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BEHAVIOUR AND MOTILITY OF CULTURED CHICK MESODERM CELLS IN STEADY ELECTRICAL FIELDS

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SUMMARY

The behaviour and motility of dissociated and freshly dissected mesoderm from early chick embryos placed in culture under electrical stimulation from extracellular fields was studied in a variety of conditions. It was established that it is not possible to set up a voltage gradient steep enough for individual cells to respond in a polar way to physiological levels of voltage drop. In addition, a gradient of adhesiveness of the substrate can be set up in the absence of cells, to which cells can respond by a different rate of spreading at opposite ends of the 3 cm long field. The observations are discussed in relation to the migration of mesoderm out of the primitive streak and to the possible roles of extracellular materials and extracellular current pathways in the early embryo.

During the process of gastrulation in the early chick embryo, the upper layer (or epiblast) invaginates to form the primitive streak, which is the focus for the formation of the middle layer (or mesoderm). This tissue migrates out of the primitive streak at right angles to its main axis, and moves towards the lateral margins of the embryo [1]. The controls of this orderly migration of the mesoderm out of the primitive streak are as yet unknown, although many hypotheses have been suggested.

In an earlier study [2] we have shown the existence of extracellular electrical current pathways which leave the primitive streak region dorsally in all directions, and it was suggested that these currents were caused by a pump operating in the epiblast, accumulating ions, probably sodium and water, in the cavity under this tissue. It was inferred that these currents return to the epiblast via the primitive streak in central areas of the blastoderm. Thus the direction of current flow dorsal to the epiblast was opposite to the direction of migration of this tissue, whereas flow around the mesoderm was opposite to the direction of its migration under the epiblast.

It was therefore tempting to connect these two phenomena causally. The present study was designed to investigate the possible types of responses which mesoderm cells could show in reaction to physiological levels of applied extracellular current fields either by means of direct effects on the cells themselves (electrophoresis or galvanotaxis), or via effects on the substrate on which the cells are moving (electrophoresis plus haptotaxis). The mesoderm in situ migrates on the basal lamina underlying the epiblast. The basal lamina is composed of various glycoproteins and associated fibronectin [3–5]. Effects of electrical fields on these substrates could be

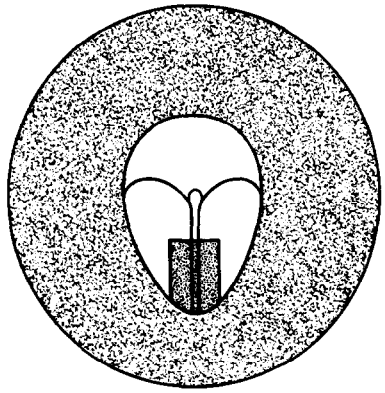

Fig. 1. Schematic diagram of a stage 4 blastoderm showing the area from which mesoderm was dissected.

envisaged as acting by electrophoresis or differential adsorption of adhesive molecules such as fibronectin onto the substrate, creating a gradient of substrate adhesiveness along which the cells could move [3, 6–8].

These and other hypotheses were tested in the present investigation, using an in vitro model of the migrating mesoderm cells on various types of substrates in a variety of experimental conditions, under applied steady extracellular current fields.

MATERIALS AND METHODS

Dissociation of cells

Lateral plate mesoderm pieces were dissected from chick embryos between Hamburger & Hamilton [9] stages 3 and 4 using fine tungsten needles sharpened in molten sodium nitrite. Dissections were carried out with the embryos submerged in Tyrodes' solution; only mesoderm from the primitive streak and adjacent areas in the posterior half or so of the streak was used (fig. 1). The dissected pieces were collected in siliconised glass centrifuge tubes containing ice cold Tyrodes' solution. Each sample contained mesoderm pieces pooled from at least twelve blastoderms. The Tyrodes' was then withdrawn from the tubes as far as possible, and the pieces placed into 5 ml Ca^{2+}-, Mg^{2+}-free Tyrodes' solution (CMF), and incubated in an agitating water bath at 37°C for 45 min, during which period the pieces were pipetted up and down through a flamed siliconised Pasteur pipette at the beginning, middle and end of the incubation period. The samples were spun in a bench centrifuge for 5 min at 1500 rpm, and the pellet resuspended in 2 ml CMF. The samples were subsequently recentrifuged for 5 min at 1500 rpm, the CMF removed and the pellets resuspended in 0.5 or 1 ml medium.

Media

For most experiments, a medium containing 9 ml 199 (Wellcome): 1 ml fetal calf serum (FCS) (Gibco): 0.5 ml penicillin (5000 IU/ml stock) and streptomycin (500 μg/ml stock) (Gibco). Experiments with serum-free medium were done using 10 ml 199 : 0.5 ml penicillin and streptomycin stock. These media were sterilized by filtration through a Millipore type GS (0.22 μm) filter.

Experiments with higher viscosity medium were done using the above medium containing 0.3, 0.5 or 0.6% w/v Methocel (methylcellulose), which had been autoclaved dry before adding the medium aseptically. The methylcellulose was allowed to dissolve overnight under constant stirring at 4°C. In these cases, the final pellet of dissociated cells was resuspended directly in the higher viscosity medium.

Substrates

For most experiments, glass coverslips 32×32 mm (No. 1, Chance Propper Ltd) were used, after having been cleaned by boiling, sulphuric acid treatment and alcohol rinsing before being allowed to dry.

Collagen was obtained from six rat tails, extracted in acetic acid, centrifuged for 2 h at 2300 rpm and dialysed for 12 h at 4°C using Visking dialysis tubing (Visking 2-18/32). The dialysate was then spread on clean glass coverslips with a glass rod and allowed to gel for 5–10 sec while exposed to ammonia vapour to increase the pH. The coverslips were then rinsed thoroughly in distilled water and allowed to soak overnight in the medium to be used.

Basal laminas from *Xenopus laevis* tadpole tail fins (stage 49–52) were prepared by alcohol fixation followed by trypsin digestion at room temperature as described by Overton [10]. Basal laminas from the under-surface of the epiblast of the chick were prepared by 10–15 min Triton-X100 (1%) treatment of the exposed ventral surface of unfixed Eyal-Giladi & Kochav [11] stage XIV to Hamburger & Hamilton stage 3 epiblast from which the endodermal and mesodermal (if any) layers had been dissected manually. These basal laminas were held firmly against the base coverslip of the experimental chambers by means of small pieces of coverslip glass.

Stimulation

All stimulation experiments were carried out in the electrophoresis chamber shown in fig. 2, provided with two bridges of 2% agar in Tyrodes' solution containing two wide-surfaced foil or Ag/AgCl electrodes (Clarke Electromedical Co.). The agar bridges were 3 cm wide, 3 cm long and 1 mm deep, and the distance between the two bridges was about 3 cm. Currents of between 100 μA (10 $mV \cdot cm^{-1}$) and 40 mA

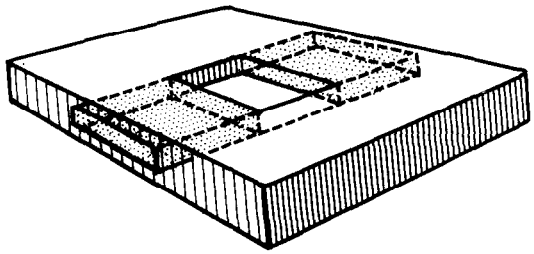

Fig. 2. Diagrammatic representation of the electrophoresis chambers used in the present investigation. The stippled areas represent the agar bridges. The central cavity was fitted with coverslips to enclose the medium containing the cells.

(4 $V \cdot cm^{-1}$) were delivered via a simple feedback-operated current clamp device driven by a type 741 operational amplifier. The resistivity of all media used was of the order of 100 $\Omega \cdot cm$. The experimental set-up just described proved to be a good source, the resistance of the system being stable over the range of currents used for periods well exceeding 18 h of current flow and with minimal if any signs of electrolysis. The long distance between the electrodes (some 10 cm) was intended to prevent electrode products from affecting the response of the cells in the chamber.

The setup described, when assembled, was calculated to provide an essentially uniform field within the central $\frac{1}{3}$ (1 cm) of the width of the chamber. This was confirmed by plotting the direction of electrophoretically-induced cell translocation: cells within this 3×1 cm band moved parallel to the direction of the field and to each other.

For experiments stimulating the substrate-attached materials, the entire setup was operated without cells, but with serum-containing medium for periods from 10 min to 5 h, after which period the chambers were washed thoroughly by flushing with serum-free medium with the current still on; the stimulation was then switched off and the cells added onto the system. Except in experiments where the stability of the gradients was being tested, no current was delivered after addition of the cells.

Filming

Most experiments were filmed using a Bolex cine camera with Wild Variotimer time-lapse attachments fitted to a Zeiss Standard WL microscope with Nomarski optics using 6.3× or 16× objectives, on Ilford 16 mm Pan-F Type 752 film. The microscope was fitted with a temperature-maintaining Perspex chamber which was kept at 37°C or room temperature (22–26°C) in control experiments.

Sign convention

According to the accepted conventions, current is represented as flowing from the positive pole (anode) to the negative pole (cathode) of a field. Electrophoretic mobility is usually given a conventional sign indicating the direction of movement in the field; a negative sign preceding a mobility value implies a net negative charge for the moving particle, which will therefore move towards the positive pole (anode).

Analysis of results

Direction of cell movement. In general, this was obvious from direct observation of the time-lapse films. In a few cases, however, the direction of movement of cells with respect to the field orientation was assessed as follows: the area around a given cell or group was divided into four quadrants upon projection of the film, which were aligned parallel to the field direction; a given number of frames (about 50–100) were then advanced and the new position of the cell plotted once more. Cells were asigned a + value if they moved towards the anode, a − value if they moved towards the cathode, and 0 if they moved normal to the axis of the field.

Assessment of spreading. The measurements were only made within the central $\frac{1}{3}$ (1 cm) of the width of the experimental chambers, where the field is most uniform. This band 3 cm long by 1 cm wide was divided into three equal portions 1×1 cm and the percentage of spread cells counted within each of these squares. Attachment in unspread cells on substrates

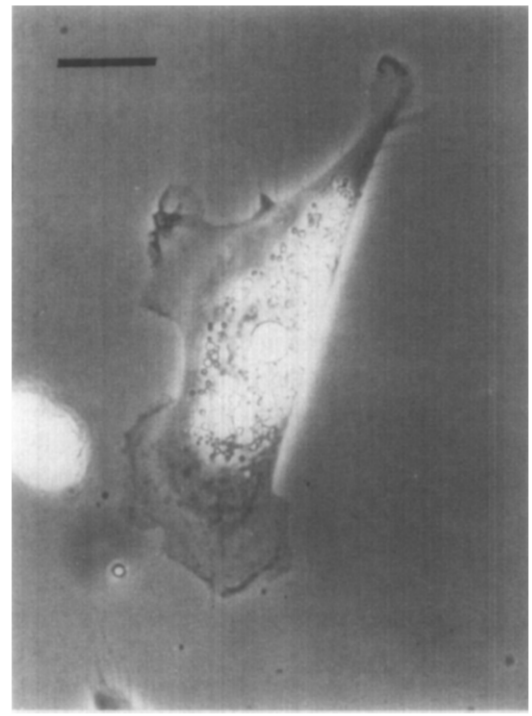

Fig. 3. Spread mesoderm cell on a glass substrate. Bar, 20 μm.

Table 1. *The figures represent the percentage of spread cells (attached but unspread cells in brackets) in three different regions of various substrates which had been stimulated in the presence of serum as described in the text*

n, No. of cells scored (no. of separate experiments in brackets). Attachment of unspread cells was assessed by inverting the substrate after counting the total number of cells in each region, and then counting the number which remained in position

Experiment	Anode	Centre	Cathode	*n*
Serum-coated glass	81	31	0	527 (3)
Collagen	10	2	0	168 (2)
Tadpole tail B. L.	(92)	(87)	(50)	341 (3)
Chick blastoderm B. L.	(77)	(70)	(43)	115 (1)

other than glass was assessed by inverting the substrate briefly after counting the total number of cells in the given position and then counting the number of cells remaining. When tadpole tail or chick blastoderm basal laminas were being used, three of these were set up in each chamber, each within one of the 1×1 cm marked squares as described above.

RESULTS

Attachment and spreading on the substrate

In serum-containing medium, dissociated mesoderm cells tended to attach relatively slowly, but most cells had usually spread on glass substrates by 10 h incubation. These spread cells tended to be fibroblast-like in morphology, with ruffled membrane activity, lamellipodia and other cytoplasmic extensions, and very motile (fig. 3). Cells which remained unspread seemed to form transient adhesions to the glass substrate, with abundant blebbing and some adhesion of filopodia. On collagen-coated glass and tadpole tail or chick blastoderm basal laminas, cells tended to attach more rapidly and more strongly than to glass, but they did not usually spread on these substrates. Cells which had attached to these substrates were motile and filopodia were sometimes visible, and they tended to bleb almost constantly. Ruffling activity and lamellipodia were not apparent on basal lamina, and only occasionally on collagen-coated glass.

In serum-free medium, dissociated cells tended to attach to the glass very rapidly and very strongly, but they did not spread as readily as with serum-containing medium (see [12]). In higher viscosity media, cells tended to attach and spread as on glass, but ruffling was absent and the cells were less motile than in ordinary medium.

Freshly dissected pieces of mesoderm explanted on glass substrates usually spread well, but as more or less single cells which were very motile. If the medium contained serum, the explants were more epithelial, but the frequency of spreading was lower than when serum-free medium was used [12]. The more motile cells showed clear contact-induced spreading in both dissociated cells and explants but the resulting groups of epithelioid spread cells were transient and tended to break up. Spreading of these pieces on all substrates occurred radially from the centre of the explant and was not usually asymmetric along any one axis. Thus, the spreading of the piece appeared to reflect the sum of all the local "flattenings" (i.e., spreading of its individual cells) on the substrate and not any active process such as cell locomotion.

Electrophoretic mobility of cells

This was determined from time-lapse films, averaging a minimum of 50 cells in at least

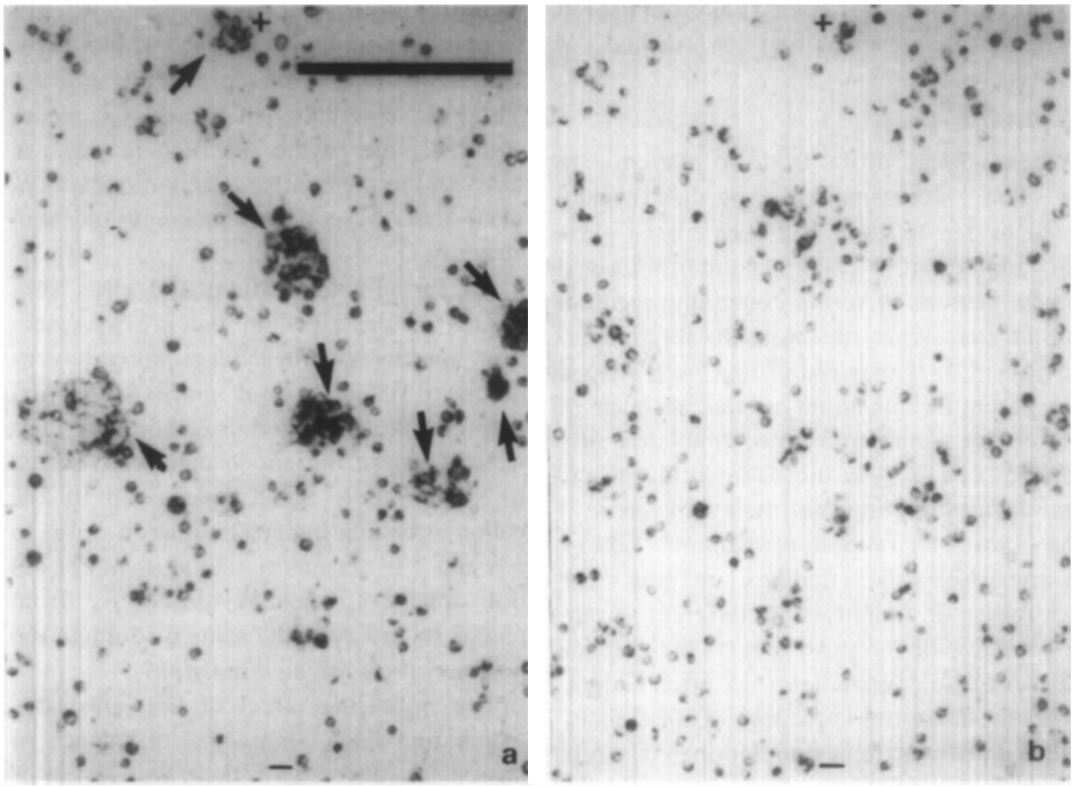

Fig. 4. Dissociated cells after spreading for 5 h on a glass coverslip while a field of 100 $mV \cdot cm^{-1}$ was applied to the chamber. (*a*) Cells 1.0 cm from the centre of the field towards the anode. Note spread groups of cells (arrows). (*b*) The same coverslip in an area 1.0 cm from the centre of the field towards the cathode. Note the absence of spread cells. Bar, 250 μm.

two separate experiments. At pH 7.2–7.8, the dissociated cells showed an electrophoretic mobility of -2.0 μm/sec/V/cm. In 0.3% Methocel, mobility was of the order of -8 μm/min/V/cm. At viscosities greater than that of 0.5% Methocel no mobility was observed. Mobility in serum-free medium of normal viscosity was of the order of -2.5 μm/sec/V/cm.

These electrophoretic mobilities were determined using relatively low levels of voltage drop (from 500 mV/cm^{-1} to 1 V/cm^{-1}). The mobility of the cells in these experiments was not altered at room temperature, which is a strong indication that it was solely due to electrophoresis and not to any active movement of the cells.

Active responses to the applied field

In the present experiments, no evidence was found for positive or negative galvanotaxis of attached cells to applied fields within the ranges discussed above, on any of the substrates used at any of the medium viscosities used and regardless of whether or not the medium contained serum. All observed migration of the cells was apparently random in orientation, accompanied by cytoplasmic extensions such as ruffles and could be inhibited by either room temperature or urea treatment. The only movement observed which was aligned with the field was clearly due to electrophoresis of the cells only and could not be inhibited by room temperature or urea treatment.

The cells which were being electrophoresed were always unspread and not attached.

Substrate effects

When voltage drops from 50 mV/cm^{-1} to 4 V/cm^{-1} were applied for periods from 10 min to 5 h to glass, collagen-coated glass or tadpole tail or chick blastoderm-derived basal laminas in serum-containing medium of different viscosities (total 16 different experiments), it was found that cells placed in serum-free medium in the absence of electrical stimulation onto stimulated substrates of any type did not migrate in any prevalent direction, but their movement, if any, was of a random orientation. Table 1 summarises the findings relating cell spreading or attachment to different serum-coated stimulated substrates, in the absence of serum or stimulation, 5 h after plating. Fig. 4 shows the spreading of dissociated cells under electrical stimulation in different regions of the field.

Attached and spread cells in areas in the vicinity of where the anode had been behaved as if in serum-containing medium on glass substrates which had been stimulated, even though the cells were surrounded by serum-free medium. The application of small (from 10 to 50 mV/cm^{-1}) "maintenance" voltage drops after plating the cells in serum-free medium onto stimulated substrates did not induce cell alignment, and the behaviour of cells under these conditions did not differ visibly from that described above, when no current was passed after plating the cells.

Effects on pieces of tissue

Pieces of freshly dissected lateral plate or primitive streak mesoderm (stages 3–4, Hamburger & Hamilton [9]) containing some 500–1 000 cells and having a diameter of up to 100 μm did not exhibit any electrophoretic mobility of the whole piece when 100 to 800 mV/cm^{-1} were applied. The lack of mobility of these large pieces could be due to the resulting increase in mass and to the comparatively rapid attachment that these pieces made to the substrate.

On serum-coated stimulated glass substrates as described in the previous section, pieces of mesoderm spread very quickly. When several pieces were explanted onto the same coverslip, the ones nearest where the anode had been started to spread first. Small pieces (up to 200 cells) spreading under stimulation of up to 1 V/cm^{-1} in serum-containing medium did not appear to behave differently from pieces spreading in the same medium in the absence of electrical stimulation.

The polarising effect of the substrates which had been stimulated on pieces of mesoderm placed upon them was lost after trypsin digestion or if serum was added before the pieces were placed on the stimulated coverslips. The effect was not lost, however, if the stimulated coverslips were stored in serum-free medium in the absence of a maintenance current for up to two days in a refrigerator at 4°C.

DISCUSSION

Electrophoretic mobility

The extent of electrophoretic mobility displayed by dissociated mesoderm cells in the present experiments is slightly less than that described by Zalik et al. [13] for EDTA and CMF-dissociated whole chick blastoderms in a cylindrical cell electrophoresis apparatus. The slight discrepancy (about a factor of 1.8) can be accounted for by several factors. The differences in the dissociation technique could be a factor, as the use

of 2×10^{-3} M EDTA in their experiments could affect surface charge through making the cell membrane leaky. We have found the present CMF-based technique adequate for all tissue types from these early embryos [14]. The close proximity of the substrate in the present experiments is also likely to lead to transient localised adhesions being formed by the cells, thus increasing the resistance to movement. A third factor could be the difference in cell types, as we have found that dissociated cells from the epiblast and lower layer tissues have greater mobility than mesoderm in the present setup (unpublished observations).

Determination of the minimum voltage drop required to dislodge a still spherical cell from recently formed adhesion to a substrate in experimental chambers, such as those described here could provide a simple method to estimate the strength of initial adhesions to the substrate.

Substrate effects

The present results indicate that:

(*a*) Several types of substrates can be polarised by applied electrical fields through effects on substrate-attached materials (see [12]), as determined by differences in the behaviour of cells in different regions of the 3 cm long field and by the spreading of freshly dissected tissue on stimulated substrates.

(*b*) This effect of the stimulation on the substrate is stable and persists after washing for at least two days in the absence of a maintenance current. This polarisation is lost if serum is added or after trypsin digestion, suggesting that a serum-derived protein is responsible for this polarisation. Fibronectin may be a likely candidate for this role (see [12]).

(*c*) Single dissociated mesoderm cells cannot respond in a polarised way to the substrate in the present experiments, probably owing to the comparatively shallow slope of the induced adhesiveness gradient in relation to cell size.

(*d*) The shallow slope of the adhesiveness gradient could not be significantly increased neither by an increase in the viscosity of the medium nor by the application of small maintenance currents after plating the cells (both would reduce back-diffusion and therefore 'fuzzing' of the slope of the gradient).

It can therefore be concluded that the inability of single spreading cells to respond in a polarised manner to the substrate in the present experiments is probably due to the relatively low levels of voltage drop applied to the system. Since voltage drops greater than about 1 V/cm^{-1} are unlikely to be present along an axis perpendicular to the axis of the primitive streak in the embryo [2, 17] it could be inferred that individual mesoderm cells migrating out of the primitive streak in the embryo would not be able to respond directly to electrophoretically established gradients of substrate adhesiveness unless very long filopodia were present [3] or the lateral plate tissue behaved as a unit rather than as individually responding cells.

Conclusions

The present experiments suggest that lateral electrical currents flowing in the extracellular space surrounding the mesoderm do not play a role in the orientation of migration of individual mesoderm cells out of the primitive streak. Electrophoresis of the cells laterally out of the streak is also unlikely, as cells in situ appear to move faster [15] than would be expected from electrophoresis of whole cells (see [2] and MacKenzie, unpublished observations). In-

hibition of the extracellular electrical current pathways in embryos using strophanthidin (an inhibitor of the sodium pump) or by short-circuit with saline or albumen all seem to interfere seriously with mesoderm migration out of the primitive streak (unpublished observations).

It thus appears that the extracellular electrical controls of mesoderm migration are only indirect, if any, perhaps through effects upon ingression from the epiblast or through maintenance of the primitive streak region. These possibilities will be examined in another publication [16]. It seems likely at this stage that the migration of mesoderm laterally is guided by mechanical constraints imposed at the streak by cells accumulating there as they ingress.

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