Organization of connective tissue patterns by dermal fibroblasts in the regenerating axolotl limb

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Summary
A set of tendons, aponeurotic sheets and retinaculae, which transduce muscle action from proximal limb levels to flexion and extension of the digits, is found in limbs of many vertebrates. This set of structures, here termed the digit tendon complex, is described for the axolotl forelimb. We show that the complex forms autonomously in muscleless axolotl limb regenerates produced from a cuff of unirradiated dermis surrounding an irradiated limb stump, and persists for up to a year after amputation. The pattern of other connective tissue structures, including the skeleton, is also normal. Fibroblast condensations that may represent sets of these cells normally associated with muscles in the extensor and flexor compartments of the carpal region also form in muscleless limbs. The results are discussed in terms of the importance of the dermis in pattern regulation, selforganization of connective tissues in general and autonomous development of the digit tendon complex in particular.

Key words: limb regeneration, connective tissue, fibroblasts, pattern formation.

Introduction
When the limb of a urodele amphibian is amputated, a new limb regenerates which comprises exactly those parts that were removed. A number of recent studies indicate that the positional information necessary for pattern regulation to occur during regeneration lies within the connective tissue of the limb. Such studies have focused primarily on the connective tissue dermis, the mesodermal component of the skin. Surgical manipulations in which the limb skin is rotated (Carlson, 1974, 1975), made symmetrical with respect to the transverse limb axes (Tank, 1979; Maden & Mustafa, 1982; Slack, 1980, 1983), or used as positionally mismatched implants (Tank, 1981; Rollman-Dinsmore & Bryant, 1984) all demonstrate that the dermis dramatically affects the pattern-regulation process. Taken together with the results of these experimental procedures perhaps the strongest evidence for a central role for the dermal connective tissue cells in pattern regulation is the observation that this tissue contributes relatively more cells to the blastema than would be expected from relative cell numbers in the stump (Muneoka et al. 1986; see also Tank & Holder, 1979).

Consistent with this notion of the importance of connective tissues in pattern formation are the observations that tendons can form autonomously in chick wings devoid of muscle (Kiény & Chevallier, 1979), that the connective tissue in such limbs is able to organize grafted myogenic cells into recognizable muscles (Chevallier & Kiény, 1982), and that the pattern of wing muscles is determined by the somatopleure from which connective tissues differentiate (Chevallier et al. 1977). The tendons that form in muscleless chick wings are those associated with flexion and extension of the digits by muscles originating at more proximal segments, from the humerus or radius and ulna (Kiény & Chevallier, 1979; Fig. 1). Although clearly described by these authors, the fact that tendons for muscle attachment within the fore and upper limb levels failed to form was not discussed. In this paper, the distal limb tendons, here referred to as the digit tendon complex, is considered as a separate set of structures the development of which may be autonomously controlled. In all vertebrate limbs, such a group of tendons is present, often in combination with specialized connective tissue structures such as expanded fascial sheets, retinaculae and aponeuroses (Fig. 1). Tendons within these complexes may produce very complicated patterns with branching, overlapping, fusion and, as is the case with tendons in the human hand, situations where one tendon pierces another before insertion.

The digit tendon complex provides an excellent system for study of the pattern-organizing characteristics of connective tissue cells. In the present study, we examine the ability of connective tissues to undergo morphogenesis in regenerating axolotl limbs which are deprived of muscle, looking in detail at the formation of the digit tendon complex. Muscleless regenerates were created by amputating limbs in which all tissues except...
Fig. 1. The digit tendon complex varies in extent in different vertebrate groups. (A) In humans, the complex comprises 22 tendons from 14 muscles which are evident in this schematic cross section through the wrist (after Snell, 1986). The tendons are grouped together in synovial sheaths and surrounded by flexor and extensor retinaculae. These retinaculae and the tendons are black in this drawing. In the chick, fewer tendons are evident than in the human in the extensor (B) and flexor (C) compartments, and they appear separate, not associated with any fascial sheets. The only tendons to form in developing muscleless chick wings (Kieny & Chevallier, 1979) are those of flexor digitorum superficialis (fdst) and profundus (fdpt) and extensor digitorum communis (edct) and extensor metacarpales ulnares (emut). These tendons and others in the wrist area are highlighted in black. hth.m, hypothenar muscles; th.m, thenar muscles; ham, hamate; cap, capitatet; traz, trapezoid; trap, trapezium. 2, 3, 4 represent digit numbers.

the skin had been X-irradiated. This method has been used previously to examine questions of metaplasia (see, for example, Dunis & Namenworth, 1977; Lheur-eux, 1983) but little attention has been paid to pattern regulation in such limbs. We demonstrate that patterning of the connective tissue components of muscleless regenerates is remarkably normal in a number of respects, including the overall pattern of the skeleton and the structure of the digital tendon complex which, unlike the case in the chick wing where tendon primordia degenerate before hatching, remains well formed for a long period. These results add considerable support to the hypothesis (Bryant, 1978; Bryant et al. 1981) that fibroblasts (and principally dermal fibroblasts) play a pivotal role in limb pattern regulation and that they are able to selforganize into complex tissue patterns.

Materials and methods

General
All experiments were performed on larval axolotls (Ambystoma mexicanum) which were spawned at King's College. Animals were maintained in individual plastic containers in standing tap water through the duration of the experiment and were fed twice weekly on chopped heart. During surgery animals were anaesthetized in MS222 (Sigma).

Experimental
Operations were carried out on the upper arm region from which a complete skin cuff (epidermis and dermis) was removed from approximately the mid two thirds. The cuff was laid aside while the remaining limb tissues received 2500 rads of soft X-rays. Any adhering muscle fibres visible in the dissecting microscope were cleaned from the skin which was then returned to the upper arm and sutured into place in a normal orientation (Fig. 2). Following a period of between 8 and 16 days for skin healing, the limb was amputated through the distal edge of the skin cuff and the protruding humerus trimmed flush with surrounding soft tissues. Control operations involved the same procedure except the limb was not X-irradiated. In addition, a number of normal limbs were fixed for wax histology (see below) to help assess the normal pattern of tendons and fascial sheets.

After a period of between 3 and 12 months the limbs were fixed for light and electron microscopic analysis.

(1) Analysis of skeletal and tendon patterns
In order to reconstruct the overall skeletal and tendon pattern in the regenerated limbs they were fixed in Bouin's fluid, decalcified in EDTA, dehydrated, stained with Victoria blue and cleared in methyl salicylate. At this point, the overall skeletal pattern was drawn with a camera lucida and the gross
Limb regeneration from dermal fibroblasts

Table 1

<table>
<thead>
<tr>
<th>No. of digits</th>
<th>Muscle-less</th>
<th>Partly muscled</th>
<th>Normal muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>30</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>Spike</td>
<td>2</td>
<td>2</td>
<td>3*</td>
</tr>
<tr>
<td></td>
<td>12 (40%)</td>
<td>8 (26%)</td>
<td>7 (23%)</td>
</tr>
</tbody>
</table>

Experimental: 7 7 2 2 3
Control: 7 7 - - -

*Not included in muscle pattern analysis.

appearance of muscle was assessed using cross polaroid filters set to pick up birefringence in myotubes. The limbs were then returned to alcohol and processed for wax histology. Serial transverse 10 μm sections were cut on a rotary microtome and stained either with haematoxylin and eosin or Sirius red, a stain that reveals collagen. Camera-lucida drawings of every tenth section were then made of selected specimens and the tendon and fascial pattern reconstructed.

(2) Electron microscopy

A number of digits were removed from operated and control limbs and fixed in 2.5% glutaraldehyde. The tissue was postfixed in osmium tetroxide, dehydrated and embedded in Araldite. Sections for light microscopy were cut at 1 μm and stained with toluidine blue, and the block was then trimmed and ultrathin sections taken.

Results

General

A total of 30 experimental limbs were produced, 25 of which regenerated 4-digit limbs, two were 3-digit limbs and three were short spikes (Table 1). Seven operated controls all regenerated 4-digit limbs. The detailed structure of the experimental limbs and the normal anatomy derived from the operated controls and five normal limbs are presented below.

(1) Overall appearance of regenerates and skeletal patterns

The great majority of regenerates (25 of 30–83%) had grossly normal appearance with 4 digits. Of the 30 experimental limbs, 11 were stained with Victoria blue to reveal the skeleton to ascertain whether it could form normally in the absence of muscle. Of the 11 cleared specimens, four proved to be muscleless after subsequent sectioning, five had partial muscle patterns, one had a normal musculature, and one was a spike. The ten cases with digits all had essentially normal skeletons (Fig. 3); minor variations in carpal number and position were seen, and two cases had a fused elbow joint (Fig. 3D).

(2) Muscle patterns

Muscle patterns were examined in each of the 27 experimental regenerates with digits and three classes of muscle distribution were evident. Twelve limbs had no muscle distal to the amputation plane; seven had normal muscle patterns and eight had muscle present only in part of the regenerates. The structure of muscleless limbs will be described in detail in the next section, where a comparison is made with the normal limb; however, the structure of the partly muscled limbs is worthy of comment. In six of these eight cases, muscle was present in a restricted position within the forearm in either the extensor (dorsal) or flexor (ventral) compartment (Fig. 6A). When present it was continuous with stump musculature and formed identifiable forearm muscles when sufficient myofibres were present. The muscle in all of these examples petered out at more distal levels and in only one regenerate did it reach the wrist. In all six cases, therefore, no muscle was present in the proximal carpals and hand. The remaining two regenerates showed a different pattern, with small amounts of muscle present only in the flexor (ventral) compartment of the metacarpals. Thus, in

Fig. 3. Skeletal patterns of four regenerates, three of which (B,C,D) were shown to be muscleless upon subsequent histological analysis. A is a normal regenerate from a control operation showing the normal axolotl limb skeleton. Magnification is ×44 in all cases.
these examples, cells of the myogenic lineage may have been carried distally during regeneration and differentiated in the absence of more proximal muscle.

(3) Patterns of tendons, aponeurotic sheets and fascia
In this section, we concentrate on muscleless limbs and describe the normal connective tissue pattern assessed from normal and control limbs only where necessary to clarify these. Throughout the muscleless regenerates the mesodermal limb regions where muscle would normally exist contained blood vessels, peripheral nerves and accumulations of fibroblasts arranged in condensations of various sizes and densities. The nerve pattern will be discussed in detail elsewhere. No tendons were evident at upper or forearm levels; however, fibroblast condensations were present in the forearm although they did not have any obvious pattern from limb to limb excepting the presence of a thin expansion in the flexor (ventral) compartment in the distal third, which corresponds to the beginning of the palmar aponeurosis, a specialization of the tendon of the large digital flexor muscle, palmaris superficialis (ps – Fig. 4; muscles named after Francis, 1934 and Grim & Carlson, 1974). This structure is the first part of a complex arrangement of connective tissues comprising the tendons, aponeurosis and fascia that transduce muscle action from the forearm, carpus and metacarpals into flexion or extension of the digits; the digit tendon complex (Fig. 5A). To be able to describe the appearance of this connective tissue complex in muscleless limbs, it is necessary first to clarify its normal structure.

In the axolotl, extension of the digits is brought about...
by two principal muscles; the large extensor digitorum communis (edc), originating from the humerus, and the smaller distal extensor brevis digitorum (edb – associated with abductor digit for digit I) which arise from the carpals (Fig. 4A,C,D). The edc myofibres end at the midcarpal level and form a set of three tendons, one for each of digits 2, 3 and 4; two lateral tendons insert into the proximal region of the metacarpals and a large medial tendon attaches to the dorsal side of the ebd fascia. The ebd tendons form at the distal metacarpal level where they merge fully with the middle edc tendons.

As mentioned above, the flexor component of the digit tendon complex begins with the formation of the palmar aponeurosis at the beginning of the carpals (Fig. 4A). Palmaris superficialis fibres have run out by the midcarpal and several muscles originate and insert into the aponeurosis within the carpal area. Two muscles, flexor digitorum brevis superficialis (f dbs) and flexor digitorum brevis medi (fd bm), originate from the aponeurosis and insert into the proximal phalanx at the metacarpophalangeal joint (Fig. 5B). With the passage of the ps tendon, which inserts into the distal phalanx, the metacarpophalangeal joint has a particularly complex set of tendon insertions on the flexor side (Figs 4D, 5B). In addition to the muscles mentioned, a number of other muscles are found in the carpal and digit regions that insert into either the palmar aponeurosis or the metacarpals (see Grim & Carlson, 1974). These muscles are not described here because they have very short tendons or fleshy insertions and, for the sake of simplicity, are not considered part of the digit tendon complex.

The digit tendon complex in muscleless limbs
The digit tendon complex was present in each of the 12 muscleless limbs (Fig. 6). In the flexor compartment, it comprised the palmar aponeurosis, the tendons of ps extending to the distal phalanx and the tendons of f dbs and fd bm at the metacarpophalangeal joint. A capsule was also present at each of the digital joints. In the extensor compartment, the long tendons of edc and the shorter tendons of ebd were clearly present. The middle edc tendon (Fig. 5A) of each digit began in the midcarpus and appeared continuous with that of ebd, forming a long tendon running to the distal phalanx, exactly as it does in the normal limb. In the case of both the palmar aponeurosis and the tendons of edc, they begin at the appropriate proximodistal level, forming in ‘mid-air’ with no obvious structure or connective tissue cells located more proximally.

Structure of the tendons in muscleless limbs
In order to ensure that the tendons seen in muscleless limbs at the light microscopic level were of normal structure, thin sections from digits from muscleless limbs and control limbs were examined in the electron microscope. Normal tendons of the ps and ebd muscles comprise stellate fibroblasts in a sea of oriented collagen fibrils, typical of tendons in other vertebrates (Fig. 7A and see for example Chaplin & Greenlee, 1975; Birk & Trelstad, 1986). The same tendons in muscleless limbs have an identical ultrastructure and appeared as well formed in all respects as their normal counterparts (Fig. 7B).

Additional connective tissue condensations
In all of the muscleless limbs, two additional condensations of fibroblasts were present within the carpal region which were not identifiable as part of the digit tendon complex (Fig. 8). They are worthy of note because of their consistent appearance and the possibility that they represent fibroblasts normally constituting the peri-, endo- and epimysia of muscles associated with the complex. The first appears in the flexor compartment of the carpus (Fig. 8B–D). In the normal limb, the flexor compartment contains more muscles than the extensor compartment (Grim & Carlson, 1974) and, in muscleless limbs, the ventral side of the carpals contains more connective tissue cells than the dorsal side. Within this ventral region, a single clear oblique band of fibroblasts was seen emerging from the surface of the ulnare and intermediate carpal elements and joining the dorsal side of the palmar aponeurosis. No single muscle normally lies in this location; however, three muscles, palmaris profundus I, II and III (pp I–III) (Grim & Carlson, 1974; Fig. 8A), originate from the posterior carpal and distal ulna and run obliquely, pp III and II inserting into the dorsal palmar aponeurosis and pp I inserting into the first metacarpal. The oblique connective tissue band, therefore, lies in the overall position of these three muscles and may represent the fibroblasts normally associated with them. The second fibroblast condensation was located dorsal to the anterior carpal, the radiale and prepollicis, in the normal location of abductor minimi I (Grim & Carlson, 1974; Fig. 8A,E).
Fig. 6. The digit tendon complex in muscleless limbs shown through transverse sections stained with haematoxylin and eosin. (A) A partially muscled limb with dorsal muscle masses (arrows) in the extensor compartment which ran out prior to the wrist. (B–F) A sequence of sections of a completely muscleless regenerate at progressively more distal levels. (B) No muscle is evident at the forearm level. However, peripheral nerve fascicles (arrowed in the higher power view of the same section shown in C) are clearly evident in dorsal and ventral positions. (D) In the midcarpus, the palmar aponeurosis is present (arrows). (E) The metacarpal level showing the principal tendons, pst ventrally and edbt dorsally (arrows). (F) The metacarpophalangeal joint showing the tendons of the complex on extensor and flexor sides of the proximal end of the third phalanx of digit 3. The central tendons of fdbs and fdbm are arrowed. Abbreviations as for Figs 4 and 5. Bar, 10 μm.
Discussion

The differentiation of the digit tendon complex in the muscleless axolotl limb regenerates confirms and expands the original observations of Kieny & Chevallier (1979), who described tendon formation in developing muscleless chick wings. In the chick, the tendons are transient primordia, surviving a couple of days, whereas in the axolotl the digit tendon complex survives for at least a year. In either case, however, the formation of tendons in the absence of muscle indicates the piece-meal manner in which limb patterns form; parts of the pattern develop independently and then connect together. The organization of connective tissues into recognizable patterns in muscleless limb regenerates adds to the growing body of evidence implicating the fibroblast as a cell type of major importance in the patterning process. Evidence is strong that in the chick wing muscle patterns are determined by somatopleurally derived connective tissue (Chevallier et al. 1977) and, although such a role for fibroblasts has not been demonstrated directly in the axolotl limb, these cells have been implicated in pattern regulation in a number of ways including positional mismatch and cell contribution experiments (see Introduction).

It is intriguing that only the tendons and aponeurosis of the digit tendon complex formed and not tendons within the forearm. This result is exactly that described by Kieny & Chevallier (1979) in the chick, although these authors did not place any significance in their observation that the only tendons to form were those of the digit tendon complex (refer to Fig. 1B,C where the tendons formed in the Kieny and Chevallier experiments are indicated). The similar results in the comparable experiments in chick and axolotl indicate that not all tendons may be formed in the same way; the long and complex tendons, aponeurotic sheets and retinaculae typical of digit tendon complexes may be considered as a separate and identifiable part of the limb pattern in terms of the information necessary to build them during development and the cellular mechanisms involved in morphogenesis.

A unique feature of the results is the appearance of connective tissue bands which may represent the fibroblasts normally associated with particular muscles (Fig. 8). Like the tendons, these do not form at forearm levels and, in the case of the ventral oblique band, approximates the position of a group of three muscles, those of the palmaris profundus set; however, they were present in each limb examined, suggesting that they are examples of sets of fibroblasts fated to package myoblasts into recognizable muscles during normal regeneration. Their presence is, therefore, consistent with a pattern-organizational role for these cells. Such bands were not seen in muscleless chick wings (cf. Kieny & Chevallier, 1979).

The present experiments provide strong evidence for a controlling role of fibroblasts in pattern regulation because these cells, as the major component of the dermis, give rise to the whole limb regenerate. In those limbs that are completely muscleless, the skeleton forms essentially normally as well as the digit tendon complex. This result extends the initial findings of Dunis & Namenworth (1977) and Lheureux (1983) who first created muscleless limbs using non-irradiated dermis as a sole source of cells for regeneration although these authors were concerned with metaplasia rather than the pattern per se and did not discuss the overall organization of the regenerates. It is interesting to note that the success rate for producing muscleless limbs (40% - Table 1) is approximately that achieved in these other studies. It is unclear why partial or completely muscled limbs should regenerate. In previous studies (for example Maden, 1979; Holder et al. 1979), 2500 rads have been shown to prevent limb regeneration; but the conditions may be different if a population of non-irradiated cells is present at the stump. It is interesting to note that in partially muscled regenerates, in the majority of cases, muscle was found proximally in the regenerate and was continuous with the stump musculature in the appropriate flexor or extensor compartment. This arrangement suggests that a subpopulation...
of cells from the muscle lineage escaped the immediate effects of X-irradiation (which damages DNA – Maden & Wallace, 1976) but could only undergo a limited number of cell divisions. In two cases, myotubes were seen in distal limb regions in the absence of muscle at more proximal levels of the regenerate. The origin of these rare cells is unclear but their existence raises the possibility that small numbers of myoblasts exist in distal locations in other, apparently muscleless, regenerates. The detection of a low number of myoblasts at a density too low to promote fusion into myotubes is beyond the resolution of the histological methods used in this paper and would require a myoblast-specific cell marker. We cannot completely rule out the possibility, therefore, that the presence of unfused myoblasts may influence the organization of connective tissue patterns in some way.

Finally, the organization of tendons and fascial sheets in the absence of external mechanical influences normally provided by muscle contraction highlights the
self-organizational capacities of connective tissue cells. The normal appearance of organized collagen fibrils and the normal stellate appearance of tendon fibrils in the ps tendon of muscleless limbs (Fig. 7) is reminiscent of the behaviour of fibroblasts in culture, where fibroblast traction models collagen matrices into organized arrays (Harris et al. 1981; Stopak & Harris, 1982). The results support the notion that the organization of collagen matrices within the tendons and fascial sheets of the digit tendon complex is a property of the fibroblasts that generate that matrix. This view is entirely consistent with the conclusions of Stopak et al. (1985) who used labelled injected collagen to assess the principal factors affecting its morphogenesis in chick limbs.

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References


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