLB) and hindlimbs (HLB).

These modifications were first detectable at 15 dpc. In the FLB of Triparanol-treated embryos the major defect was the reduction of the interdigital region between digits 2 and 3. In all control FLB examined (n = 35), digits 2, 3 and 4 were regularly spaced and the distance between the tip of digits 2 and 3 was equal to the distance between the tip of digits 3 and 4 (Fig. 2A). In 78% of the limbs of embryos treated at 9 dpc (n = 103), the distance between digits 2 and 3 was less than the distance between digits 3 and 4 (Fig. 2B). In the HLB the reduction of the anterior autopod was even more pronounced. In 35% of treated limbs, the size of digit 2 was reduced (n = 91; Fig. 2F and G). The condensation of the digit appeared shorter and the indentations between digits 1/2/3 were less marked. As a result digit 3, which is normally located in the middle of the plate, was positioned in the anterior half of the plate, the middle being marked by the webbing between digits 3 and 4 (Fig. 2G). In 27% of the limbs the deficiency was even more severe with no external appearance of digit 2 (Fig. 2H). Instead there was one large primordium, covering the anterior half of the plate. Similar anomalies were induced by treatment at 10 dpc, with similar frequencies (data not shown).

Two days later at 17 dpc, the dysmorphogenesis of the limbs was even more apparent and 97% of the treated embryos had at least a malformation at one limb (n = 31). The reduction of interdigital region 2/3 observed at 15 dpc in the FLB of treated embryos resulted in syndactyly at 17 dpc (Fig. 2D). Although different limbs were affected at different extent, the syndactyly of digits 2/3 was observed in 87% of treated embryos. At that stage, we also observed in two embryos an ectopic digit on the posterior side of the FLB (Fig. 2D). This digit was clearly shorter, and located proximally. In the HLB, the deficiency of the anterior region also resulted in syndactyly, but in this case of digits 1/2 (Fig. 2K). In the most extreme cases ectrodactyly was observed with only four digits individualized (Fig. 2L). Less affected limbs could also be recognized by a shortening of

Figure 1. Steroids levels in maternal serum (A) and fetal tissues (B) assayed by GC/MS at different stages after exposure to 200 mg/kg of Triparanol at 9 dpc (T9). Chol, cholesterol; Desmo, desmosterol; Zymo, zymosterol.
digit 2 and by less pronounced indentations between digits 3, 2 and 1 (Fig. 2I).

We also examined limbs at 19 dpc. At that stage we were not able to analyze syndactyly in the FLB due to the secondary fusion of the epidermis between digits, that brings digits together and parallel to each other (28). However we could still clearly identify a deficiency in the anterior half of the HLB, with a varying degree of severity, extending from a shortening of digit 2 to syndactyly of digit 1/2 and to ectrodactyly (Fig. 2N–P).

**Limb dysmorphogenesis is associated with skeletal abnormalities**

Since the morphology of the autopod was abnormal in treated embryos, we analyzed to what extent the cartilage was also affected. For this analysis the right limbs of 17 and 19 dpc fetuses examined above were stained with alcian blue and cleared to reveal the forming skeletal elements.

In all the treated FLB where syndactyly of digits 2/3 was observed at 17 dpc, metacarpals and phalanges were present and similar to controls, confirming that it was a soft tissue syndactyly (data not shown). Similarly, in the treated HLB where a shorter digit 2 was observed, the underlying skeletal elements were indistinguishable from controls (data not shown). In the HLB with syndactyly of digit 1/2, all cartilage in the metatarsal region was present and the proximal phalanges of both digits were individualized (Fig. 3B). However the condensation of the medial phalange of digit 2 appeared delayed at 17 dpc and only two phalanges were present at 19 dpc, instead of the normal three phalanges (Fig. 3F). In those cases, digit 2 thus seems to acquire a digit 1 identity. In the limbs with ectrodactyly, two different cartilage phenotypes were observed. In one case, the only trace of digit 2 was a small piece of cartilage between metatarsals 1 and 3 (arrowhead in Fig. 3D). In all other cases, the metacarpal elements of digits 1 and 2 were both present, but a fusion was observed at the level of the proximal phalanges, resulting in only one digit (arrowheads in Fig. 3C, G and H).

In addition to the skeletal defects underlying the morphological anomalies, the alcian blue staining revealed other cartilage phenotypes in the handplate. Analysis revealed a noticeable enlargement in the width of metacarpal 5 (m5) in 87%
of the FLB examined at 17 dpc \( (n = 31) \); compare m5 in Fig. 3I and J). A basal cartilaginous nodule was frequently observed, that could be an ectopic carpal, but was always connected to m5 (arrow in Fig. 3J). The basal enlargement of m5 was also noticed in older limbs (30%; data not shown). The whole area around m5 seemed to be enlarged: the distal carpal d4 was elongated and in some cases split in two pieces (arrow in Fig. 3J). These anomalies were present in morphologically normal limbs and in the two limbs where an ectopic digit 6 was observed. In those limbs a cartilaginous condensation was present within the ectopic posterior digit. Arrowhead points to a cartilaginous condensation in the ectopic posterior digit. (K) Tarsal region of a control HLB at 15 dpc. (L) Tarsal region of a Triparanol-treated HLB. Note the slight enlargement of m5. Arrows point to the elongation of the cuboidal cartilage and to the fusion between metatarsal and tarsal cartilage. Bar = 0.5 mm.

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This skeletal analysis confirmed the deficiency in the anterior region of the autopod of treated limbs, with a clear reduction of the area of digits 1, 2 and 3. In addition it revealed an enlargement of the posterior region of the FLB, that led to minor alterations of cartilage in most cases but also in ectopic digit in more severe cases. A similar but apparently less severe posterior alteration could be observed in some HLB.

**Triparanol treatment interferes with gene expression in the digital and interdigital regions**

To understand the molecular mechanism underlying the dysmorphogenic cascade leading to syndactyly and polydactyly, gene expression in the limbs was examined by whole-mount *in situ* hybridization (WM-ISH) at 15 dpc. We studied genes that are expressed in the cartilaginous condensations, in the perichondrium and in the interdigital mesenchyme. In the condensations, we examined the expression of *Ihh*, and in the perichondrium we analyzed the expression of two of its target genes, *Ptc1* and *Gli1*. These three genes are key actors in the process of endochondral bone formation (29,30). In the interdigital mesenchyme we studied the expression pattern of *Bmp2* and *Bmp7*, which participate to the process of programmed cell death in digit formation (31).

In the HLB, *Ihh* was expressed in the entire mesenchymal condensations of the forming digits 2, 3 and 4. A smaller and faint labeling was also visible in the condensation of digit 5, but no labeling was detected in digit 1 (Fig. 4A). In the FLB, the interzone (future joint area) has appeared and the mesenchymal condensation was segmented in the metacarpals and proximal phalanges cartilaginous elements (Fig. 4J). *Ihh* expression was restricted to the middle of the metacarpal elements of all digits, and it was weakly expressed in the condensations of proximal phalanges of digits 2, 3 and 4. About 20 and 35% of embryos exposed to Triparanol at 9 and 10 dpc, respectively, had an abnormal expression of *Ihh* in the HLB, in the condensation of digit 2 \( (n = 32, n = 11) \). The signal was either twice as intense than in digits 3 and 4 (Fig. 4B), or reduced in size and/or distorted (not illustrated). In more severely affected HLB, where the anterior area was so reduced that proper formation of all digit condensations was compromised, the domain of expression was reduced to a small point located...
proximally next to digit 3 (Fig. 4C). In the treated FLB, increase in the intensity of expression of Ihh was also observed in the condensation of m2 (arrow in Fig. 4K).

Gli1 and Ptc1 were both expressed in the perichondrium region flanking the Ihh expressing domain, although the expression of Gli1 appeared more sharply defined than the expression of Ptc1. Exposure to Triparanol, either at 9 or 10 dpc, resulted in modifications of the expression of Gli1 in the anterior region of the HLB. In half of the HLB examined (19 out of 38), the domain of expression of Gli1 in the perichondrium of digit 2 was not sharply defined, but extended in the interdigital towards digit 1. In 20% of the limbs this signal also extended on the other side, in the interdigit 2/3 (Fig. 4E). In those cases, there was no unlabeled region between the condensations of digits 2 and 3, and a high labeling covered the region between digits 1 and 3. On the contrary, in the most severely affected limbs the intensity of the signal in the perichondrium of digit 2 was clearly reduced (Fig. 4F). Similarly, in more than 20% of the HLB examined after exposure to Triparanol, the level of expression of Ptc1 was increased around the mesenchymal condensation of digit 2 (data not shown).

We next examined the expression of two Bmp genes, Bmp2 and Bmp7, that are expressed at high level in the interdigital region at that stage of development. Their pattern of expression was very similar. In the HLB of control embryos, the expression was present at very high level in the interdigits 1/2, 2/3, 3/4 and 4/5 (Fig. 4G). This expression extended from the proximal to the distal part of the autopod, although it appeared excluded from the most distal region (the progress zone). In the FLB the signal seemed to decrease in the center of the interdigit region but remained intense around cartilage and around the future joints (Fig. 4M). Expression of these two genes was also modified in the anterior region of Triparanol-treated HLB and FLB, in the interdigit 2/3. The defects varied from a narrower domain of expression to an interrupted domain with unlabeled areas that appeared as fusion between the mesenchymal condensations of digits 2 and 3 (Fig. 4I and N). In the most extreme case a large domain of expression was present between unlabeled condensations of digits 1 and 3, with no unlabeled areas corresponding to the condensation of digit 2 (data not shown). In more than 20% of the limbs hybridized with Bmp2, the domain of expression was restricted to the proximal part of the autopod and the unlabeled distal region was larger than in control embryos (Fig. 4H).

As Bmp proteins are required for cell death in the interdigital region, we examined if the modifications of their expression pattern in treated embryos could perturb the process of apoptosis in this area. Apoptotic cells were detected in treated limbs at 15 dpc by whole-mount TUNEL staining. At that stage in the FLB, apoptotic cells can be seen at the distal end of the interdigit, in a triangular-shaped area between digits 1/2, 2/3, 3/4 and 4/5, extending into the interdigital mesenchyme (Fig. 4L). In the treated FLB, although a few labeled cells...
could be detected in the interdigit 2/3 just beneath the surface ectoderm, they were much reduced in number as compared with the interdigit 3/4, and did not extend proximally (Fig. 4O).

These data reveal modifications in gene expression in the forming cartilage elements of digit 2, associated with modifications of gene expression in the interdigital regions around digit 2 and with reduced apoptosis in the interdigit 2/3. These events are likely to precede the apparition of the syndactyly observed in the anterior part of the autopod of treated limbs.

**Triparanol treatment interferes with Shh signaling in the developing limb**

We finally wanted to identify the early defect that leads to the deficiency of the anterior part of the limb at 15 dpc. It is known that Shh, produced by the zone of polarizing activity (ZPA), is the primary determinant of the antero-posterior polarity of the vertebrate limb field (32,33). Shh acts as a morphogen, and it has been suggested that digits 5 and 4 arise in the posterior domain of high signaling activity, digits 3 and 2 arise from more anterior mesenchymal cells, where Shh activity is low, while the most anterior digit seems to develop in a Shh-independent way (34). To study the range of Shh signaling in the limb of Triparanol exposed embryos, we examined the expression of Shh target genes earlier in development, at 13 dpc.

We first analyzed the expression of *Ptc1*, which encodes the Shh receptor. Its expression in the limb at 13 dpc is Shh-dependent and closely correlates with the activation and anterior movement of N-Shh within the limb field (34). Its expression is dynamic: it is first detected at the posterior margin of the limb bud (ZPA) then extends in a domain larger than, but overlapping with, the ZPA. Later, *Ptc1* expression is lost in the ZPA, when Shh expression is strongest (35,36). In the control limbs, we observed *Ptc1* expression in the ZPA and in the mesenchyme around the ZPA, covering the posterior two-thirds of the limb bud. Its expression is graded, decreasing from posterior to anterior (Fig. 5A and C). In the FLB of treated embryos, two modifications of this pattern of expression were observed. First, its expression was downregulated in the ZPA (Fig. 5B). Second, its expression was reduced in the anterior part of its domain of expression, at the level of future digit 3. Instead of forming a dome-shaped domain of expression, the labeling appeared as an inverted S-shaped (~) domain (arrow in Fig. 5B). In the HLB, a consistent downregulation of the expression was observed at the level of the ZPA, and the domain of expression in the mesenchyme was reduced in the distal part of the limb, under the AER (arrow in Fig. 5D). The gradient of signal intensity, from high posterior to low anterior, was less pronounced in treated HLB, although the intensity around the ZPA (the level of future digit 4) was similar to in control limbs.

As *Ptc1* was downregulated in the ZPA, we next examined the domain of expression of *Shh*, which coincides with the ZPA (Fig. 5E and G). In addition, *Shh* expression is dependent upon its own activity, through a Shh/Fgf4 feedback loop (see below). In the FLB of treated embryos, the intensity of the signal was reduced as compared to control embryos, and the domain of expression appeared thinner along the posterior margin of the

**Figure 5.** Expression of Shh’s target genes in the limbs of control and Triparanol-treated embryos at 13 dpc. (A, C) Control FLB and HLB illustrating the normal pattern of expression of *Ptc1*. (B, D) In the FLB of Triparanol-treated embryos *Ptc1* expression was reduced posteriorly in the ZPA, as well as in the anterior distal region (arrow). (D) In the HLB of treated embryos, the expression was reduced posteriorly and distally, under the AER (arrow). (E, G) Control FLB and HLB illustrating the normal pattern of expression of *Shh*. (F) In the FLB of Triparanol-treated embryos the intensity of expression of *Shh* was reduced. (H) In the HLB of treated embryos the intensity of expression was slightly reduced and the expression was graded proximally (arrow). (I) Control HLB illustrating the normal pattern of expression of *Fgf4*. (J) The intensity and domain of expression of *Fgf4* in Triparanol-treated embryos were similar to controls. (K–N) TUNEL staining of 14 dpc FLB. (K) Ventral view of a control FLB showing the presence of apoptotic cells along the AER, from the posterior edge until a clear border at the anterior edge (arrow). (L) In treated limbs there is an increase in the density of labeled cells in the anterior part of the AER, in the area of digits 1/2 (between arrow and arrowhead). (M) Magnification of the anterior border of the AER, view from the top. Apoptotic cells are restricted to ectodermal ridge. (N) In treated limbs the density of apoptotic cells is increased in the ridge and scattered cells are also present ventrally and proximally (arrows). Bars = 500 μm.
In the HLB, the intensity of the signal was similar or slightly lower in treated and control limbs; however, the domain of expression was more elongated along the posterior margin, extending more proximally than in controls (Fig. 5H). At that level, the expression appeared to fade progressively, from distal to proximal (arrow in Fig. 5H), while a clear border was apparent in control limbs.

We also examined the expression of the BMP antagonist, Gremlin, and the Fibroblast growth factor Fgf4. As an antagonist of BMPs signaling, Gremlin appears to protect the undifferentiated mesoderm from the apoptotic influence of the BMP proteins (37). Gremlin also participates in relaying the Shh signal from the mesenchyme to the apical ectodermal ridge (AER), expressing Fgf. In turn, Fgf4 signaling by the posterior AER maintains the Shh signal in the ZPA, thus establishing a Shh/Fgf feedback loop (38). Expression of Gremlin in the mesenchyme underlines the expression of Fgf4 in the AER, with their anterior border of expression coinciding. By comparison of control and treated limbs, we were unable to detect consistent differences in the intensity of expression, nor in the anterior extend of the domain of expression of these two genes (Fig. 5I and J and data not shown).

As these signals are known to be involved in programmed cell death and cell proliferation, two processes that could participate in the deficiency of the anterior part of the autopod, we analyzed cell proliferation and apoptosis in the limbs one day later, at 14 dpc. Apoptosis was assayed by whole-mount TUNEL and cell proliferation by the immuno-detection of an antigen (Ki-67) expressed in all active stages of cell cycle. At that stage in mammalian limbs apoptosis is prominent in the AER and patchy labeled cells were detected all along the ridge (Fig. 5K). In treated FLB, two modifications of the labeling were observed. First, in about 60% of the limbs examined (n = 12), the number of labeled cells was increased in the anterior part of the ridge, covering the region of future digits 2 and 3. Such increase in apoptosis in the anterior part of the ridge could also be observed in some HLB, albeit it was less obvious due to a higher density of apoptotic cells present in the control HLB (data not shown). Second, in 75% of the treated FLB, scattered cells were observed at the anterior border of the limbs, extending either ventrally or proximally (arrows in Fig. 5N), as opposed to the precisely defined border observed in control limbs (Fig. 5M). This probably reflects a loss of integrity of the anterior margin of the AER. At 14 dpc, cell proliferation was present mainly in the distal mesoderm, underneath the AER, the so-called progress zone, and was absent from the core of the limb. No significant and reproductive differences were observed between control and treated limbs (data not shown).

**DISCUSSION**

This study uncovers the disruption of limb development in cholesterol-deficient embryos, and reveals the importance of cholesterol for proper limb patterning. By using a distal inhibitor of cholesterol biosynthesis in rodent, we were able to reproduce limb anomalies frequently observed in human syndromes, thus providing a valuable animal model to understand the molecular mechanisms underlying the appearance of these limb defects. The analysis of this animal model at the molecular level extends our knowledge of the importance of cholesterol for the activity of Hedgehog proteins during vertebrate development. We show that cholesterol has a unique role for some particular activities of Shh during limb patterning, a role that cannot be fulfilled by any of the other sterols detected in the fetal tissues.

**Triparanol treatment reproduces limb defects observed in human syndromes of cholesterol biosynthesis defects**

Experimental inhibition of cholesterol biosynthesis in animals using pharmacological inhibitors such as Triparanol, AY9944 and BM15.766, has been used for many years as a model for human syndromes, such as the SLOS (reviewed in 16,26). These teratogenic models are essential to our understanding of the molecular mechanisms involved in the physiopathology of human disorders, since genetic models are unable to restitute the human phenotype. In the past, these studies have focused mainly on the anomalies of the central nervous system, of the holoprosencephalic realm. Those anomalies, although spectacular, are not the most frequently associated with defects in cholesterol biosynthesis in humans. Until very recently, few studies have been concerned by the developmental defects of the limbs. To further our understanding of the functions of cholesterol in limb patterning and differentiation, we used here a protocol with late administration of Triparanol, either at 9 or 10 dpc (40). We observed a high frequency of limb defects, similar to those described in SLOS patients, but also in CDPX2 and Greenberg skeletal dysplasia patients (see Table 1). In the FLB autopod, pre-axial syndactyly of digits 2 and 3 was found most commonly, but also post-axial polydactyly, varying from minor cartilage changes to the presence of an ectopic digit. In the HLB autopod, pre-axial syndactyly with variable expressivity was seen at very high frequency, ranging from syndactyly of digits 1 and 2 to ectrodactyly. Although postaxial polydactyly was not observed in the HLB, minor cartilage changes were detected in the posterior region. It is noticeable that these defects were induced in a sterol environment where cholesterol deficiency is associated with accumulation of desmosterol and zymosterol, while in SLOS patients, the steleome is characterized by accumulation of 7-DHC and 8-DHC. It thus seems that for the limb patterning anomalies, as was shown previously for the central nervous system anomalies (18,41), the critical event is the cholesterol deficiency, which cannot be substituted by any of the accumulated precursors.

**Limb patterning defects: from anterior deficiency of the limb bud to reduced interdigital apoptosis**

The first visible defect in the treated limbs was a deficiency in the anterior part of the autopod at 15 dpc. Indeed, the extent of the anterior area of forming digits 1–3 was reduced and the middle of the plate was located at the interdigit 3/4, instead of being at the level of digit 3. Based on our results of ISH at 15 dpc, we can propose a model for the genesis of the limb patterning defects, which is illustrated in Figure 6. As a result of the deficiency of
the anterior patterning, mesenchymal condensations of the future cartilage appeared to form with the correct shape and number, although they were located closer to each other in the anterior part of the autopod, as revealed by Ihh expression. In those cases, there seemed to be a dosage imbalance in the Ihh signal received by the perichondrial cells. Owing to their proximity, mesenchymal cells between the two condensations received Ihh signal from both sides, and expression of genes such as Gli1 was highly increased. The upregulation of Gli1 in the perichondrium of digits 1, 2 and/or 3 was accompanied by a loss of Bmp2 and Bmp7 expression in the corresponding interdigital region. We were able to show in the FLB that these modifications of gene expression were associated with a reduction of apoptosis in the interdigital mesenchyme, thus explaining the phenotype of pre-axial syndactyly observed later in development. However in a few cases, the deficiency of the anterior area was such that mesenchymal condensations were not able to form in the correct number and only four condensations were seen. Gene expression in these condensations appeared normal and the result was the formation of four well-formed digits and ectrodactyly.

All together, these data unravel the molecular mechanism underlying the apparition of the morphological limb defects in this animal model, and most likely in the human syndromes. However they also suggest that cholesterol deficiency interferes with a patterning event occurring earlier than 15 dpc.

**Anterior deficiency of the limb bud: alteration of Shh signaling**

Inhibition of cholesterol biosynthesis at a critical time for limb development did not seem to affect limb bud outgrowth, and proximo-distal as well as dorso-ventral polarity appeared normal. In contrast the antero-posterior development is disrupted and digit number and identity is affected. As the antero-posterior patterning of the limb bud is under the control of Shh, the initial defect could be an altered activity of Shh in the cholesterol-deficient limb. However, our results contrast with what is observed in Wnt7a KO mice, where decreased expression of Shh is accompanied by the absence of posterior digits (42). Indeed, digits 5 and 4 always formed properly in all Triparanol-treated limbs examined. As posterior digits are induced by short-range signaling, this activity of Shh is not affected in our model. On the other hand, our results are comparable with the loss of anterior digits accompanying overexpression of Ptc1 (43). One explanation for this phenotype was that overexpression of Ptc1 in the limb changed the range of action of Shh, as Ptc1 is known to sequester free Hh proteins, thereby regulating its movement in its target field (44). Loss of long-range activity of Shh was also reported in limbs using an engineered protein Shh-N without cholesterol modification (34). The consequence was a loss of anterior digits 2 and 3. Triparanol-induced cholesterol deficiency thus reproduces a mild phenocopy of the loss of Shh long-range activity. In order to demonstrate this modification in treated limbs, we analyzed the expression of different targets of Shh signaling. We show here that Ptc1 expression, a marker of the level and range of activity of Shh in the limb bud, was affected in the cholesterol-deficient limbs. Its expression in the posterior domain located around the ZPA was as high in treated as in control embryos (35,36). A possible explanation for this premature downregulation in the treated limbs would be that overexpression of Ptc1, it would set up the feedback loop between Ptc1 and Shh earlier than normal, leading to the attenuation of the Shh signaling cascade. In support of this hypothesis, the level of Shh expression was almost similar in treated and control HLB, but it was clearly reduced in treated FLB, which reflect a later developmental stage.

The proximo-distal outgrowth of the limb is also regulated by Shh from the ZPA. At that level, an autoregulation loop has been demonstrated via Fgf4 and the intermediate Gremlin (38).
As mentioned previously, the proximo-distal outgrowth of the limbs does not seem to be affected in the treated limbs. Indeed, normal levels of expression of Fgf4 and Gremlin were observed in the AER and in the mesenchymal cells. In this process Shh is acting at short range, and this activity does not seem to be altered as a consequence of cholesterol deficiency.

At the cellular level, we have tried to understand what mechanisms could link the alteration of Shh long-range signaling and the anterior deficiency of the limb observed at 15 dpc. Cell proliferation does not seem to be involved as we did not detect any alteration at 14 dpc, although we cannot exclude a short and temporary block in cell proliferation between 13 and 14 dpc. On the opposite, an increase of cell death was observed in the anterior part of the AER of the FLB, covering the area of digits 1/2. Moreover, an increase of apoptotic cells and a loss of integrity of the anterior end of the ridge was detected in 75% of the FLB. AER maintenance and AER length are important in controlling the width of the bud outgrowth, which is central in specifying digit number (reviewed in 45). Factors involved in the maintenance of the ridge are FGFs and factors involved in the regression of the AER are BMPs. For example, inhibition of Bmp signaling in the anterior part of the limb leads to a decrease of cell death in the anterior necrotic zone, to an ectopic anterior expression of Fgf4 and to soft tissue outgrowth (46), a phenomenon opposite to the one observed in the present paper. Although we have not detected changes in the anterior extend of Gremlin expression, it is likely that alteration of Shh long-range signaling has disrupted the balance between BMPs and FGFs, resulting in the regression of the anterior part of the AER and in an alteration of the anterior end of the ridge, thereby limiting the outgrowth of the limb in the anterior region. This process is thus probably part of the cascade that leads to the anterior deficiency of the autopod.

**Posterior enlargement of the limb bud: relation to the posterior necrotic zone**

Altered Shh signaling can also explain the post-axial polydactyly and the posterior enlargement of the autopod, so it was shown in chick embryos that Shh affects cell death in the posterior necrotic zone (47). This function is unusual as Shh is known to act as a survival factor in other locations, but acts as an inducer of apoptosis at that precise level. This activity of Shh seems to be part of a ‘buffering system’ required to regulate the number of Shh-expressing cells and by this way, regulating signaling in the limb. A modification of this particular activity in cholesterol-deficient embryos may partially disrupt this buffer, thus leading to a decrease of apoptosis in the posterior necrotic zone. As the posterior and anterior necrotic zone have been suggested to play a role in the control of the number of mesenchymal cells available to form digits, such a defect would explain our phenotype. The observation of a gradient of expression instead of a sharp border at the proximal side of the expression domain of Shh supports the idea that the buffering system is modified. However, because the number of apoptotic cells in the posterior necrotic zone of rodent embryos are intrinsically low, we have not been able to demonstrate this hypothesis by TUNEL labeling.

**Unique role of cholesterol for proper activity of Shh in the limb**

Our data confirm that cholesterol is required for the long-range activity of Shh during limb patterning but not for the short-range signaling at that level. In addition, we suggest that cholesterol is also required for the proper regulation of the posterior necrotic zone (Fig. 6). The mechanisms by which cholesterol-modified Hedgehog peptides are able to diffuse and to signal at long-range are still unknown. However recent studies suggest that they could diffuse in the form of a multimeric complex (48). The alteration of proper formation of such complexes in our model could result in an abnormal movement of the signal, or to an abnormal presentation/reception of the complexes at the level of the target cells.

Our results also bring new lights in the understanding of how cholesterol biosynthesis defects affect Hh signaling. First, the present data show that the important event following inhibition of cholesterol biosynthesis is the deficiency of cholesterol, and not the accumulation of precursors. Indeed, similar limb defects are observed in different animal models and human syndromes, that share a common cholesterol deficiency but differs in the sterol precursors that accumulate. Second, we show that the inhibition of cholesterol biosynthesis mimics the phenotype of a engineered N-Shh peptide without cholesterol, thus suggesting that the defective step in our model is the biogenesis of fully active peptide, and not the blockage of the signaling cascade. Indeed, locally, the peptide produced is able to signal properly to target cells to induce posterior digits identity. This contrasts with previous data that suggested that the mechanisms of action of inhibitors of cholesterol biosynthesis would be the production of a teratogenic precursors that would act as cyclopamine or jervine to block the signaling at the level of the target cells (9,10). We can thus conclude from this study that cholesterol is required for two specific activities of Shh in patterning of the limb, and that these particular roles cannot be fulfilled by any other sterol present in the fetal tissues.

**MATERIALS AND METHODS**

**Rat treatment**

Wistar rats (Ifca Credo, France) were housed under standard conditions in a 12h light/dark cycle. Males and females were paired for a short period (8 to 10 a.m.), after which females were checked by examination of a vaginal smear. The day of positive smear was designated as 0 dpc. A single dose of 200 mg/kg of Triparanol (4-chloro-α-[4{-2-(diethylamino)ethoxy}phenyl]-α-(4-methylphenyl)benzeneethanol) dissolved in sunflower oil was administrated by gavage on either 9 or 10 dpc. According to the experiments females were sacrificed on 13, 15, 17 or 19 dpc and embryos/fetuses were recovered in phosphate-buffered saline (PBS) and processed according to the following procedures. After sacrifice of the dam, maternal blood was collected at the level of the jugular vein. Serum was then stored at −20°C for sterol analysis. At 17 and 19 dpc, the limbs of fetuses were isolated and the rest of the body (head, trunk and tail) was stored at −20°C for dosage of sterols.
Triparanol (MER-29) was a gift of Marion Merrel Dow Research Institute (Cincinnati, OH).

**Lipid extraction and gas chromatography–mass spectrometry (GC/MS)**

Maternal blood and fetal tissue were processed as described elsewhere (41).

**Morphology and cartilage staining**

Limbs collected at 17 and 19 dpc were fixed in Bouin solution overnight at room temperature. They were rinsed and stored in 70% ethanol solution. At that stage the morphology of the limbs was evaluated under dissecting microscope. In *toto* staining of the cartilage with alcian blue was performed according to the procedure previously published (49). At the end of the procedure, limbs were cleared with benzyl benzoate and benzyl alcohol and analyzed under a dissecting microscope.

**Whole-mount *in situ* hybridization (WM-ISH)**

Embryos collected at 13 and 15 dpc were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C. The following day, they were washed in PBS with 0.1% Tween 20 (PBT), dehydrated through a graded series of ethanol, and stored in 100% ethanol at −20°C. WM-ISH was carried out on isolated limbs using digoxigenin-labeled riboprobes according to Wilkinson (50). At 15 dpc limbs were isolated and two probes were used for a single embryo: the anterior and posterior left limb were hybridized with a probe while the right limbs were hybridized with another probe. Plasmids for hybridization were kindly provided by the following: Shh, Ihh, A. McMahon; Ptc, G.M. Morriss-Kay; Gli1, Gli3, C. Hui; Bmp2, Bmp7, B. Hogan; Fgf4, J. Heath; Gremlin, R. Zeller.

**Detection of apoptotic cell death in whole embryos**

Apoptotic cells were detected in whole embryos at 13 dpc and in isolated limbs at 14 and 15 dpc using the deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) procedure as described (51,52).

**Analysis of cell proliferation**

Cell proliferation was assayed by immuno-detection of the nuclear cell proliferation-associated antigen Ki-67, expressed in all active stages of cell cycle (BD Biosciences). Whole-mount immunohistochemistry was performed as described (53). Briefly, 14 dpc isolated limbs were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C. Endogenous peroxidase activity was blocked by immersing the limbs in 3% hydrogen peroxide in methanol for 1 h. The mouse monoclonal antibody Ki-67 was detected with the Vectastain Elite ABC kit using DAB (Vector).

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**ACKNOWLEDGEMENTS**

The authors wish to thank Dr R. Rezsohazy, Dr F. Clotman and R. Kozyraki for their support and their helpful comments on this manuscript, and V. Bonte for technical assistance. This study was supported by the Fonds National de la Recherche Scientifique from Belgium, and by the Fonds Speciaux de la Recherche from the Université Catholique de Louvain.


