The generation of cell surface polarity in mouse 8-cell blastomeres: the role of cortical microfilaments analysed using cytochalasin D

T. P. FLEMING¹,* , S. J. PICKERING¹, F. QASIM¹ AND B. MARO¹,2

¹ Department of Anatomy, University of Cambridge, Downing Street, Cambridge, CB2 3DY, UK
² Centre de Génétique Moléculaire du C.N.R.S., 91190 Gif sur Yvette, France

SUMMARY

The mechanism by which a surface pole of microvilli is generated in mouse 8-cell blastomeres has been investigated. 4-cell and 8-cell embryos (or cell couplets) were incubated for precise times during their respective cell cycles in medium containing cytochalasin D (CCD) to disrupt the microfilament system. The blastomeres were analysed immediately for the distribution and state of organization of their microvilli, using three morphological techniques. The results indicate that the surface pole, characterized by microvilli containing CCD-resistant core filaments, is not generated by the gradual segregation of stable microvilli to the apical surface. An alternative model is proposed, based upon (a) the stabilization of the apical cytocortex prior to the elongation of apical microvilli and (b) the destabilization of cytocortical elements in contact areas and the shortening and loss of basolateral microvilli.

INTRODUCTION

During the process of compaction at the 8-cell stage of mouse development, blastomeres become closely apposed along their basolateral surfaces (Lehtonen, 1980) concurrent with an extensive reorganization of their cytoplasmic and membrane components which become polarized (reviewed by Johnson & Maro, 1986). There is now much evidence suggesting that the radial asymmetry of the embryo generated at compaction provides the foundation for the subsequent formation of two cell populations that ultimately give rise to the trophectoderm and inner cell mass tissues of the blastocyst (Johnson & Maro, 1986).

Our attention is currently focused on the process of polarization itself, the sequence of its constituent events, and the underlying mechanisms controlling its organization. Recently, it has been shown that the contact-mediated restriction of microvilli to the apical cell surface is preceded by the polarization of specific components within the cytoplasm (Johnson & Maro, 1984; Fleming & Pickering, 1985; Maro, Johnson, Pickering & Louvard, 1985). However, this temporal sequence is unlikely to be causal since drugs that destabilize the cytoskeleton are

* To whom reprint requests should be sent.

Key words: mouse embryos, microvilli, microfilaments, cell polarity, cytochalasin D.
potent inhibitors of cytoplasmic events but do not prevent the expression of surface microvillous polarity (Johnson & Maro, 1984, 1985; Fleming, Cannon & Pickering, 1986). Indeed, if surface polarity is allowed to form in the presence of such drugs, and then the drugs are removed, cytoplasmic polarity may develop in certain cases subjacent to the surface pole (Johnson & Maro, 1985). Taken together, these results suggest that (a) polarization within the cytocortex may precede and act as a focus for cytoplasmic polarity and (b) the earliest events of cytocortical polarity may have so far gone undetected. We have called these early, presumptive events 'covert polarity' (Johnson & Maro, 1985, 1986; Fleming et al. 1986).

In the present paper, we describe experiments designed to uncover evidence of covert, cytocortical polarity. In these experiments cytochalasin D (CCD) has been employed to destabilize microfilaments. Previously, it has been shown that microvillous poles within late 8-cell embryos are stable following exposure to CCD (Handyside, 1980; Pratt, Ziomek, Reeve & Johnson, 1982; Sutherland & Calarco-Gillam, 1983) and contain microvilli with intact core filaments (Pratt, Chakraborty & Surani, 1981), presumably due to a low turnover of monomeric actin, (cf. brush border microvilli: Mak, Trier, Serfilippi & Donaldson, 1974; Stidwell, Wysolmerski & Burgess, 1984). The generation of such poles can also occur at the normal developmental time when CCD is present from the beginning of the 8-cell stage although the location and detailed organization of the poles may be highly modified (Johnson & Maro, 1984, 1985; Fleming et al. 1986). From these results, it seemed possible that the cytocortical transition from the non-polar to the polar state might be achieved in two steps: (a) the segregation of stable, CCD-resistant microvilli from unstable, CCD-sensitive microvilli, and the localization of the former at the cell apex, and (b) the production of a global rise either in the threshold for actin polymerization or the actin turnover rate in the blastomere, leading to the loss of all basolateral microvilli. The first step might constitute covert polarization and the second, overt polarization. Indeed, some evidence that CCD-resistant microvilli might gather together in advance of the late 8-cell stage has been presented previously (Pratt et al. 1982).

In order to test this possibility, we have placed embryos in CCD at various time points during the third and fourth cell cycles and examined the resulting distribution pattern of CCD-resistant microvilli soon afterwards. Our results indicate, contrary to the prediction, that the restriction of stable microvilli to the polar region occurs concurrent with the development of overt polarity and not significantly in advance of it. A detailed analysis of the results leads us to propose a mechanism by which the elaboration of a pole of microvilli might be achieved.

MATERIALS AND METHODS

Embryo recovery and staging

Female MFI (Olac Ltd) mice were superovulated (5 i.u. PMS followed 45–48 h later by 5 i.u. hCG, Intervet) and placed overnight with HC-CFLP (Hacking & Churchill Ltd) males; a vaginal
plug indicated successful mating. Late 2-cell or early 4-cell embryos were flushed from oviducts at 48–54 h post-hCG using Hepes-buffered Medium 2 + 4 mg ml\(^{-1}\) bovine serum albumin (M2+BSA; Fulton & Whittingham, 1978) and cultured in drops of Medium 16 + 4 mg ml\(^{-1}\) BSA (M16+BSA; Whittingham, 1971) in dishes under oil at 37°C and 5 % CO\(_2\) in air.

Experiments were performed either on intact embryos or on isolated cell couplets. Intact embryos were staged from the beginning of the third or fourth cell cycles as follows. Stock cultures at 48–50 h post-hCG (mainly late 2-cell stage) or at 58–60 h post-hCG (mainly late 4-cell stage) were cleared of any early developing 4-cell or 8-cell embryos respectively, and examined hourly for evidence of recent division to the appropriate cell stage. Newly formed 4-cell or 8-cell embryos thus derived were pooled and either used immediately in experiments or cultured as a separate group until required. Staging of 8-cell couplets from the start of the cell cycle was performed as follows. Late 4-cell embryos were isolated from their zonae and disaggregated to single blastomeres (1/4 cells) using a flame-polished micropipette, following incubation in Ca\(^{2+}\)-free medium. Small groups (four or five) of 1/4 cells were transferred to M16+BSA drops with the cells spaced apart and examined hourly for division to 2/8 couplets. These were collected, subsequently maintained as isolated pairs in microdrops of medium, and used in experiments at the appropriate time postdivision.

Embryo manipulations

Removal of the zona pellucida was achieved by brief exposure (15–20 s) to prewarmed acid Tyrode’s solution containing 4 mg ml\(^{-1}\) polyvinyl pyrrolidone (Nicholson, Yanagimachi & Yanagimachi, 1975) followed by extensive washing in M2+BSA.

Embryo decompaction was carried out by incubation in Ca\(^{2+}\)-free M2 or M16 + 6 mg ml\(^{-1}\) BSA for 10–15 min at 37°C.

Cytochalasin D incubation protocols

Experiments were performed using the microfilament-disrupting drug, cytochalasin D (CCD, Sigma) at a working concentration of 0.5 \(\mu\)g ml\(^{-1}\) and derived from a stock solution in dimethylsulphoxide (1 mg ml\(^{-1}\); DMSO) stored at \(-20°C\). 4-cell and 8-cell embryos and in vitro-derived 2/8 couplets at selected times postformation were incubated in M16+BSA containing CCD (or DMSO in controls) for 3 h or longer at 37°C in 5 % CO\(_2\) in air. Subsequently, embryos were isolated from their zonae before analysing the effects of drug treatment on microvillous conformation and distribution. In some experiments, zonae were removed prior to drug treatment without apparently altering the effects of CCD exposure. Three techniques were used for analyses: surface labelling by FITC–concanavalin A (FITC-Con A) binding, transmission and scanning electron microscopy. In all cases up until the time of fixation, treatment of experimental and control embryos/cells included the presence of CCD or DMSO respectively.

FITC–concanavalin A labelling

Intact 4-cell embryos, small cell clusters from partially disaggregated 8-cell embryos, or 2/8 couplets, were stained with FITC–Con A (Polysciences) at 0.7 mg ml\(^{-1}\) in M2+BSA+0.02 % azide for 5–10 min to label the cell surface, washed three times in M2+BSA+azide, fixed in 3 % paraformaldehyde in phosphate-buffered saline for 15 min, and stored for up to 12 h in M2+BSA. Embryos and cells were transferred to wells of a tissue-typing slide (Baird & Tatlock) in drops of M2+BSA under oil. FITC–Con A labelling was examined using a Zeiss Universal photomicroscope fitted with a Zeiss filter set 487709. Photomicrographs were taken on Kodak Tri-X 35 mm film.

Transmission electron microscopy (TEM)

Intact embryos were fixed in 3 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 15–30 min, washed in buffer and postfixed in 1 % osmium tetroxide in 0.1 M cacodylate (20 min), dehydrated in an ethanol series and embedded in Spurr’s resin. Ultrathin sections were cut on an LKB Ultrotome III, stained with alcoholic uranyl acetate and lead citrate and viewed with an AEI 6B or a Philips 300 electron microscope at 80 kV.
Scanning electron microscopy (SEM)

The procedure used was that of Johnson & Ziomek (1982). Briefly, embryos were fixed in 6% glutaraldehyde in 0-1 M-cacodylate buffer (pH 7-3) for 1 h, washed in buffer, attached to poly-L-lysine-coated coverslips for dehydration through an alcohol series, critical-point dried via carbon dioxide in a Polaron E3000 drying apparatus, coated with a 50 nm layer of gold in a Polaron E5000 diode sputtering system and examined in a SM-35CF Jeol scanning microscope at 20 kV.

RESULTS

FITC—Con A labelling

4-cell and 8-cell embryos at different stages during their respective cell cycle were treated with CCD (or DMSO in controls) for 3 h before immediate analysis of surface morphology using FITC—Con A labelling (Table 1). At the 4-cell stage, control blastomeres from each of the three treatment periods (0–3 h, 3–6 h, 6–9 h postformation) showed uniform (apolar) staining (Fig. 1A; Table 1, column 3, lines 1–3). CCD-treated cells, however, were stained in a heterogeneous manner, usually with small (measuring <15 μm), randomly placed but sharply defined foci of label (Fig. 1B,C; Table 1, column 5, lines 4–6). On occasions, larger (>20 μm) zones of randomly positioned fluorescent binding sites were present (Fig. 1D; Table 1, column 6, lines 4–6). Evidence of polar labelling, comparable with control late 8-cell blastomeres, was not found in CCD-treated 4-cell embryos.

The staining of control 8-cell blastomeres was mainly apolar during the early period of the cell cycle, but changed to a predominantly polar pattern at later time

Table 1. FITC—Con A labelling patterns in blastomeres from 4-cell and 8-cell embryos following treatment with CCD (or DMSO alone in controls) for 3 h prior to analysis. 8-cell embryos were partially disaggregated to small cell clusters before surface labelling.

<table>
<thead>
<tr>
<th>Embryo stage and treatment time (h postformation)</th>
<th>No. cells scored (2)</th>
<th>FITC labelling pattern (%)</th>
<th>Heterogeneous staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Apolar (3)</td>
<td>Polar (4)</td>
</tr>
<tr>
<td>4-cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) 0–3 h DMSO</td>
<td>101</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>(2) 3–6 h DMSO</td>
<td>62</td>
<td>95·2</td>
<td>0</td>
</tr>
<tr>
<td>(3) 6–9 h DMSO</td>
<td>108</td>
<td>91·7</td>
<td>0</td>
</tr>
<tr>
<td>(4) 0–3 h CCD</td>
<td>155</td>
<td>5·8</td>
<td>0</td>
</tr>
<tr>
<td>(5) 3–6 h CCD</td>
<td>155</td>
<td>13·5</td>
<td>0</td>
</tr>
<tr>
<td>(6) 6–9 h CCD</td>
<td>101</td>
<td>5·9</td>
<td>2·0</td>
</tr>
<tr>
<td>8-cell</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(7) 0–3 h DMSO</td>
<td>114</td>
<td>72·8</td>
<td>27·2</td>
</tr>
<tr>
<td>(8) 3–6 h DMSO</td>
<td>99</td>
<td>45·5</td>
<td>54·5</td>
</tr>
<tr>
<td>(9) 6–9 h DMSO</td>
<td>134</td>
<td>24·6</td>
<td>75·4</td>
</tr>
<tr>
<td>(10) 0–3 h CCD</td>
<td>144</td>
<td>4·9</td>
<td>5·6</td>
</tr>
<tr>
<td>(11) 3–6 h CCD</td>
<td>146</td>
<td>0·7</td>
<td>24·7</td>
</tr>
<tr>
<td>(12) 6–9 h CCD</td>
<td>330</td>
<td>3·0</td>
<td>54·2</td>
</tr>
</tbody>
</table>
Fig. 1. 4-cell embryos labelled with FITC-Con A.
(A) Control embryo (incubated in DMSO, 0–3 h postdivision) showing a homogeneous (apolar) distribution of label on the cell surface.
(B) CCD-treated embryo (3–6 h postdivision) where surface labelling is concentrated in small, randomly distributed, heterogeneous zones (arrows).
(C) CCD-treated embryo (0–3 h postdivision) with a staining pattern similar to (B) but photographed at a higher focal plane to show the small discrete zones of label (arrows) en face.
(D) CCD-treated embryo (6–9 h postdivision) showing the rarer pattern of large heterogeneous zones of surface label. ×250.

points (Fig. 2A,B; Table 1, compare columns 3 and 4, lines 7–9). In rare cases, labelling was concentrated in a ring or halo at the periphery of the pole (Fig. 2C). CCD-treated 8-cell blastomeres were mainly labelled heterogeneously at the earlier treatment times, but with the size of the randomly placed foci of label being larger in the 3–6 h group than in the 0–3 h group (Fig. 2D–F; Table 1, compare columns 5 and 6, lines 10 and 11). In the latter cases, intervening regions of unlabelled membrane were also larger in size (Fig. 2F). The incidence of polar FITC labelling increased progressively during the cell cycle at similar times, but at a lower percentage value, to control cells (Fig. 2G; Table 1, column 4, lines 10–12). Surface poles in CCD-treated 8-cells, unlike controls, were usually sharply delineated from non-polar regions of the cell surface (Fig. 2H); in addition, non-polar membrane often contained patches of heterogeneous labelling.

Couplets of 2/8 blastomeres, timed from division in vitro of 1/4 cells and incubated for specific periods during the cell cycle in CCD or DMSO, were also analysed by FITC–Con A labelling (Table 2). In control cell pairs, incubated in DMSO alone either for a 3 h period similar to that used in experiments on whole
Fig. 2. Disaggregated cell clusters derived from 8-cell embryos and labelled with FITC–Con A.

(A) Control cells (embryo incubated in DMSO, 3–6 h postdivision) with homogeneous (apolar) surface labelling.
(B) Control cells (6–9 h postdivision) with polarized surface labelling.
(C) Polarized control cells (6–9 h postdivision) showing a polar ring of bright fluorescence.
(D) CCD-treated cells (0–3 h postdivision) with small heterogeneous zones of surface labelling.
(E) CCD-treated cell (0–3 h postdivision) showing small heterogeneous zones of fluorescence viewed en face.
(F) CCD-treated cells (3–6 h postdivision) with larger zones of heterogeneous labelling.
(G) CCD-treated cells (6–9 h postdivision) with surface poles.
(H) CCD-treated cell (6–9 h postdivision) showing the discrete nature of the surface pole, viewed en face. ×250.

embryos (0–3 h, 3–6 h, 6–9 h, 8–11 h postformation) or for the complete period from division of 1/4 cells (0–6 h, 0–9 h postformation), the incidence of polar labelling increased with time postdivision (Fig. 3A, B; Table 2, column 4, lines 1–6). In nearly all cases, surface polarity developed opposite to the point of contact with the sister blastomere (apical region of cell, see Table 2, column 5, lines 1–6) as described previously (Ziomek & Johnson, 1980). Couplets treated with CCD were mostly labelled heterogeneously at 3 h and 6 h postdivision with a predominance of small, discrete clusters of stain occurring at the earlier time point (Fig. 3C) and larger foci with wider intervening regions of unlabelled membrane occurring later (Fig. 3D). The transition to the latter pattern of heterogeneity was not sensitive to CCD treatment and was evident at a similar frequency in both 3–6 h and 0–6 h CCD groups (Table 2, columns 8 and 9, compare lines 7, 8 and
Table 2. *FITC–Con A labelling patterns in 2/8 pairs of blastomeres following treatment with CCD (or DMSO alone in controls) for varying times before analysis*

<table>
<thead>
<tr>
<th>Treatment (h post formation) from 1/4 cell</th>
<th>No. cells scored (2)</th>
<th>Apolar (3)</th>
<th>Polar*</th>
<th>Heterogeneous staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>total (4)</td>
<td>apical (5)</td>
<td>lateral (6)</td>
</tr>
<tr>
<td>(1) 0–3 h DMSO</td>
<td>78</td>
<td>87·2</td>
<td>12·8</td>
<td>10·2</td>
</tr>
<tr>
<td>(2) 3–6 h DMSO</td>
<td>44</td>
<td>61·4</td>
<td>38·6</td>
<td>36·3</td>
</tr>
<tr>
<td>(3) 6–9 h DMSO</td>
<td>72</td>
<td>38·9</td>
<td>61·1</td>
<td>61·1</td>
</tr>
<tr>
<td>(4) 8–11 h DMSO</td>
<td>62</td>
<td>16·1</td>
<td>83·9</td>
<td>82·3</td>
</tr>
<tr>
<td>(5) 0–6 h DMSO</td>
<td>103</td>
<td>66·0</td>
<td>32·0</td>
<td>30·1</td>
</tr>
<tr>
<td>(6) 0–9 h DMSO</td>
<td>97</td>
<td>38·1</td>
<td>61·9</td>
<td>61·9</td>
</tr>
<tr>
<td>(7) 0–3 h CCD</td>
<td>74</td>
<td>9·5</td>
<td>2·7</td>
<td>0</td>
</tr>
<tr>
<td>(8) 3–6 h CCD</td>
<td>58</td>
<td>0</td>
<td>12·0</td>
<td>3·4</td>
</tr>
<tr>
<td>(9) 6–9 h CCD</td>
<td>71</td>
<td>2·8</td>
<td>52·1</td>
<td>35·2</td>
</tr>
<tr>
<td>(10) 8–11 h CCD</td>
<td>61</td>
<td>0</td>
<td>59·0</td>
<td>57·4</td>
</tr>
<tr>
<td>(11) 0–6 h CCD</td>
<td>100</td>
<td>1·0</td>
<td>19·0</td>
<td>9·0</td>
</tr>
<tr>
<td>(12) 0–9 h CCD</td>
<td>87</td>
<td>0</td>
<td>59·7</td>
<td>24·1</td>
</tr>
</tbody>
</table>

*Polar labelling, where fluorescence is concentrated to one zone on the cell surface, is subdivided into apical (directly opposite to contact point with sister blastomere), lateral or basal.*
Fig. 3. *In vitro* cultured 2/8 couplets labelled with FITC-Con A.

(A) Precompact control couplet (incubated in DMSO, 0–6 h postdivision) showing surface poles positioned opposite to contact zone.

(B) Control couplet (6–9 h postdivision) that has compacted and with surface poles positioned opposite to contact point.

(C) CCD-treated couplet (0–3 h postdivision) with small heterogeneous zones of surface label.

(D) CCD-treated couplet (3–6 h postdivision) with larger zones of heterogeneous labelling.

(E) CCD-treated couplet (0–9 h postdivision) where both cells have polarized either opposite (left cell) or lateral (right cell) to contact point.

(F) CCD-treated couplet (0–6 h postdivision) where both cells show polar labelling at the contact zone.

(G) CCD-treated couplet (6–9 h postdivision) with surface poles positioned opposite to the contact point, from which an additional zone of fluorescence also extends. ×250.

11). Surface polarity developed in CCD-treated couplets at similar times but at lower frequencies than control pairs (Table 2, column 4). However, in agreement with previous findings (Johnson & Maro, 1984, 1985; Fleming *et al.* 1986), polar labelling tended to be randomly placed with respect to the cell contact point in all groups where CCD was present during the early period postdivision, but was predominantly apical in 6–9 h and 8–11 h CCD groups, thus comparable with controls (Fig. 3E,F; Table 2, columns 5–7, compare lines 7–12). Where apical poles developed, the contact region between blastomeres often retained a bright fluorescence indicative of a second microvillous population (Fig. 3G).

**Scanning electron microscopy (SEM)**

SEM provided the most reliable and stringent method for analysing surface morphology following CCD treatment. Control 4-cell embryos displayed an even (apolar) distribution of microvilli at each of the three time points studied (Fig. 4A;
<table>
<thead>
<tr>
<th>Treatment time (h postformation)</th>
<th>No. cells scored</th>
<th>Apolar (%)</th>
<th>Polar (%)</th>
<th>Heterogeneous</th>
<th>Elongate 'spaghetti-like' microvilli present (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 0–3 h DMSO</td>
<td>115</td>
<td>100</td>
<td>0</td>
<td>68.0</td>
<td>10.7</td>
</tr>
<tr>
<td>(2) 3–6 h DMSO</td>
<td>76</td>
<td>100</td>
<td>0</td>
<td>66.4</td>
<td>3.9</td>
</tr>
<tr>
<td>(3) 6–9 h DMSO</td>
<td>62</td>
<td>100</td>
<td>0</td>
<td>63.3</td>
<td>0</td>
</tr>
<tr>
<td>(4) 0–3 h CCD</td>
<td>75</td>
<td>0</td>
<td>0</td>
<td>59.6</td>
<td>0</td>
</tr>
<tr>
<td>(5) 3–6 h CCD</td>
<td>128</td>
<td>0</td>
<td>0</td>
<td>70.7</td>
<td>8.6</td>
</tr>
<tr>
<td>(6) 6–9 h CCD</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>64.2</td>
<td>35.8</td>
</tr>
<tr>
<td>(7) 0–6 h CCD</td>
<td>52</td>
<td>0</td>
<td>0</td>
<td>59.6</td>
<td>0</td>
</tr>
<tr>
<td>(8) 3–9 h CCD</td>
<td>58</td>
<td>0</td>
<td>0</td>
<td>70.7</td>
<td>29.3</td>
</tr>
<tr>
<td>(9) 0–9 h CCD</td>
<td>53</td>
<td>0</td>
<td>0</td>
<td>64.2</td>
<td>35.8</td>
</tr>
</tbody>
</table>
Table 3, column 3, lines 1–3). Lateral microvilli, forming bridges between adjacent cells, were evident within the intercellular furrows (Calarco & Epstein, 1975). In CCD-treated 4-cell embryos, microvilli were usually short or stubby in appearance and were distributed in random clumps over the exposed cell surface. Microvillous aggregates were mostly of small size (Fig. 4B) in all experimental groups, but in some cases covered larger areas of the cell surface with intervening regions of smooth membrane (Fig. 4C; Table 3, columns 5 and 6, lines 4–9). Rarely, extremely elongate microvilli, lying parallel to the cell surface (‘spaghetti-like’ microvilli) were detected (Table 3, column 7); these structures, more commonly seen in experimental 8-cell embryos, are shown in Fig. 5G. Lateral microvilli linking adjacent cells were usually retained following CCD treatment (Fig. 4B).

At the early 8-cell stage, most control embryos contained a homogeneous distribution of microvilli (Fig. 5A), but by 9 h postdivision the majority had compacted and polarized with microvilli restricted to the apical surface and absent from regions bordering cell contacts (Fig. 5B; Table 4, columns 3 and 4, lines 1–4) (see also Reeve & Ziomek, 1981). The boundary between these two zones, more clearly revealed in decompacted embryos, was usually gradual and appeared to contain shorter microvilli than those within the apical pole (Fig. 5C).

8-cell embryos were treated with CCD for a duration of 3–9 h in seven experimental groups and examined at four time points during the cell cycle (Table 4, lines 5–11). In those exposed during 0–3 h postformation, the cells contained a heterogeneous distribution of predominantly stubby microvilli usually

---

Fig. 4. 4-cell embryos examined by scanning electron microscopy.
(A) Control embryo (incubated in DMSO, 0–9 h postdivision) with an even (apolar) distribution of microvilli. Lateral microvilli linking adjacent cells can be seen in the intercellular furrows.
(B) CCD-treated embryo (0–3 h postdivision) showing small heterogeneous clusters of predominantly stubby microvilli separated by narrow zones of microvillus-free membrane. Lateral microvilli between adjacent cells are still present.
(C) CCD-treated embryo (6–9 h postdivision) with larger heterogeneous zones of modified microvilli and larger intervening regions of smooth membrane. ×3900.
aggregated in small, randomly placed clumps (Fig. 5D). At later time points (examined at 6 h or 9 h postformation) microvilli remained heterogeneous in their distribution but tended to occur in fewer, larger-sized aggregates on the exposed cell surface with intervening regions being microvillus-free (Fig. 5E; Table 4, column 6, lines 6–11). This transformation took place whether or not CCD was present throughout the cell cycle up until the time of fixation. In an increasing proportion of cells analysed at later time points (6 h or 9 h postformation) the large aggregates of microvilli also included highly elongate ‘spaghetti-like’ microvilli lying in a prostrate position and often forming a reticulate configuration (Fig. 5F,G; Table 4, column 7). When present, these modified microvilli were localized consistently over the region of apical membrane normally occupied by polar microvilli in control cells. The incidence of ‘spaghetti-like’ microvilli was generally lower if CCD was present early in the cell cycle. Although in many cases by 9 h postformation, microvilli were localized predominantly in a single large cluster on one region of the apical cell surface (e.g. arrowed cells in Fig. 5E,F), such cells were not classified as polar due to the irregular outline of the microvillous zone and the abnormal morphology of the microvilli when compared with controls (see Fig. 5C). Only in those groups where CCD was absent during the initial 6 h of the cell cycle (6–9 h, 8–11 h CCD) were a significant number of cells with a definitive polar morphology recorded (Fig. 5H; Table 4, column 4, lines 7 and 8). Of these, a small proportion (12%, 6–9 h group; 11%, 8–11 h group) displayed poles that were not orientated centrally on the apical surface (see Johnson & Maro, 1984) and, in some cases, a ring of more prominent microvilli was present at the periphery of the pole (Fig. 5H, arrows). ‘Spaghetti-like’ microvilli were not detected in the 8–11 h group.

**Transmission electron microscopy**

CCD- and DMSO-treatment times corresponded with those employed in the FITC–Con A labelling analysis of intact embryos (Table 1), but included an additional 0–9 h treatment of 8-cell embryos. Control 4-cell embryos, throughout the cell cycle, possessed microvilli distributed evenly over both exposed and intercellular membrane faces (Fig. 6A). At high magnification, core filaments were detectable within each slender microvillus (approx. 6 μm long, 0.05 μm diameter) and a moderately dense, filamentous cytoskeleton was evident on the cytoplasmic side of intervening regions of membrane (Fig. 6B). In 4-cell embryos treated with CCD for 3 h, microvilli occurred in aggregates of varying size located intermittently on exposed membranes, as reported by Pratt et al. (1981); in contrast, microvilli were present consistently in areas of cell contact (Fig. 6C). Both at contact zones (Fig. 6D) and elsewhere (Fig. 6E), microvillous profiles were predominantly swollen or bleb-like but also included some with a more typical configuration. In many instances, normal microvilli containing core filaments were found to originate from the bleb-like structures and occasionally to project horizontal to the cell surface (Fig. 6F), similar to the ‘spaghetti-like’ microvilli detected by SEM. Bleb-like microvilli contained denser cytoplasm than
within the adjacent cell cortex (Fig. 6F) and the membrane-associated cytoskeleton was less evident than in control sections.

At the early 8-cell stage, control embryos (DMSO, 0–3 h postformation) were precompact with microvilli distributed homogeneously over all regions of the cell
surface. By 9 h postdivision, most control embryos had compacted and microvilli were localized almost exclusively in a polar cluster on the apical membrane; areas of contact and peripheral regions of the apical surface were predominantly microvillus-free (Fig. 7A). At high magnification, all microvilli examined contained typical core filaments. In most sections of CCD-treated early and mid 8-cell embryos (0–3 h, 3–6 h postformation), the apical membrane was either microvillus-free or contained isolated and apparently randomly placed clusters of modified (bleb-like) microvilli (Fig. 7B). Microvilli were present consistently in areas of cell contact (Fig. 7B). At 9 h postdivision, cells with apically polarized microvilli were present in both 0–9 h and 6–9 h CCD groups. In the latter group, microvilli were often of normal morphology and tended to be absent or in reduced numbers in regions of cell contact. Conversely, all polarized cells examined in the 0–9 h CCD group contained a varying proportion of microvilli at the basolateral sites of membrane apposition (Fig. 7C). In a small proportion of 6–9 h CCD-treated embryos, the polarized cell surface was particularly rich in microvilli with the majority sectioned transversely and each containing core filaments (Fig. 7D). These may represent the polar aggregates of ‘spaghetti-like’ microvilli seen by SEM.

**DISCUSSION**

The three techniques used to analyse surface morphology following CCD treatment in general produced consistent results and revealed a microvillous distribution pattern that changed according to the stage of development reached at the time of fixation and the specific period during which drug treatment was carried out. The principal exception to this pattern of consistency was the low scoring of surface polarity in drug-treated 8-cell embryos following SEM examination. We feel that this exception reflects more closely the superiority

---

Fig. 5. 8-cell embryos examined by scanning electron microscopy.

(A) Control embryo (incubated in DMSO, 0–6 h postdivision) with an apolar distribution of surface microvilli and lateral microvilli linking adjacent cells.

(B) Compacted control embryo (6–9 h postdivision) with a polarized distribution of microvilli. Note that microvilli are essentially absent from the regions bordering cell contacts.

(C) Control embryo (0–9 h postdivision) that has been decompacted prior to fixation to reveal more clearly the apical poles of microvilli.

(D) CCD-treated embryo (0–3 h postdivision) with small heterogeneous clusters of stubby microvilli and smooth membrane elsewhere.

(E) CCD-treated embryo (3–6 h postdivision) showing large heterogeneous zones of stubby microvilli (arrow) with intervening smooth membrane.

(F) CCD-treated embryo (0–9 h postdivision) with large clusters of microvilli. Note that these zones contain elongate, ‘spaghetti-like’, microvilli (arrows) lying prostrate to the cell surface.

(G) Region of the apical surface of a cell from a CCD-treated embryo (0–9 h postdivision) showing ‘spaghetti-like’ microvilli at higher magnification.

(H) CCD-treated embryo (8–11 h postdivision) showing a polarized distribution of microvilli with a normal morphology. Arrows denote the periphery of the pole where microvilli are sometimes more prominent. A–F, H, x3900; G, x9600.
Table 4. *Outer surface morphology of zona-free intact 8-cell embryos incubated in CCD (or DMSO in controls) for varying periods prior to analysis by scanning electron microscopy*

<table>
<thead>
<tr>
<th>Treatment time (h postformation)</th>
<th>No. cells scored</th>
<th>Apolar (%)</th>
<th>Polar (%)</th>
<th>Heterogeneous small patches (%)</th>
<th>Heterogeneous large patches (%)</th>
<th>Elongate 'spaghetti-like' microvilli present (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 0–3 h DMSO</td>
<td>237</td>
<td>90.7</td>
<td>9.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(2) 3–6 h DMSO</td>
<td>338</td>
<td>33.1</td>
<td>66.9</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(3) 6–9 h DMSO</td>
<td>393</td>
<td>26.2</td>
<td>73.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(4) 8–11 h DMSO</td>
<td>42</td>
<td>9.5</td>
<td>90.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(5) 0–3 h CCD</td>
<td>149</td>
<td>0</td>
<td>2.6</td>
<td>52.3</td>
<td>45.0</td>
<td>10.7</td>
</tr>
<tr>
<td>(6) 3–6 h CCD</td>
<td>262</td>
<td>0</td>
<td>3.4</td>
<td>19.5</td>
<td>77.1</td>
<td>31.7</td>
</tr>
<tr>
<td>(7) 6–9 h CCD</td>
<td>177</td>
<td>0</td>
<td>37.3</td>
<td>8.5</td>
<td>54.2</td>
<td>53.1</td>
</tr>
<tr>
<td>(8) 8–11 h CCD</td>
<td>110</td>
<td>0</td>
<td>70.0</td>
<td>16.4</td>
<td>13.6</td>
<td>0</td>
</tr>
<tr>
<td>(9) 0–6 h CCD</td>
<td>163</td>
<td>0</td>
<td>0</td>
<td>32.5</td>
<td>67.5</td>
<td>16.6</td>
</tr>
<tr>
<td>(10) 3–9 h CCD</td>
<td>126</td>
<td>0</td>
<td>4.8</td>
<td>23.0</td>
<td>72.2</td>
<td>38.1</td>
</tr>
<tr>
<td>(11) 0–9 h CCD</td>
<td>168</td>
<td>0</td>
<td>3.0</td>
<td>7.7</td>
<td>89.3</td>
<td>32.1</td>
</tr>
</tbody>
</table>
of this technique over others for topographical analysis (by combining high resolution with depth of focus) rather than an indication of artifacts introduced during processing. Thus, for example, the high incidence of large heterogeneous clusters of microvilli recorded on the apical membrane of CCD-treated late 8-cell embryos by SEM (Table 4, column 6, lines 7; 10 and 11) will include those blastomeres where microvilli have localized predominantly into a single focus. However, they are distinguishable from cells with a definitive polar phenotype on the basis of their abnormal microvillous morphology and the absence of a clear relationship between the focus and the points of cell contact. Such blastomeres, especially those containing ‘spaghetti-like’ microvilli where an excess of membrane is localized on the cell surface, could be classified as polar when examined by an alternative technique (e.g. FITC–Con A labelling) and hence may explain the discrepancy in scoring patterns between the different methods of analysis. The status, in terms of cell polarization, of blastomeres from the SEM analysis with modified, but localized, microvilli is considered later in the Discussion.

For each of the techniques used, polarized cell surfaces were not detected earlier in CCD-treated embryos than in controls. Thus, our data do not support the hypothesis that surface polarization is initiated far in advance of overt expression via the gradual segregation of microvilli containing cytochalasin-resistant core microfilaments to the apical membrane (see Introduction). However, we consider that the temporal and spatial patterns of microvillous localization that we have detected following CCD treatment provide evidence in favour of an alternative mechanism to explain surface polarization. An analysis of this evidence is given below.

Each technique demonstrated that throughout the 4-cell stage and during the early period of the 8-cell stage, CCD treatment resulted in the formation of small heterogeneous clusters of microvilli that appeared randomly positioned on the outward-facing cell membrane. Such clusters could either signify localized domains of the cell surface where cortical cytoskeletal elements and their association with the membrane are more stable than elsewhere or they may arise as a direct result of disrupting the microfilament system of the cell with CCD. There is no evidence to favour the first explanation as the apical membrane and associated cytoskeleton appears to be structured homogeneously during early cleavage (Lehtonen & Badley, 1980; Sobel, 1983a,b; Johnson & Maro, 1984; Pratt, 1985) and the modified bleb-like microvilli that predominate following CCD treatment do not appear to contain a stable microfilament organization when examined by TEM. One way cytochalasins are thought to act in vivo is to sever filaments and their network associations (Schliwa, 1982) which, in cultured cells, can lead to an energy-dependent contraction, or release of tension, of the cytoskeleton and the formation of random foci rich in disorganized microfilaments (Weber, Rathke, Osborn & Franke, 1976; Godman, Woda, Kolberg & Beri, 1980; Schliwa, 1982). TEM examination of the heterogeneous clusters of microvilli on experimental embryos showed that a number of the bleb-like protrusions contained disorganized microfilaments and were in fact elevated platforms upon
which slender microvilli with a normal morphology were positioned (see also Pratt et al. 1981). This configuration strongly suggests a destabilization and contraction of the submembraneous cytoskeleton comparable with the effects described above on cultured cells exposed to cytochalasins. Thus, we conclude that the small heterogeneous clusters of modified microvilli detected in 4-cell and early 8-cell embryos are induced by drug treatment and do not represent an inherent asymmetry in the organization of the cytocortex.

During the mid to late 8-cell stage, FITC-Con A labelling and SEM analyses demonstrated that both the heterogeneous zones of microvilli and the intervening regions of smooth membrane became fewer and increased in size in drug-treated embryos whether or not CCD was present from the time of division. This transformation occurred only slightly before or coincident with the time that control embryos began to polarize and would therefore appear to represent an integral stage in the process. The simplest interpretation would be that actin filaments within certain regions of the cytocortex become further stabilized whilst elsewhere they become destabilized, so that in the former regions CCD-induced fragmentation of the cytoskeleton is less extensive than at earlier stages. Stabilization could be achieved either by (a) a shift in the equilibrium between monomeric actin and microfilaments favouring the polymerized state or by (b) the incorporation of actin-binding protein(s) to cross-link elements within the cytocortex and/or to slow down actin turnover within microvillus core filaments. Both α-actinin (Lehtonen & Badley, 1980) and a protein that is cross-reactive with an antibody to avian erythrocyte β-spectrin (Sobel & Alliegro, 1985) have been localized in cortical and cytoplasmic domains during cleavage. Although changes in the distribution of these or other actin-binding proteins have not been detected concurrent with microvillous polarization, a detailed examination specifically at this time has not so far been carried out. The large heterogeneous zones of microvilli observed in CCD-treated cells were distributed randomly on cell surfaces without reference to cell contact points, and would appear to be an intermediate

---

**Fig. 6.** Regions of 4-cell embryos examined by transmission electron microscopy.

(A) Control embryo (3–6 h postdivision in DMSO) showing a homogeneous distribution of microvilli on outward-facing and apposed cell surfaces. Bar, 5 μm.

(B) Region of cell surface from control embryo showing core microfilaments within microvilli (arrowheads) and a reticulate filamentous cytoskeleton associated with the cell membrane (arrow). Bar, 0-1 μm.

(C) CCD-treated embryo (3–6 h postdivision) with microvilli restricted to the contact zones (arrows) and localized regions of the exposed cell surface indicated by arrowheads. Bar, 5 μm.

(D) Intercellular region of CCD-treated embryo showing many of the microvillous profiles to be swollen or bleb-like (arrows). Bar, 1 μm.

(E) Region of outer surface of CCD-treated embryo where microvilli and bleb-like protrusions (arrow) are present. Bar, 1 μm.

(F) Region of exposed surface of CCD-treated embryo at higher magnification. The bleb-like protrusions possess a dense filamentous material whilst elsewhere a submembraneous cytoskeleton is less-clearly discerned. Microvilli, containing core filaments, are shown to originate from the protrusions (arrows), and may project horizontal to the cell surface. Bar, 0.5 μm.
stage in the formation of the polarized FITC–Con A labelling pattern. Thus, whilst the process of cytoskeletal reorganization that leads to progressively larger and finally polar microvillous domains appears to be insensitive to drug treatment, the process by which the orientation of the developing polar focus is regulated is, in contrast, disrupted by drug action. A disturbed orientation of surface poles generated in the presence of CCD has been reported previously (Johnson & Maro, 1984, 1985; Fleming et al. 1986) and may result from the inhibition of cell flattening (Johnson & Maro, 1986; Johnson, Maro & Takeichi, 1986).

The low incidence of surface polarity recorded in drug-treated 8-cell embryos examined by SEM was due in part to the altered morphology of the cell surface and specifically in many cells to the occurrence of elongate ‘spaghetti-like’ microvilli. In TEM sections, microvilli of comparable morphology lying horizontal to the cell surface were found to contain core microfilaments. Similar long microvillus-like processes have been observed after exposure of chick embryonic duodenum to cytochalasins (Noda, 1985) and the presence of long microvilli on cytochalasin-treated late 8-cell mouse embryos has been recorded by Sutherland & Calarco-Gillam (1983). A clear interpretation of the origin and mode of formation of these structures cannot be presented but some clues as to their significance in relation to cell polarization are at hand. ‘Spaghetti-like’ microvilli predominated at a time when control cells had begun to polarize, were localized on the apical membrane in the position normally occupied by polar microvilli, and their incidence was reduced when CCD incubation was initiated soon after division. These facts indicate that their formation may represent a cytochalasin-induced modification of the normal polarization process that unfolds once the axis of polarity has been established free of drug interference. The restriction of microvillous elongation to the apical surface suggests that the generation of surface polarity may involve a localized regulation of microvillous core filament growth and turnover of actin. This view is supported by the SEM observations of Johnson & Maro (1985) indicating that surface polarity may be brought about by the shortening of basolateral microvilli and the lengthening of apical microvilli. However, in the present context it is difficult to reconcile microvillar growth in terms of core filament assembly with the mode of action of CCD in vivo. Cytochalasins prevent polymerization by binding to the ‘barbed’ end of

---

Fig. 7. Regions of 8-cell embryos examined by transmission electron microscopy.

(A) Control embryo (6–9 h postdivision in DMSO) showing polarized microvilli. Apposed membranes (arrows) and the peripheral apical surface appear microvillus free. Bar, 5 μm.

(B) Region of CCD-treated embryo (0–3 h postdivision) showing bleb-like microvilli along the cell contact zone (arrows) but restricted to a small patch on the exposed membrane. Bar, 5 μm.

(C) Blastomere from CCD-treated embryo (0–9 h postdivision) showing evidence of a microvillous pole on the apical membrane. Note the presence of additional microvilli along regions of cell contact (arrows). Bar, 5 μm.

(D) Apical pole region of CCD-treated embryo (6–9 h postdivision) with a prominent population of microvilli that are orientated parallel to the cell surface and containing core filaments (inset, arrowheads). Bar, 1 μm. Inset, 0.5 μm.
microfilaments where monomers normally become incorporated more rapidly, and hence induce a net depolymerization in filaments that are rapidly turning over (Lin, Tobin, Grumet & Lin, 1980; MacLean-Fletcher & Pollard, 1980). However, addition of monomers can still take place in the presence of cytochalasins by binding to the ‘pointed’ end of F-actin both in microvilli (Pollard & Mooseker, 1981; Mooseker, Pollard & Wharton, 1982) and elsewhere (MacLean-Fletcher & Pollard, 1980; Wilkins & Lin, 1981). It is possible, therefore, that the localized increase in the G-actin pool, brought about by CCD-induced depolymerization of more dynamic cortical microfilaments, could initiate uncontrolled growth within the core filaments of apical microvilli that would normally be undergoing assembly as the cell polarizes. Thus, although the SEM analysis, in contrast to FITC–Con A staining, resulted in a low scoring of definitive microvillus poles following extended drug treatments, we consider that the altered morphology of the apical membrane may in fact represent features of surface polarity that have become highly modified.

The ring-type pattern of microvillous pole, detected more frequently in CCD-treated cells (Fig. 5H; see also Johnson & Maro, 1985) than in DMSO-treated controls (Fig. 2C) or completely untreated cells (T. Fleming, unpublished observations), may represent an alternative defect arising from the polarization process. Here, microvilli are restricted primarily to the boundary between proposed regions of microvillous elongation (apical surface) and shortening (basolateral surface) and therefore may result from a spatial aberration in the process regulating core filament length.

The TEM analysis showed that microvilli were present consistently at cell contact sites in precompact CCD-treated embryos. Since all 4-cell categories as well as early 8-cell embryos showed this feature, it is unlikely to relate to cell polarization but rather to processes involved in cell apposition. Contact sites during early cleavage exhibit a specificity in their membrane composition and lipid organization (Izquierdo, Lopez & Marticorena, 1980; Izquierdo & Ebensperger, 1982; Pratt, 1985) and show a contact-dependent depletion of cytoskeletal and cytoplasmic elements compared with the outer surface (Sobel, 1983a, b; Johnson & Maro, 1984; Maro et al. 1985). Thus, the contact-specific zones of bleb-like microvilli would appear to derive from a CCD-induced destabilization and contraction of the modified cytoskeleton in this region of the cell cortex. In contrast to earlier stages, compacted embryos treated with CCD (8–11 h post-division) retained their polar organization and, in TEM sections, were essentially microvillus-free on their basolateral surfaces. However, cells that generated a polar phenotype in the presence of CCD in some cases displayed a microvillus population at and adjacent to cell contacts after TEM (Fig. 7C) and FITC–Con A (Fig. 3G) examination. Thus, one component of the polarization process, the reduction in basal microvilli, can be perturbed by CCD and may involve a local restriction in core filament depolymerization.

In summary, our results indicate that reorganization of the actin cytoskeleton associated with cell surface polarity occurs approximately at the same time that
polarity is expressed overtly, rather than at an earlier period during development. The modifications to surface morphology induced by CCD cannot be interpreted with certainty but provide evidence that the polarization process comprises at least three separate events: (a) the laying down of the orientation of the axis of polarity occurring early during the 8-cell stage (Johnson & Ziomek, 1981) by a mechanism sensitive to CCD treatment, probably as a result of the drug's antiflattening action (Johnson & Maro, 1986; Johnson et al. 1986); (b) the subsequent destabilization of microvilli in the contact areas and their stabilization on the exposed cell surface induced by CCD-insensitive changes in the organization of the underlying cortical cytoskeleton; (c) the expression of the overt polar phenotype controlled by the reciprocal alteration of microvillar length at apical and basolateral surfaces; the mechanism allowing elongation of apical microvilli by net growth of core microfilaments may be triggered in the presence of CCD but does not seem to be regulated properly leading to abnormal polar morphologies. Although this model for cell surface polarity is based upon indirect evidence, it provides a foundation for more directed experimental approaches, such as an analysis of the temporal and spatial expression of actin-regulating proteins. These studies are now underway in our laboratory.

We wish to thank Dr M. H. Johnson and our research colleagues for their helpful criticisms of the manuscript, Roger Liles, Tim Crane and Ian Edgar for photographic work and Sheena Glenister for typing the manuscript. The study was supported by grants from the Medical Research Council to Dr M. H. Johnson and Dr P. R. Braude, the Cancer Research Campaign to Dr M. H. Johnson, Dr H. P. M. Pratt and Dr R. T. Hunt, the Foundation pour la Recherche Medicale to B. M., and the H. E. Durham Fund, King's College, Cambridge to F. Q. B. M. is an EMBO Research Fellow.

REFERENCES


*(Accepted 4 February 1986)*