Myoblasts and notochord influence the orientation of somitic myoblasts from *Xenopus laevis*

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SUMMARY

The orientation of developing myoblasts extending a bipolar axis in the presence of explanted myoblasts or whole notochord has been studied *in vitro*. Myoblasts tended to elongate perpendicular to the lines of diffusion of substances from these tissues. A slow-release source of agar impregnated with medium conditioned by segmented somitic myoblast or notochord also caused myoblasts to elongate perpendicular to the lines of diffusion from the source. Medium conditioned by neural tube cells or unsegmented mesoderm cells did not influence the orientation of myoblasts.

It is concluded that somites and notochord release diffusible substances *in vitro* which are capable of directing the orientation of developing myoblasts. *In vivo*, a somite-derived material could play a role in determining the direction of myoblast elongation in the presomitic mesoderm. An interaction between somite and notochord-derived secretions could influence the rotation of presomitic myoblasts to form a segmented somite.

INTRODUCTION

To form a somite, the presomitic myoblasts of *Xenopus laevis* first elongate perpendicular to the neural tube and notochord, before a fixed number rotate *en bloc* through 90° to lie parallel to these structures (Hamilton, 1969; Cooke & Zeeman, 1976). The mechanisms controlling the direction of cell elongation and cell rotation are unknown. Somites in *Xenopus* thus contain a two-dimensional stack of elongated myoblasts with the long axis of each cell extending from the rostral to the caudal end of the somite. In chick embryos, where the cells of the somite form into a rosette rather than a stacked array, apical somites may induce the formation of the next somite to arise caudally (Lanot, 1971). There is evidence also that the notochord produces a diffusible substance which can induce somite formation (Nicolet, 1971; Hornbruch, Summerbell & Wolpert, 1979).

The role of notochord and apical somites in the formation of new somites in *Xenopus* has not been investigated. Diffusible substances from these sources, conceivably could be involved in determining the direction of myoblast elongation and subsequent rotation.

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I have cultured *Xenopus* myoblasts in the presence of a unilateral source of notochord or somitic myoblasts and of media conditioned by these tissues. Myoblasts tended to elongate perpendicular to the lines of diffusion from these sources.

**MATERIALS AND METHODS**

Embryos of *Xenopus laevis* at stages 19/20 or stages 23/24 were used (Nieuwkoop & Faber, 1956). The method of culturing myoblasts was essentially that reported previously (Hinkle, McCaig & Robinson, 1981) and is modified from Jones & Elsdale (1963). The dorsal third of an embryo was excised in Steinberg's solution (composition mmol^{-1}: NaCl, 58; KCl, 0.67; Ca(NO_3)_2, 0.44; MgSO_4, 1.3; Tris, 4.6, pH 7.9) and transferred to a Steinberg's solution containing 1 mg ml^{-1} of collagenase (Type 1; Sigma Ltd) for 10–15 min. This facilitated dissection of specific somites or other tissues which in most cases were transferred to a Ca^{2+}/Mg^{2+}-free Steinberg's solution containing 0.4 mM EDTA (Sigma) for 20–30 min. The dissociated tissues then were drawn up through a fine, flame-drawn Pasteur pipette and dispersed into localized areas of culture medium in a plastic tissue culture dish (Falcon: Type 3003F). Culture medium was Steinberg's solution supplemented with 20% L15 Liebowitz solution, 1% foetal bovine serum and 2% penicillin 5000 i.u.ml^{-1}/streptomycin 5000 µg ml^{-1} (all from Flow Laboratories, Irvine) and used at pH 7.9.

This solution lay in the trough formed by two strips of No. 1 cover glass (64×10 mm) which were glued to the dish with silicone rubber; 1 cm apart. Two types of experiment were performed as outlined in Fig. 1. (A) A mass of myoblasts or a whole (undissociated) notochord was plated to form a longitudinal column of tissue and allowed to grow for 1 h. A column of myoblasts then was dispersed 1–2 mm away from the explanted tissues. (B) In other experiments, one half of each culture chamber was filled with a thin layer of 1.1% agar, impregnated with media which had supported the growth of myoblasts or notochord cells. This constituted a slow-release, unilateral source of any substances released by these cells (see below). Culture medium was plated into the other half of the chamber and contacted the agar slab. One hour later, myoblasts from stage 19/20 embryos were dispersed in a column about 1 mm away from the agar–culture medium interface. In all cases a roof of No. 1 cover glass was applied with silicone grease, making the final chamber dimensions 64×10×0.5 mm and the volume around 300 µl. Spherical myoblasts were left to develop for 18–20 h before the orientation of their elongation was assessed. Bipolar myoblasts were counted as elongating at an angle less than or greater than 45° to the horizontal, long axis of the chamber and thus to the lines of diffusion from the test sources. Only myoblasts develop from cultures of somites at these stages and these are identified by their bipolar axis and central phase-bright yolk granules.

**Preparation of conditioned media (CM)**

All CM were prepared from tissues of embryos at stage 19/20. The following tissues were excised and cultured in individual chambers containing 1.5–2 ml of culture medium for 20 h. (a) Somitic myoblasts (SMCM) – somite pairs 2–5 from two embryos (dissociated); (b) Notochord (NotoCM) – two complete notochords (dissociated or whole explants); (c) Neural tube (NTCM) – two complete neural tubes (dissociated); (d) Unsegmented mesoderm (UMCM) – two caudal slabs of unsegmented mesoderm from two embryos (dissociated).

For each experiment medium from five culture chambers was pooled, filtered (0.2 µm mesh; Nalgene) and added in equal volume to a 2-2% agar solution in Steinberg's or culture medium at 38°C. The agar was plated out as a thin layer (1 mm) in one half of a culture chamber (Fig. 1).

Slow release from the 1.1% agar slabs was checked by adding a few drops of India ink to a test CM and preparing agar slabs with this. After 1 h a front of ink had extended about 1 mm into the culture medium. After 17 h, this had advanced 9–10 mm from the agar–culture medium interface and the intensity of dye diminished with distance from the agar slab. Gradients of diffusible colloidal substances therefore are set up within the time course of these experiments and myoblasts plated about 1 mm from the agar–culture fluid interface will differentiate and
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somites, notochord and myoblast orientation

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Myoblast orientation assessed Tissue pieces. Myoblasts or notochord

Fig. 1. Design of experiments. (A) Strips of myoblast or of notochord are preplated as outlined in Materials and Methods. (B) A slow-release agar source of CM fills half of the chamber.

Myoblasts elongate while exposed to such gradients. Myoblast elongation begins 1–3 h after plating. A similar experimental design has shown that an agar source of β-nerve growth factor establishes a gradient within 24 h such that only 10% of the source concentration is present at a distance of 10 mm (Letourneau, 1978).

RESULTS

(A) Myoblast orientation in control cultures

The direction of bipolar elongation of myoblasts from spherical myoballs was assessed in cultures of somites 6 and 7 from stage 19/20 embryos. Fig. 2 shows that as many cells elongated at less than 45° to the horizontal as elongated at greater than 45° to the horizontal. Myoblast orientation was random.

(B) The influence of pre-plated myoblasts and notochord on myoblast orientation

Myoblasts from somites 2–7 of stage-22 embryos were dissociated and plated as a broad column across a chamber (Fig. 1). In other experiments, the whole notochord was explanted and similarly aligned. One hour later the rostral, unsegmented myotomal mesoderm was dispersed in a parallel column, 1–2 mm away. (Such cells are in the process of elongating and segmenting in vivo.) The orientation of these myoblasts as they elongated was assessed relative to the horizontal axis of the chamber. This is also the vector of diffusion from the tissue sources or agar slabs. Myoblasts tended to elongate perpendicular to this vector with 66% and 72% of cells developing a bipolar axis at more than 45° to the lines of diffusion from myoblasts or notochord masses respectively (Figs 2,3,4).

(C) The effect of CM on myoblast orientation

Appearance of cultures used to condition culture medium

(a) Segmental myoblasts. Myoblasts differentiated well into bipolar muscle cells in vitro and 3000–5000 grew per ml of culture fluid.
(b) Notochord. Dissociated notochord cells did not spread well on tissue culture plastic. Only about 30 vacuolated cells per dish had spread and continued to differentiate. The others remained as spherical cells, some adhering to the plastic substrate, others only loosely attached.

Whole explants of notochord usually adhered firmly to the dish, since cells at several points migrated out from the explant and spread onto the plastic.

(c) Neural tube. Although about 3000 cells per ml attached to the plastic surface, only about 10% differentiate by sending out neurites (305 ± 30: n = 4).

(d) Unsegmented mesoderm. If the most rostral area of somite forming myoblasts is removed most of the dissociated cells attach to the plastic substrate but remain as undifferentiated, spherical myoballs throughout the 20 h culture period.

(i) SMCM. This was tested on three different categories of myoblast: (1) segmenting myoblasts, those in the process of elongating and rotating to form a somite i.e. the most rostral area of unsegmented mesoderm, (2) newly segmented, uninnervated myoblasts; from somites 6 and 7 of stage 19/20 embryos and (3) denervated myoblasts; dissociated from somites 1 and 2 of an older embryo, stage

Fig. 2. Direction of elongation of myoblasts, source shown, in control cultures and relative to (1) a preplated (1 h) mass of myoblasts grown from somites 2–6, stage 22, and (2) a whole notochord. Open columns – elongation at >45° to horizontal. Hatched columns – elongation at <45° to horizontal.
Fig. 3. Myoblasts from segmenting mesoderm tend to elongate parallel to a preplated mass of myoblasts, out of picture to the left. Scale bar, 200 μm.

Fig. 4. Myoblasts from segmenting mesoderm tend to elongate parallel to an explanted whole notochord, out of picture to left. Scale bar, 200 μm.

Fig. 5. Direction of elongation of myoblasts source shown relative to a source of SMCM or of NotoCM (stage 19). Open columns – elongation at >45° to the horizontal, long axis of the chambers. Hatched columns – elongation at <45° to the horizontal.

24, in which around 9 of the 15 somite pairs have become innervated. Figs 5 and 6 show that in each of the above cases, myoblasts tended to elongate perpendicular to a unilateral source of SMCM, with roughly two thirds of cells developing a bipolar axis at more than 45° to the horizontal.
Even when all serum-derived proteins were removed from the conditioning medium, to eliminate their non-specific involvement in this response, myoblasts still tended to elongate perpendicular to the agar source of SMCM (Fig. 5).

(ii) *NotoCM*. The effect of agar slabs impregnated with NotoCM on myoblast orientation also was tested. Myoblasts from somites 6 and 7 tended to develop their axis of elongation perpendicular to the lines of diffusion from the agar slab (Figs 5, 7).

(iii) *UMCM, NTCM and nerve growth factor (NGF)*. A source of UMCM or NTCM and agar slabs impregnated with 5000 ng ml⁻¹ of 7S-NGF did not affect the direction of myoblast elongation. In each chamber myoblasts from the most recently formed somites, 6 and 7 stage 19/20, were dispersed about 1 mm from the agar source to be tested. The same number of myoblasts developed a bipolar axis at less than or greater than 45° to the horizontal axis of the culture chamber. Cell orientation was random (Fig. 8). This indicates that neither NGF nor any substances released by these cell types, nor some feature of the design of the experiment could induce order in the orientation of developing myoblasts.

These data are summarized in Table 1 which gives a measure of the moderate degree of order imposed on myoblasts by myoblasts and notochord-derived substances.
DISCUSSION

This study shows that substances released from myoblasts and from notochord cells can influence the initial axis of development of myoblasts and thus provides evidence for a chemically induced orienting response of myoblasts.

Several cell types including newborn rat skeletal muscle release NGF (Murphy et al. 1977). Developing myoblasts in *Xenopus* also may release NGF. However, 7S-NGF at a concentration that orients nerve (Letourneau, 1978) did not affect myoblast orientation and therefore is unlikely to be the active orienting agent released by myoblasts or notochord cells.

The nature of the myoblast-orienting substance released by myoblasts and notochord is not known although specific proteins or peptides are likely candidates. A gradient of the protein nerve growth factor can orient nerve growth (Gundersen & Barrett, 1980), while oriented cell shape changes and directional migration of leucocytes, for example, are induced by chemoattractant peptides released by bacteria (e.g. Schiffman, 1982).

*Xenopus* myoblasts also orient in response to a small applied electric field; their bipolar axis develops perpendicular to the lines of force of the electric field (Hinkle et al. 1981). [It was shown in that study that once myoblasts elongate *in vitro*, they remain fixed firmly to the substrate and do not rotate.] Furthermore, endogenous electric fields do exist at these stages; current is driven across the skin, through the internal tissues and exits at the blastopore (Robinson & Stump, 1984). Together, these observations of chemically and electrically-mediated myoblast orientation may play a role in determining the initial orientation of presomitic myoblasts in *Xenopus laevis* and their rotation through 90° to form a somite.

Fig. 9 shows schematically the anatomical arrangement of somitic and presomitic myoblasts and notochord. A myoblast-orienting substance released by
newly formed somites and diffusing into the presomitic mesoderm would be expected to cause cells to elongate perpendicular to the long axis. That is in fact the observed direction of initial elongation. A notochord-derived orienting substance which would be expected to cause myoblasts to align parallel to the notochord could instigate myoblast rotation to form a somite by competing with a somite-derived orienting factor (Fig. 9).

Both presomitic and somitic myoblasts lie perpendicular to the expected lines of skin current flow through subjacent tissues. Thus the anatomical organization of these tissues is consistent with their morphology having been determined both by the existing endogenous electric fields and by the release of myoblast and notochord-derived orienting substances.

There is no evidence that any of these influences on myoblast orientation in vitro play a role in the organization of tissues in vivo. Moreover, most vertebrate species have somites formed by cells arranging themselves in a rosette pattern around a central lumen. The role played by any cell-orienting influence from preformed

<table>
<thead>
<tr>
<th>Origin of myoblasts</th>
<th>Test condition</th>
<th>Number of cultures</th>
<th>D ( \frac{n &gt; 45^\circ - n &lt; 45^\circ}{n &gt; 45^\circ + n &lt; 45^\circ} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presomitic elongating</td>
<td>SOM 2–7: Stage 22</td>
<td>7</td>
<td>0.33</td>
</tr>
<tr>
<td>Presomitic elongating</td>
<td>SMCM</td>
<td>12</td>
<td>0.25</td>
</tr>
<tr>
<td>Newly formed som. 6+7 Stage 19</td>
<td>SMCM</td>
<td>14</td>
<td>0.24</td>
</tr>
<tr>
<td>Denervated som. 1+2 Stage 24</td>
<td>SMCM</td>
<td>2</td>
<td>0.31</td>
</tr>
<tr>
<td>Newly formed Stage 19</td>
<td>SMCM (−serum)</td>
<td>4</td>
<td>0.28</td>
</tr>
<tr>
<td>Presomitic elongating</td>
<td>Notochord: whole explant</td>
<td>8</td>
<td>0.43</td>
</tr>
<tr>
<td>Newly formed Stage 19</td>
<td>NotoCM</td>
<td>14</td>
<td>0.19</td>
</tr>
<tr>
<td>Newly formed Stage 19</td>
<td>NTCM</td>
<td>4</td>
<td>0.02</td>
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<tr>
<td>Newly formed Stage 19</td>
<td>UMCM</td>
<td>8</td>
<td>0.05</td>
</tr>
<tr>
<td>Newly formed Stage 19</td>
<td>7S-NGF</td>
<td>7</td>
<td>0.06</td>
</tr>
<tr>
<td>Som. 6+7 Stage 19</td>
<td>None Control</td>
<td>4</td>
<td>0.01</td>
</tr>
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The degree of orientation is represented by the ratio D. If all myoblasts aligned at >45° to the horizontal long axis of the chamber, D would be 1. If the same number of myoblasts orient at >45° and at <45° to the horizontal, D would equal 0.

A ratio of 0.2–0.4 is obtained when 60–70% of cells align at >45° to the horizontal.
somites, notochord or endogenous electric current in these cases would have to be more complex.

Other evidence impinges on such a scheme. Segmentation occurs normally in the posterior half of *Xenopus* embryos bisected before or as the first somites are forming (Deuchar & Burgess, 1967; Pearson & Elsdale, 1979). Thus segmentation occurs autonomously without the influence of a signal from preformed somites. But what of cell orientation? Myoblast orientation in the first autonomously forming somite in a posterior half has not been described. In the absence of already formed anterior somites, these cells may not elongate perpendicular to the notochord. Myoblast elongation in all but the first somite could be influenced by each autonomously segmented somite. The final orientation of myoblasts parallel to the long axis could then arise under the influence of the notochord.

In u.v.-irradiated *Xenopus* larvae with no notochord, somite pairs often fuse across the midline below the neural tube (Youn & Malacinski, 1981). If the notochord played a role in the 90° rotation of myoblasts one would expect its loss

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**Fig. 9.** (A) Schematic horizontal, longitudinal section through the notochord and somites of *X. laevis*. The myoblasts of preformed somites lie perpendicular to the lines of diffusion of putative substances from the notochord (arrows). Segmenting myoblasts lie perpendicular to the lines of diffusion of putative substances from the already formed somites (arrowheads). If notochord and somite-derived myoblast-orienting substances co-exist *in vivo*, then a competition between them to determine myoblast orientation might be responsible in part for initiating myoblast rotation. The direction of the rotation that occurs *in vivo* is shown. A notochord-derived factor may have to be more active and active slightly later at the same rostrocaudal level than the somite-derived factor to bring about a full rotation, since myoblasts in a newly formed somite then lie in register with those of more mature somites. (B) The myoblasts of already formed somites and of segmenting somites lie perpendicular to the lines of current flow through the skin and into the internal tissues of the embryo (see Robinson & Stump, 1984).
to disrupt severely this process. Disruption did occur particularly in the central fused region of the somite although some of the more dorsal and ventral cells still rotated through 90° (Youn & Malacinski, 1981). This finding suggests that the notochord can not be the only determinant of myoblast rotation.

Possible mechanisms of cell orientation

Both an applied electric field and a chemical gradient cause myoblasts to orient perpendicular to the stimulus vector. Perhaps related mechanisms are involved. In the case of an electric field, the anodal-facing membrane of a spherical myoblast hyperpolarizes while the cathodal-facing membrane depolarizes (Jaffe & Nuccitelli, 1977). These events may lead to local increases in Ca$^{2+}$ influx, which by locally activating the contractile apparatus would limit cell spreading in lateral directions and lead to a perpendicular orientation of the cell (Cooper & Keller, 1984). Calcium is important also in chemotaxis. Local changes in cytoplasmic Ca$^{2+}$ and cell contractility have been suggested to impart both a change in cell shape and the vector component to granulocyte chemotaxis (Gallin & Rosenthal, 1974). Increases in membrane permeability to Na$^+$ and in the exchangeable pool of cytoplasmic Ca$^{2+}$ have been observed in leukocytes exposed to a chemoattractant peptide (Naccache, Showell, Becker & Sha’afi, 1977).

An asymmetric distribution of both the cytoskeleton and of integral membrane proteins has been observed in several cell types in response to an applied electric field (Luther, Peng & Lin, 1983; Poo & Robinson, 1977; Poo, 1981); events which may induce perpendicular alignment. It remains to be investigated whether myoblast or notochord-derived substances influence cell orientation by mechanisms involving any of the above.

The significance of these observations could extend beyond a possible role in somite organization. Adult skeletal muscle when minced up can regenerate into an organized functional unit (Carlson, 1968). If adult muscle cells also secrete a substance which can act to orient other muscle cells, similar to that released by innervated and uninnervated embryonic myoblasts, this could play a role in re-establishing order in regenerating muscle.

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REFERENCES


Somites, notochord and myoblast orientation


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