INVASIVE BEHAVIOUR OF MOUSE PRIMORDIAL GERM CELLS IN VITRO

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SUMMARY

We have isolated migrating primordial germ cells (PGCs) from 10.5-day mouse embryos and studied their behaviour when cultured on a mouse embryo fibroblast (STO) cell line. Living and fixed PGCs were identified by fluorescent labelling with a monoclonal antibody specific for PGCs in the culture system used. The behaviour of the cells was studied using interference reflection microscopy (IRM) and time-lapse video cinematography. The IRM pattern displayed by PGCs is typical of highly motile cell types, the cells lack focal contacts and possess large areas of close contacts indicative of weak membrane to substrate interaction. The PGCs exhibit relatively high rates of translocation and lack contact inhibition. They were observed to underlap STO cells in subconfluent monolayers and to penetrate between the cells of confluent monolayers, becoming located between the monolayer and its substrate. These observations support the hypothesis that migrating mouse PGCs are inherently motile and are able transiently to disrupt the adhesion of surrounding cells. These results suggest that PGCs actively migrate to the developing gonad in vivo.

INTRODUCTION

Migrations of individual cells during vertebrate morphogenesis have been documented for several cell types (see Armstrong, 1985; Le Douarin, 1984; Thiery, 1984; Trinkaus, 1984, for reviews). The routes taken by different migratory cells have, in many cases, been found to possess specializations that may assist in directional movements. For example, amphibian mesoderm cells migrate over an array of extracellular matrix (ECM) fibrils on the blastocoel roof (Nakatsuji & Johnson, 1984), neural crest cells of axolotl embryos require changes in ECM composition to initiate migration (Lofberg et al. 1985) and avian neural crest cells move through transient ECM-filled spaces after leaving the neural tube (Duband & Thiery, 1982; Thiery et al. 1982). However, the route taken by the primordial germ cells (PGCs) of mouse embryos (Chiquone, 1954; Mintz & Russell, 1957; Ożdżęński, 1967) shows no obvious specializations that may assist the movements of the cells and, whilst it has been assumed that the PGCs reach the site of gonadal development due to intrinsic motile capabilities, this has never been demonstrated directly, due to lack of a suitable experimental system. Indeed, some workers have postulated the progressive change in location of the PGC during development to be mainly due to gross morphogenetic tissue rearrangements (Snow, 1981). However, evidence from electron-microscope studies suggests that migratory mouse PGCs

Key words: primordial germ cell, cell migration, cell interactions, cell invasiveness.
move through highly cellular embryonic tissues and may be capable of distorting and displacing cells that they encounter (Clark & Eddy, 1975).

We have recently demonstrated that mouse embryo PGCs isolated during their migration display motile behaviour when cultured on monolayers of mouse embryo fibroblasts (Donovan et al. 1986). In this paper we describe some of the properties of migratory PGCs in vitro and present evidence suggesting that they are intrinsically highly motile and capable of invasive activity in the system we have used.

MATERIALS AND METHODS

Cell culture

STO cells (Martin & Evans, 1975) were grown, harvested and irradiated as described (Donovan et al. 1986). Subconfluent or confluent monolayers were produced by adding 2 ml of irradiated STO cells (7.5 x 10⁴ or 2 x 10⁵ ml) in bicarbonate-buffered Dulbecco’s modified Eagle’s medium (DMEM; Flow Laboratories), supplemented with 10% foetal calf serum (FCS; Tissue Culture Services), 4 mM-glutamine and antibiotics to 22 mm diameter acid-washed glass coverslips in 35 mm diameter Petri dishes. The monolayers were maintained at 37°C in 10% CO₂ for 24 h prior to addition of PGCs.

PGC containing cell suspensions were prepared from 10.5-day MF1 mouse embryos as described (Donovan et al. 1986) and added to STO cell monolayers at 1 embryo equivalent per dish.

Labelling and observation of PGCs

Histochemical and immunocytochemical procedures used on living and fixed cells, and time-lapse video recording were as described (Donovan et al. 1986). In order to facilitate immunostaining of cells beneath confluent STO monolayers, some cultures were permeabilized by treatment with 0.1% Triton X-100 for 10 min prior to staining. IRM was performed using a Zeiss photomicroscope equipped with an epi-illuminator 100 and HBO 50 W lamp. A IIIRS epi-fluorescence condenser was used, equipped with a BP 546 green interference exciter filter, FT 510 chromatic beam splitter and a green interference barrier filter set 520/560. A polar protected by a heat-reflecting filter was placed between the epicondenser field diaphragm and the exciter filter, and a second polar was placed above the barrier filter. Cells were observed with a ×40 Planapo objective (numerical aperture 1.0) and the position of the polar and the field diaphragm setting adjusted to give the best image contrast. Photographs were taken on either Kodak Tri-X pan or Technical pan 2415 film.

RESULTS

Morphology of migratory-stage PGCs in vitro

The morphology of migratory-stage PGCs and their interaction with STO cells were studied by seeding suspensions containing germ cells prepared from 10.5-day embryos onto confluent or subconfluent monolayers of gamma-irradiated STO cells on glass coverslips. Cultures were fixed after 2 or 20 h and PGCs were identified by staining either for alkaline phosphatase (AP) activity or TG1 monoclonal antibody reactivity. AP has been shown to be a marker of PGCs in early mouse embryos (Chiquone, 1954; Mintz & Russell, 1957) and we have shown that both AP and TG1 staining are specific for PGCs in the culture system used (Donovan et al. 1986; Fig. 1). TG1 monoclonal antibody recognizes a surface determinant on PGCs
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Fig. 1. Alkaline phosphatase (A,C) and TG1 immunofluorescent (B,D) double-labelling of PGCs cultured on subconfluent STO cell monolayers for 2 (A,B) or 20 (C,D) h. The PGCs appear highly polarized, possessing well-defined uroids (u) and leading lamellae (l). Underlapping of STO cells by PGCs occurs during the 20-h period, leading to reduced penetration of immunostaining reagents (D). Bar, 10 μm.

and so is useful both in demonstrating surface morphology of PGCs and in labelling living PGCs for behavioural studies (see below).

On subconfluent monolayers, PGCs fixed 2 h after seeding and stained with both AP and TG1 possess several characteristics typical of motile cells (Fig. 1A,B). The cells usually appear elongate with pseudopodia and fine filopodial extensions. Sometimes a distinct uroid is present, though most cells are not obviously polarized. After 20 h in culture, PGCs retain these features and are often found partially underlapping STO cells, resulting in areas of decreased fluorescent staining due to reduced antibody penetration (Fig. 1C,D).

On confluent STO cell monolayers, AP was less useful in demonstrating PGC morphology due to localized diffusion of the reaction product into neighbouring cells. In cultures fixed 2 h after seeding and stained with TG1, PGCs were found on top of the monolayer and appeared less elongate than those observed on subconfluent monolayers, often possessing fine extensions, which projected either over the surface of the monolayer or down between the STO cells (Fig. 2A,B). After 20 h in culture, PGCs cultured on confluent STO monolayers were found beneath the monolayer.
and appeared to have spread between the STO cells and the coverslip, having lost the fine extensions observed at 2 h after seeding (Fig. 2C, D). It seems that the PGCs invade the STO monolayer and, having done so, assume a different morphology. The appearance of these cells suggests they are no longer motile. However, direct investigation of their motility was precluded by our inability to live-label cells beneath confluent monolayers with TG1.

Contact pattern of migratory stage PGCs in vitro

PGCs isolated from 10.5-day embryos were live-labelled with TG1 between 2 and 6 h after seeding onto subconfluent STO cell monolayers. The label was rapidly capped (visible within 10 min) and internalized by the cells, and their subsequent morphology was indistinguishable from that of cells fixed prior to exposure to the antibodies. The TG1 antigen is present on the cells after live-labelling, as demonstrated by fixing and restaining the cells (Fig. 3). Thus, it appears unlikely that the identification procedure interferes with the subsequent behaviour of the cells.

Fig. 2. Phase-contrast (A, C) and TG1 immunofluorescent staining (B, D) of PGCs cultured on confluent STO cell monolayers for 2 (A, B) or 20 (C, D) h. After 2 h culture, PGCs possess fine extensions, which often penetrate into the monolayer (arrowhead in B indicates such a process leaving the plane of focus). After 20 h in culture, PGCs are found beneath the monolayer and lack polarization and fine processes. Bar, 10 μm.
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Fig. 3. Phase-contrast (A) and fluorescence (B) micrographs of a PGC seeded onto a subconfluent STO monolayer, fixed 30 min after live-labelling and restained with TG1. The original label is capped to a large patch near the uroid (arrowhead in B). The TG1 antigen is present on the remainder of the cell surface. Bar, 10 μm.

The contact pattern of both STO cells and live-labelled PGCs was studied using interference reflexion microscopy (IRM). STO cells exhibit a substrate contact pattern typical of fibroblastic cell types, with large pale areas presumed to represent regions with a relatively large membrane to substrate gap (Verschueren, 1985), and small dark areas representing focal contacts (FCs), usually distributed near the periphery of the cells (Fig. 4). In contrast, PGCs possess an entirely different substrate contact pattern when viewed by IRM. Large, mid-grey areas predominate, representing close contacts (CCs), with occasional darker and lighter grey areas towards the centre of the pattern. Neither the large pale areas nor FCs typical of fibroblasts were observed in the PGC pattern (Fig. 4).

The behaviour of migratory-stage PGCs in vitro

Live-labelled PGCs were studied by time-lapse video recording over periods of up to 4 h for each cell studied. The observed activities of the PGCs are dependent upon their interactions with STO cells during the period studied, two types of behaviour predominating. In the first type, PGCs exhibit randomly orientated locomotion either on cell-free areas of substrate, or on top of spread STO fibroblasts. Cell tracks were produced by tracing the position of the visually estimated centre of the labelled cell on a video monitor. Two examples of such tracks are shown in Fig. 5. It can be seen that frequent, apparently random, changes in direction are made by the cells, which exhibit rapid net translocation over short distances, interspersed with turning periods during which little translocation occurs and extensions are produced in different directions. Overall rates of translocation measured over periods greater than 4 h were 53.6 μm h⁻¹ for cell A and 50.2 μm h⁻¹ for cell B. PGCs were observed to move at considerably faster speeds during prolonged periods between turns. One cell was observed to travel 92.5 μm in 41.6 min, a velocity of 133.4 μm h⁻¹. During translocation of PGCs over STO cells, membrane ruffling continued at the periphery of the STO cell. As far as we could tell, no localized paralysis of membrane ruffling
occurred and so it appears that PGC-STO cell encounter results in a reciprocal failure of contact inhibition, as judged by this criterion. The STO cells do not display extensive over- or under-lapping of each other when cultured as a subconfluent monolayer.

The second type of behaviour frequently observed was underlapping of STO cells by the PGCs, which results in little net translocation by the cells. PGCs adjacent to STO cells were located and identified by TG1 live-labelling and were photographed

Fig. 4. Interference reflexion micrograph of a PGC identified by live-labelling with TG1, 3 h after seeding onto a subconfluent STO monolayer. Focal contacts (fc) are seen beneath STO cells, whilst the PGC (pgc) displays large areas of close contacts (cc). Bar, 10 μm.

Fig. 5. Tracks of PGCs moving on STO cells in a subconfluent monolayer. Filled circles indicate the positions of the centre of the cell at 20-min intervals. The PGCs remained on STO cells for the entire period recorded for each track. Bar, 20 μm.
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at intervals of 10–20 min using IRM and phase-contrast optics. An example of underlapping of a STO cell by a PGC is shown in Fig. 6. In this sequence, a live-labelled PGC (Fig. 6A–C) is seen to underlap a STO cell, which initially loses contact with the substrate in the region of the advancing PGC (Fig. 6D,E). Subsequently, the STO cell re-establishes substrate contact around the PGC, so that the PGC is completely enveloped by the STO cell (Fig. 6F,G). The PGC then begins to spread beneath the STO cell, becoming surrounded by an area devoid of membrane–substrate interaction (Fig. 6H,I).

DISCUSSION

In very few cases of cell migration during vertebrate development is it possible to observe directly the behaviour of the migrating cells in situ (see Nakatsuji et al. 1986; Trinkaus & Erikson, 1983). In cases where this is not possible, it has been necessary to resort to in vitro systems that allow isolated migratory cells to mimic their normal activities (Heasman et al. 1977; Wylie et al. 1979; Nakatsuji & Johnson, 1982; Nakatsuji, 1984; Rovasio et al. 1983). We have previously reported the development of an in vitro system allowing observation of motile behaviour by migratory-stage mouse PGCs (Donovan et al. 1986). In the present work, we have characterized this behaviour and recorded the invasive capabilities and substrate contact pattern of the PGCs.

The observed morphology of the PGCs when plated on to subconfluent STO cell monolayers strongly suggests motile activity. Whilst some cells possessed obvious leading lamellae and retraction fibres indicative of directional locomotion, many were apparently not polarized, possibly having been fixed in the process of turning. Partial or total underlapping of STO cells by PGCs after 20 h in culture indicates that PGCs are not contact-inhibited on collision with STO cells, an observation confirmed by time-lapse filming.

On confluent monolayers, the PGCs produced fine extensions, often observed to penetrate down into the monolayer. This behaviour is comparable to that observed when a variety of cell types known to exhibit invasive properties in vivo were cultured on confluent vascular endothelial monolayers (Kramer & Nicolson, 1979). After 20 h in culture, the PGCs are found beneath the STO monolayer, and no longer possess fine extensions, suggesting they may no longer be motile. This may be a response by the PGCs to a difference in extracellular environment between the free and attached surfaces of the STO cell monolayer, as a similar phenotypic change does not occur in PGCs cultured for 20 h on subconfluent STO monolayers.

Observations on the substrate contact pattern and behaviour of living PGCs were confined to cultures on sub-confluent STO monolayers. Whilst the quantitation of cell–substrate distances from IRM images remains problematical (see Verschuren, 1985), sufficient data have been accumulated for different cell types to allow inferences about cell behaviour to be drawn. Focal contacts (FCs), typically observed with fibroblastic cell types, probably represent a cell–substrate gap of around 15 nm (Izzard & Lochner, 1976; Heath, 1982; Chen & Singer, 1982), which may
Fig. 6. Phase-contrast (B,D,F,H) and IRM (C,E,G,I) micrographs of a PGC identified by live-labelling with TG1 (A) and observed during subsequent underlapping of a STO cell (B–I). The label is seen to be patched within 10 min of completion of staining (A), when the PGC is located next to a STO cell (B) and the contact areas of the two cells are beginning to merge (C); 12 min later there is underlapping of the STO cell by the PGC (D,E) and by 40 min the PGC is almost entirely beneath the STO cell (F,G); 65 min after the initial observation, the PGC can be seen to be completely beneath the STO cell and is surrounded by an area of contact-free substrate (H,I). The STO cell periphery has re-established contact with the substrate at the position of PGC underlapping. Bar, 20 μm.
contain some cell-deposited material. This type of cell contact is associated with slowly moving or stationary cell types; the number of FCs under chick embryo fibroblasts and mesoderm cells increases as their translocation rate decreases with time in culture (Couchman & Rees, 1979a, b; Sanders, 1984). In common with several highly motile cell types, including rabbit neutrophil granulocytes (Armstrong & Lackie, 1975), amphibian leucocytes (Kolega et al. 1982) and rat leukaemia cells (Haemmerli & Ploem, 1979), migrating mouse PGCs were found to possess few, if any, FCs.

In contrast, close contacts (CCs), represented by large mid-grey areas of highly variable shape and size, often located towards the centre of cells, are thought to represent a cell–substrate gap of around 30 nm (Izzard & Lochner, 1976; Heath, 1982; Chen & Singer, 1982) and are associated with highly motile cells (Armstrong & Lackie, 1975; Kolega et al. 1982). This type of contact is invariably present beneath mouse PGCs (Fig. 4).

Comparison of a variety of cell types has revealed that CCs rather than FCs are associated with rapid cellular translocation (Shure et al. 1979; Haemmerli et al. 1980; Kolega et al. 1982; see Verschuren, 1985, for review). The IRM images of migratory mouse PGCs and their recorded in vitro translocation rates suggest that they too are highly motile. The overall rate of translocation of the PGCs was highly dependent on the outcome of collisions with STO cells. At the monolayer cell density used, PGCs on the exposed glass substrate were rarely able to move more than one cell diameter without encountering a STO cell. Often, this encounter resulted in the PGC moving over the STO cell, and a rapid rate of translocation was maintained. Underlapping of STO cells by PGCs resulted in very low rates of translocation. We have no evidence as to what determines the outcome of PGC/STO collisions.

The average translocation rate of PGCs on subconfluent STO monolayers of around 50 μm h⁻¹, with a maximum recorded rate of 133·4 μm h⁻¹, is similar to that recorded for a variety of fibroblastic cell types (Kolega et al. 1982; Couchman & Rees, 1979b). However, this is probably an underestimate of the true capabilities of mouse PGCs in vitro. The inhomogeneity of the substrate may lead to transient interactions of the PGCs with STO cells, reducing their rate of translocation. Also, it is likely that some directional cues, mimicking those presumed to be present in vivo (Rogulska et al. 1971), would reduce the rate of turning and lead to an increase in translocation rates. Of interest in this respect is the observation that the in vitro translocation rate of human neutrophils is dependent upon the culture system used (Keller et al. 1979).

Lack of contact inhibition has previously been correlated with invasive potential of cells in culture (Abercrombie, 1979). Migratory mouse PGCs appear not to be contact-inhibited on collision with STO fibroblasts. It has previously been noted that limited underlapping can occur between cells that are contact-inhibited (Erickson, 1978; Heaysman, 1978). However, in the cultures we have studied, PGCs underlap STO cells to the extent that they become located entirely beneath the STO cells. In addition, we have observed PGCs migrating freely on the upper surface of the STO cells, suggesting they are not contact-inhibited in this heterotypic encounter. In
contrast, it appears that STO cells are contact-inhibited when they encounter each other, resulting in the production of a monolayer with little overlapping of the cells. We have previously presented evidence suggesting that PGCs are not contact-inhibited when they encounter each other (Donovan et al. 1986). The lack of contact inhibition when PGCs encounter STO cells is, therefore, most probably determined by the PGCs. This lack of contact inhibition of PGCs, together with their ability to penetrate confluent monolayers of STO cells argues for an invasive capability of these cells.

The motile and invasive abilities of migrating mouse PGCs are properties that would be required by cells actively migrating through the developing embryo. These findings therefore support the conclusion that mouse PGCs arrive at the developing gonad by active migration, rather than by passive relocation dependent upon gross morphogenetic rearrangements.

We thank Linda Cairns for her skilful technical assistance and Dr Peter Beverly for the TG1 monoclonal antibody. This work was supported by a grant from the Cancer Research Campaign to Dr J. Heasman and Professor C. C. Wylie.

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*(Received 11 July 1986 – Accepted 3 September 1986)*