Differential mitosis and degeneration patterns in relation to the alterations in the shape of the embryonic ectoderm of early post-implantation mouse embryos

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SUMMARY

The shape of the embryonic ectoderm of early post-implantation mouse embryos changes greatly in the period of 6.2–7.3 days post coitum. The subcellular morphology of the embryonic ectoderm remains unchanged, except in the primitive-streak region. Cell kinetics differ between ectodermal regions. These differences may be related to the changes in the shape of the ectoderm. The increase in cell number in the lateral ectoderm (the prospective surface ectoderm) exceeds that in the frontal ectoderm (the future neurectoderm). This is not due to differences in the duration of the cell cycle. It can be explained, however, by the occurrence of different relative numbers of dividing and non-dividing cells. These numbers vary between the two regions. The percentage of non-dividing cells in the frontal ectoderm may reach 45, whereas in the lateral ectoderm this percentage is not higher than 15. Autoradiography in tritiated thymidine-treated embryos combined with the mitotic indices gave us all of the parameters necessary to present a model capable of clarifying the growth of the ectoderm during gastrulation, as well as the changes in the shape of the ectoderm.

INTRODUCTION

Between 6.2 and 7.3 days post coitum the shape of the mouse ectoderm is transformed from a cylinder consisting of an ectodermal epithelium containing the proamniotic cavity and surrounded by endoderm, into a more complex one. During this transformation, the first signs of the onset of neurectoderm, primitive streak, and neural crest become visible. Transformations of an epithelium, as described here, are thought to be evoked by structural alterations of the cells, such as the appearance and functioning of microfilaments (see Wessels et al. 1971; Karfunkel, 1971) and certain properties of cell membranes and associated structures (see Hay & Meier, 1974; Bluemink, Maurik & Lawson, 1976; Kaprio, 1977).

An alternative set of clues to the mechanisms underlying the epithelial transformation is provided by differences in cell kinetics in the various regions

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of the ectoderm. Volumetric growth of early embryonic stages has been described by Snow (1976, 1977, 1978) on the basis of studies on primary mesoderm formation related to an allometric growth of the ectoderm. Entire embryos and organs in later stages of development have been studied by Goedbloed (1972, 1976, 1977), who described discontinuities in the growth curves for both the embryo as a whole and its organs. The present study was performed in an attempt to elucidate the principle mechanism involved in the morphological transformation described above. Therefore, the data presented here concern not only details that might be involved in epithelial transformation but also differences in cell kinetics in two distinct regions of the ectoderm. An alternative model derived from cell counts, mitotic indices, and cell cycle parameters will be presented and the compatibility of the early growth of the embryonic ectoderm and the alterations in its shape will be discussed.

MATERIALS AND METHODS

Swiss mice of the CPB-S strain were used throughout this study. Males and females were kept separately and only brought together for one period of 16 h (overnight). The moment of conception (day 0) was arbitrarily put at midnight of the night preceding the morning on which the vaginal plug was found. Pregnant mice were laparotomized and perfused at room temperature via the left ventricle with the following solutions: (1) physiological saline containing 0.5% heparin and 0.5% sodium nitrite, used to remove the blood (5 min); and (2) a modified Karnovsky (1965) fixative consisting of 2% formaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate, buffered at pH 7.4 (15–20 min). In addition, the implantation sites were left in the same fixative for 4–16 h.

The implantation sites used for electron microscopy were sectioned at 150–200 μm on an Oxford vibratome; those used for light microscopy were left intact. After post fixation in 1% cacodylate-buffered OsO₄ for 1–3 h, the material was dehydrated in a graded series of ethanol and consecutively embedded in Epon. Sections were cut on an LKB III or on a Reichert Omu 3 Ultrotome. For light microscopy, serial-sectioning was performed according to Roberts & Hutcheson (1975), whole embryos being cut into 1 μm-thick sections without any noticeable loss of material. These sections were stained with hot toluidine blue.

Because mouse embryos almost always implant anti-mesometrially (Snell & Stevens, 1966; Kirby, Potts & Wilson, 1967; Gardner, 1977), the direction of sectioning is easily determined in relation to the uterine outline. Cardboard reconstructions were made of a number of embryos of each stage described in this study, to obtain correct three-dimensional images. For this purpose, use was made of the 1 μm serial sections. Ultrathin sections were contrasted with lead hydroxide and uranyl acetate and examined in a Philips 201 electron microscope.
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Colchicine treatment

Before laparotomy pregnant mice received either one intraperitoneal injection of 0.003 mg colchicine in physiological saline per gram body weight (1–8 h), or at most three injections of 0.003 mg colchicine per gram body weight at 1 h intervals.

Autoradiography

Pregnant mice received one intraperitoneal injection of [methyl-3H]thymidine, (purchased from The Radiochemical Centre, Amersham, England), with a specific activity of 45 Ci/mmol. The aqueous solution contained 100 μCi thymidine. Thirty minutes later an injection of unlabelled thymidine was given to chase the unbound labelled thymidine still present in the circulation and in the tissues. In this way a ½ h ‘pulse’ labelling was obtained. Two pregnant females were given other injections as well: the first injection of 50 μCi [3H]thymidine was followed at 30 min intervals by 25 μCi [3H]thymidine, over a 2 h period to obtain prolonged and more or less continuous labelling of the embryos. Laparatomy was performed 1–10 h after the first injection. For each group two embryos were used. Since the duplicates were quite similar (see Fig. 10) more embryos per interval were not required. In addition, two pregnant females were laparotomized 15 min after receiving 100/μCi [3H]thymidine. The embryos were processed as described above. Slides with unstained, uncoated, serial 1 μm sections were dipped in liquid K2 (Ilford) emulsion and stored at 4 °C in the dark for 3 weeks. The emulsion was developed in Kodak D19b for 4 min at 18 °C. After photographic processing the sections were briefly stained with hot (60 °C) toluidine blue. Tritium did not affect the viability of the cells, expressed as the percentage of degenerating cells, as was reported by Snow (1973a, b) for younger stages.

GENERAL REMARKS

Regions

For this study the embryonic ectoderm was divided into regions, except in preprimitive streak embryos (e.g. 6-2-day-old embryos) where the ectoderm was taken as a unit. The borders between the regions were chosen arbitrarily, but objectively. The ectoderm of 6-6-day-old embryos was divided into four regions, i.e. the frontal ectoderm, two shields of lateral ectoderm, and the primitive streak ectoderm. The borderlines between frontal and lateral ectoderm were taken at the place where the greatest diameter of the nuclei lay perpendicular to the antero-posterior axis of the ectoderm. The use of the expression frontal ectoderm is not meant to imply a prediction concerning the fate of this part of the ectoderm in neural tissue formation. It is obvious that part of this region becomes neural tissue, but which part remains unclear. In 7-3-day-old embryos
the border lies in the concavities of the ectoderm (see the figure accompanying Table 1). The border between lateral and primitive streak ectoderm was taken at the site where an interruption in the basal lamina is seen in the electron microscope. In practice, this corresponds with a line drawn under the light microscope, perpendicular to the antero-posterior axis.

The lateral ectoderm may give rise to the epidermal epithelium and the amnion, but again, no prediction concerning the fate of the lateral ectoderm is implied.

Embryonic age

The embryos used in this study were divided into three groups. The average age of each group, i.e. 6-2, 6-6 and 7-3 days, is a reasonably exact estimation, because the morphology of the embryos corresponds with embryos of the same strain used by Goedbloed (1972) who determined the age on a volumetric base. For comparison, the stages of Theiler (1972) are mentioned, but since these concern another strain, his time tables may differ somewhat from ours.

DESCRIPTION

Morphology of 6-2-day embryonic ectoderm (stage 9 of Theiler) (Fig. 1)

The ectoderm is more or less globular, giving circular contours in sections, and is clearly delimited from the surrounding endoderm by a continuous basal lamina. No regional differences in cellular morphology can be distinguished within the epithelium that might be related to the formation of the prospective primitive streak. The ectoderm contains a very small proamniotic cavity only a few microns wide, the formation of which has been described elsewhere (Poelmann, 1975).

Morphology of 6-6-day embryonic ectoderm (primitive-streak stage, stage 10 of Theiler) (Figs. 2-4)

During the next day of development the shape of the ectoderm changes from the globular via a grossly cylindrical into a more complex shape.

In transverse sections the frontal ectoderm remains semicircular, whereas the lateral ectoderm shields stretch posteriorly. These extend to the primitive streak region but remain separated from each other by the proamniotic cavity (Figs. 2 and 3).

In sagittal sections the frontal ectoderm and the primitive streak are continuous with each other. Together they comprise the axial ectoderm.

The proamniotic cavity is expanded considerably. Its contour follows the general contour of the ectoderm very closely, being wide in the anterior part and very narrow in the middle and posterior parts. The proamniotic cavity is usually asymmetric, being more pronounced at one of the anterior margins (Fig. 3). A three-dimensional representation is given in Fig. 4, which shows
Fig. 1. Micrograph of a 1 μm section of a 6-2-day-old embryo stained with toluidine blue. The direction of sectioning was slightly obliquely longitudinal. A small proamniotic cavity is present. No mesoderm is visible yet. × 320.

Fig. 2. Frontal section of a 6-6-day-old embryo shows two wings of mesoderm. The primitive streak and the frontal ectoderm are not present in this plane of sectioning. × 360.

LIST OF ABBREVIATIONS

| A  | amnion                                                      |
| Ac | amniotic cavity                                            |
| ect | ectoderm                                                  |
| e. ect | extra-embryonic ectoderm                                   |
| end | endoderm                                                   |
| Ex | exocoelom                                                  |
| fe | frontal ectoderm                                           |
| le | lateral ectoderm                                           |
| mes | mesoderm                                                   |
| mN | micronucleus                                               |
| N  | nucleus                                                    |
| Pa | proamniotic cavity                                         |
| ps | primitive streak                                           |
| troph | trophoblast                                              |
| ysc | yolk sac cavity                                           |
Figs. 3 and 4. A transversely sectioned 6-6-day-old embryo reconstructed on the basis of a projection drawing of some of the 1 μm sections. Fig. 3 is the fourth section in Fig. 4. The primitive streak is located on the left side, the neurectoderm on the opposite side. The mesoderm is lightly shaded. × 190.

Figs. 5 and 6. The same procedure as for Figs. 3 and 4 was applied to a 7-3-day-old embryo. Fig. 5 is the fourth section in the reconstruction represented in Fig. 6. In Fig. 5 the primitive streak is again on the left and the frontal ectoderm on the right. Note the expansion of the lateral ectoderm and the proamniotic cavity compared with the younger embryo of Fig. 3. For Fig. 6 the sections were turned about 90° clockwise to obtain a better view of the various layers. The bilayered amnion is visible together with a part of the extra-embryonic mesoderm and the exocoelom. The mesoderm is not shaded. × 160.
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Table 1. Dimensions of two representative embryos (see also Figs. 3–6)

<table>
<thead>
<tr>
<th></th>
<th>6-6-day-old (μm)</th>
<th>7-3-day-old (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length*</td>
<td>300</td>
<td>400</td>
</tr>
<tr>
<td>Height†</td>
<td>320</td>
<td>410</td>
</tr>
<tr>
<td>Largest circumference*</td>
<td>740</td>
<td>1280</td>
</tr>
<tr>
<td>Frontal ectoderm (fe)</td>
<td>160</td>
<td>170</td>
</tr>
<tr>
<td>Primitive streak (ps)</td>
<td>160</td>
<td>140</td>
</tr>
<tr>
<td>Lateral ectoderm (le)</td>
<td>420</td>
<td>970</td>
</tr>
</tbody>
</table>

* Measured in transverse sections.
† Number of 1 μm sections in which embryonic ectoderm is visible.

clearly the lateral mesoderm and the axial continuity of the frontal ectoderm with the primitive streak.

Morphology of 7-3-day embryonic ectoderm (head-process stage, stage 11 of Theiler) (Figs. 5 and 6)

Of the ectodermal regions, i.e. the primitive streak and the lateral and frontal ectoderm, only the lateral ectoderm has changed very much in shape. It now bulges outward, making the proamniotic cavity wider (maximally about 300 μm) compared with younger embryos. The width of the primitive streak is approximately the same as in the 6-6-day-old embryo, but it has lengthened in the antero-posterior direction. The same holds for the frontal ectoderm.

Some dimensions of 6-6- and 7-3-day-old embryos are given in Table 1. These values show clearly the considerable increase in the length of the lateral ectoderm; the frontal ectoderm and the primitive streak show no growth at all in a transverse direction. The increase in height from 320 to 410 μm affects all
regions equally. A semi-schematic reconstruction based on 1 μm sections is given in Fig. 6.

Along the lateral margins of the frontal ectoderm, the first signs of the onset of neural-crest formation are visible (Vermeij-Keers & Poelmann, 1980). During this stage only a few cells are involved and they lie in limited areas of the ectoderm, where they acquire a mesenchymatous appearance. This situation resembles a discontinuous primitive streak region on a limited scale. It is conceivable that the mesectodermal cells of the neural crest in older stages derive from these foci of ectodermal disorganization. Similar focal sites of disruption can also be found in other areas of the lateral ectoderm in 6-6-day-old embryos, but it is often difficult to ascertain the nature of the cells involved.

**Cellular and subcellular structure of the embryonic ectoderm**

The subcellular morphology of the ectodermal cells has been studied extensively. However, it is not the scope of this paper to present a detailed description of these cells. It is sufficient to state that the characteristics of the ectodermal cells did not vary between stages or regions, except the primitive streak.

**CELL POPULATION KINETICS**

Many mitoses (see also below) are encountered in the ectoderm. When a cell enters mitosis, it becomes spherical by withdrawing its basal extensions, and shifts to the apical part of the epithelium. The surrounding cells close the resulting space incompletely, leaving large intercellular clefts extending from the dividing cells to the basal lamina.

In a fast-growing tissue it is not surprising to find many dividing cells. More striking is the large number of degenerating cells. For a detailed description of the stages of degeneration and phagocytosis by neighbouring cells, the reader is referred to Poelmann & Vermeij-Keers (1976). Degenerated cells are encountered in all parts of the ectoderm and sometimes also in the mesoderm. Only in a few cases it is observed that degenerating cells fragment prior to or after phagocytosis. It is obvious that the number of the scored degenerations is enhanced, but there is no reason to assume that degenerating cells behave different in various regions. Therefore, the degeneration index of the regions is comparable.

Degenerative and mitotic figures are not evenly distributed among the interphase ectoderm cells. Mitotic figures often occur in small groups varying in size among embryos. Degenerative figures are also found in groups. In general, these groups have about the same localization in different embryos. Clusters of degenerating cells are often found in the frontal ectoderm, not far from the borderline with the lateral ectoderm. It is believed that in older stages this area of the frontal ectoderm becomes the region along which neural tube closure is established, because 7-3-day-old embryos show clumps of degenerating cells at the lateral margins of the frontal ectoderm, and during neural tube closure
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Fig. 7(a) Frequency diagram giving the distributions of maximum nuclear diameters in sections through nuclei in the frontal (line) and lateral (interrupted line) ectoderm. The two populations do not differ as to nuclear length. (b) Corresponding diagram for the distribution of minimum nuclear diameters in sections through nuclei as compared with the distributions of minimum diameters in sections through (randomly dispersed) hypothetical nuclei with a width of 10.5 μm (small dots) and 11.0 μm (large dots) respectively. The mean minimum diameter of the measured nuclei is assumed to be 10.8 μm in cross-section.

degenerating cells are still visible in the fusion zone of both neural walls (Glücksmann, 1951; Schlüter, 1973; Geelen & Langman, 1977).

Determination of mitotic index and degeneration index

The indices of mitosis and degeneration were determined in 1 μm transverse sections of untreated embryos. In these embryos the total cell number was estimated in the following way, illustrated by an embryo from the 6-6-day-old group. A large number of nuclei (in this case 162) were measured in both the lateral and frontal regions of the ectoderm, and the smallest and largest dimensions plotted as frequency diagrams (Fig. 7a, b). From these diagrams it is clear that the nuclear dimensions do not differ between the two regions. Calculations based on these frequency distributions give an average smallest diameter of 10.8 μm (Fig. 7b). Since the largest dimension of the nuclei occurs in the cutting direction, due to their orientation in the ectoderm, interphase nuclei were counted in every tenth section and the total number was considered to represent the interphase cell population. In addition, frequency distributions of different embryos were compared to check the direction of sectioning. For this purpose
Table 2. Mitotic and degeneration indices

<table>
<thead>
<tr>
<th>Embryo</th>
<th>MI</th>
<th>DI</th>
<th>Embryo</th>
<th>MI</th>
<th>DI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.0</td>
<td>11.8</td>
<td>H</td>
<td>4.9-8.0</td>
<td>12.0-6.5</td>
</tr>
<tr>
<td>B</td>
<td>3.9</td>
<td>25.8</td>
<td>I</td>
<td>7.7-14.6</td>
<td>17.5-6.0</td>
</tr>
<tr>
<td>C</td>
<td>7.0</td>
<td>3.3</td>
<td>J</td>
<td>3.1-8.9</td>
<td>18.0-7.1</td>
</tr>
<tr>
<td>D</td>
<td>5.0</td>
<td>8.8</td>
<td>K</td>
<td>4.3-7.7</td>
<td>9.7-3.0</td>
</tr>
<tr>
<td>E</td>
<td>4.9</td>
<td>9.5</td>
<td>L</td>
<td>5.4-9.5</td>
<td>16.2-3.8</td>
</tr>
<tr>
<td>F</td>
<td>5.4</td>
<td>9.8</td>
<td>M</td>
<td>7.5-13.3</td>
<td>19.9-7.5</td>
</tr>
<tr>
<td>G</td>
<td>4.3</td>
<td>7.7</td>
<td>N</td>
<td>5.9-9.2</td>
<td>16.6-5.0</td>
</tr>
<tr>
<td>O</td>
<td>7.8*</td>
<td>7.2*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>6.9-7.8</td>
<td></td>
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</tr>
<tr>
<td>Q</td>
<td>3.2-3.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>5.1-8.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>S</td>
<td>1.9-4.5</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>T</td>
<td>4.9-5.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean:</td>
<td>4.9*</td>
<td>11.0*</td>
<td></td>
<td>5.3*-8.3</td>
<td>13.5*-4.9</td>
</tr>
</tbody>
</table>

* Not significant.

MI and DI in 6-2-day-old embryos are given for the total ectoderm, in 6-6-day-old embryos they are given for the frontal and the lateral ectoderm, respectively.

7 embryos of the 6-2-day-old group and 13 of the 6-6-day-old group were used. Mitotic figures and degenerating cells were individually followed through the series of sections and in successive sections only 'new' metaphases-anaphases and degenerations were counted, which provided very exact numbers. These were compared with estimated numbers of interphase nuclei, the result giving the mitotic index (MI, i.e. the percentage of mitotic figures within a given cell population), and the degeneration index (DI, the percentage of degenerating cells). Table 2 gives the MI and DI for the total ectoderm of 6-2-day-old embryos and for the frontal and lateral ectoderm of 6-6-day-old embryos. A high percentage of degeneration in any given region always coincides with a low percentage of mitosis in the same region, and vice versa. The lateral ectoderm of 6-6-day-old embryos shows a high MI–low DI picture, whereas the frontal ectoderm of 6-6-day-old embryos and the total ectoderm of 6-2-day-old embryos is low MI–high DI.

Student's test was applied to each embryo of the 6-6-day group. In all but one of the individual embryos, the DI and MI of the lateral ectoderm proved to differ significantly from those in the frontal ectoderm. The total ectoderm of the 6-2-day group and the frontal ectoderm of day 6-6 were not significantly different. However, here again the lateral ectoderm differed significantly from the younger ectoderm. The primitive streak resembled the 6-2-day ectoderm and the 6-6-day frontal ectoderm, but absolute cell numbers in the primitive streak were too low to permit statistical analysis in this region.
Fig. 8. After prolonged (4 h) treatment with colchicine, the shape of the embryo is so severely altered that individual cell layers are hardly distinguishable. Many cells are expelled into the proamniotic cavity (arrows). ×150.

Fig. 9. Cells arrested in mitosis by colchicine treatment. Such cells characteristically do not regain their normal nuclear constitution. Around individual chromosomes or groups of chromosomes there is a 'nuclear' membrane, separating the genetic material into a large number of micronuclei. ×6600.

The cell counts showed distinct differences between the ectodermal regions with respect to mitotic and degenerative behaviour. The different percentages of mitotic (and degenerating) cells might be explained in three ways: a varying duration of the cell cycle, different mitotic times, and different subpopulations of dividing cells. Colchicine administration or autoradiography in tritiated thymidine-treated embryos was expected to provide evidence concerning predominance of one of these possibilities.

Colchicine-treated embryos

Colchicine was administered to arrest the mitotic cycle in metaphase for known periods of time. In this way the total number of cells entering metaphase during these periods could be counted. Used in low concentrations and for a limited time (up to 4 h), colchicine did not interfere with normal development, but it did not affect mitotic and degeneration indices either. After repeated injections, or extended periods of time, however, the shape of the embryo was so severely altered that the MI and DI could not be determined. The ectodermal epithelium disintegrated into globular individual cells, and a large number of cells were expelled into the proamniotic cavity. The frontal ectoderm retained its shape
to a varying degree, the other ectodermal regions expanded widely, giving rise to a large proamniotic cavity (Fig. 8). In 6·6-day-old embryos the apical membranes of the lateral ectoderm cells were far apart, which is quite different to the situation in untreated embryos.

The cells apparently did not pass through the metaphase, that is, segregation of chromosomes did not occur. Chromosomes became enclosed by a membrane resembling the nuclear envelope of normal interphase nuclei. In this way such cells acquired numerous micronuclei (Fig. 9), each containing chromatin of one or a few chromosomes.

Autoradiography

Tritiated thymidine was injected into pregnant mice about 6 days post coitum. Every hour from 1–10 h after injection, mice were killed and two embryos were excised. Counts in transversely sectioned embryos were performed as described above. Labelling was assessed in metaphase-anaphase figures (mounting up to 100% labelled figures), interphase nuclei, and degenerating cells. Background labelling was usually very low, at most 30 grains per 100 nuclei. It was determined over the non-dividing juxta-embryonic decidua. A cell was considered to be labelled when two or more grains were present above the nucleus or the chromosomes. The results for the two age groups (6·2- and 6·6-day-old embryos) were similar with respect of their time dependence and are presented together in Fig. 10. The cell cycle duration derived from the 50% intercepts amounted to 7·6 h and the S phase to 6·4 h, and is the same for embryos of both age groups and for the various ectodermal regions.

In each embryo, many interphase nuclei (varying from 30 to 50%) remained unlabelled, irrespective of the time and duration of labelling. It therefore seems reasonable to assume that a number of cells do not enter the S phase. Degenerating cells were also labelled, but the percentages were invariably below 25% and did not appear to be time dependent either. The results indicate the existence of a non-dividing subpopulation within the embryonic ectoderm of both the 6·2-day-old and the 6·6-day-old embryos. The size of the non-dividing subpopulation is not exactly known, but can be estimated with the following formula:

\[ X = \frac{C}{M} \times MI \]

in which \( X \) is the percentage of dividing cells, \( C \) the cell cycle duration, \( M \) the mitotic duration, and \( MI \) the mitotic index. The mitotic duration is not known, but is reported (see Lushbaugh, 1956) to be rather short, at most about 45 min. If the mitotic duration is the same for the lateral and the frontal ectoderm of 6·6-day-old embryos, the minimum duration is 35 min (Fig. 11).

Substitution of these two values in the above formula indicates that the size of the non-dividing subpopulation in the frontal ectoderm varies between 35 and
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Fig. 10. Tritiated thymidine-treated embryos were killed at the indicated intervals after injection. The labelled and non-labelled mitotic figures were counted separately and the results compared. The time between the first and second 50% intercepts gives the duration of the S-phase (6.4 h); the cycle time (between the first and third 50% intercepts) is 7.6 h. Solid circles: ectoderm of 6-2-day-old embryos; open circles: frontal ectoderm of 6-6-day-old embryos; squares: lateral ectoderm of 6-6-day-old embryos.

Fig. 11. The size of the non-dividing subpopulation in relation to the duration of mitosis. Since the latter is assumed to be 0.6-0.75 h (see text), the size of the non-dividing subpopulation in the frontal and lateral ectoderm can be calculated.

45% and that in the lateral ectoderm from 0 to 15% of the entire cell population (Fig. 11). Now that all parameters are known (e.g. the cell number in a region, the size of the dividing subpopulation, the duration of the cell cycle, and the estimated ages of the groups of embryos), it is possible to predict the ectoderm cell numbers of consecutive stages and to compare them with counted cell numbers of preceding or later stages of development. The 6-6-day group (all regions are developed in this stage) can serve as example. The numbers of
Table 3. Counted and predicted cell numbers

<table>
<thead>
<tr>
<th>6-2-day-old (total ect.)</th>
<th>Predicted from</th>
<th>6-6-day-old (front. ect.-lat. ect.)</th>
<th>Predicted from</th>
<th>7-3-day-old (front. ect.-lat. ect.)</th>
<th>Predicted from</th>
</tr>
</thead>
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<tr>
<td>Counted 6-6-day</td>
<td>Counted 7-3-day</td>
<td>Counted 7-3-day</td>
<td>Counted 6-6-day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>346</td>
<td>435</td>
<td>320-445</td>
<td>—</td>
<td>892-1780</td>
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<tr>
<td>459</td>
<td>504</td>
<td>356-524</td>
<td>—</td>
<td>975-2096</td>
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<td>440-605</td>
<td>369-609</td>
<td>1233-2420</td>
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<td>591</td>
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<td>474-608</td>
<td>—</td>
<td>1323-2432</td>
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<tr>
<td>—</td>
<td>639</td>
<td>516-591</td>
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<tr>
<td>—</td>
<td>776</td>
<td>551-818</td>
<td>—</td>
<td>1536-3272</td>
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<tr>
<td>—</td>
<td>825</td>
<td>623-819</td>
<td>—</td>
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<tr>
<td>1308</td>
<td>945</td>
<td>755-885</td>
<td>—</td>
<td>2106-3740</td>
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<tr>
<td>1351</td>
<td>1273</td>
<td>933-1304</td>
<td>—</td>
<td>2602-5216</td>
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</tbody>
</table>

Cell numbers of 6-6-day-old embryos are given in the fourth column (frontal ectoderm-lateral ectoderm). With these numbers, the cell numbers of older and younger embryos can be computed. For this purpose the following parameters are used: cycle length = 7.6 h; $M_{\text{front. ect.}} = 5.3\%$; $M_{\text{lat. ect.}} = 8.3\%$; duration of mitosis = 0.6 h.

The size of the dividing subpopulation is calculated with the formula: $X = (C \times M_I) / M$; the non-dividing subpopulation $Y$ equals the whole population $P$ minus the dividing population $X$. The size of an embryo, one cell cycle younger, is calculated with the formula: $\frac{1}{2}(P + Y)_{\text{(front, ect.)}} + \frac{1}{2}(P + Y)_{\text{(lat. ect.)}}$. The size of an embryo two cycles older is calculated in two steps: $2P - Y_{\text{(front, ect.)}} + 2P - Y_{\text{(lat. ect.)}}$. Only one 7-3-day-old embryo was available for calculating the size of a 6-6- and 6-2-day-old embryo.

Embryonic ectoderm cells in 6-2-day-old embryos, which are one cell cycle younger, and of 7-3-day-old embryos, which are two cycles older can be predicted (Table 3). According to Wilcoxon's test the numbers given in the first two columns of Table 3 do not differ significantly from each other.

**DISCUSSION**

Morphogenesis is the result of a number of interlocking processes, including proliferation, cell degeneration, and differentiation, although it must be kept in mind that not all of these processes may occur simultaneously. An attempt was made to describe and analyse the alterations in shape occurring in the early post-implantation mouse embryo with help of local differences in differentiation and cell population kinetics. If ultrastructural differences play a role in alterations of the shape of the embryonic ectoderm, they could be expected to occur during the transition from the 6-2- to the 6-6-day stage, in which distinct morphological alterations take place. Various organelles may be involved in differentiation, e.g. plasma membranes and attached structures, microfilaments and microtubules, and cellular junctions.

In the developing mouse embryo it is clear that changes in subcellular
structures have not been encountered during the described alterations in shape. Furthermore, no differences have been found between lateral and frontal ectoderm. Thus, the subcellular changes observed so far cannot adequately explain the changes in morphology of the embryonic ectoderm.

Other processes involved in morphogenesis include cell division and cell degeneration. The mitotic index gives a general idea of the relative increase in cell number, whereas the degeneration index gives an impression of its relative decrease. If, however, these indices are used in the calculation of the net increase in cell number, it is necessary to take into account the difference in the intervals required for completion of mitosis and degeneration, respectively. A cell cycle may take about 8 h, the process of mitosis not more than about 35-45 min. The visibility of a degenerated cell that has been phagocytosed may persist for many more hours, up to one day. Therefore, the actual number of degenerating cells may reflect the number of degenerations during a period of up to one day. Although the consecutive stages of degeneration have been described before (Poelmann & Vermeij-Keers, 1976), no time schedule was given for this process. If cell degeneration is assumed to be a random process, the number of cells degenerating per hour is very small compared with the total cell number. However, the cells that started to degenerate the day before, were part of a population comprising fewer cells. Cell degeneration has been reported to occur on an extensive scale in the earlier stages (El-Shershaby & Hinchliffe, 1974; El-Shershaby, 1974), and may contribute to the DI in our stage.

Possible roles of degeneration within the ectoderm

As early as 1926, Ernst drew attention to the process of cell degeneration. Since then, much work has been done on accumulating degenerative events (for references see Glücksmann, 1951, 1966; Saunders, 1966; Poelmann & Vermeij-Keers, 1976; Silver, 1978). The functional significance of cell degeneration is often unclear, because this process occurs very commonly during development of a great number of tissues and organs, and can seldom be related to a specific morphological differentiation. In the present case two different events could be related to cell degeneration: the formation of neurectoderm with subsequent closure of the neural tube (Menkes, 1968; Schlüter, 1973; Geelen & Langman, 1977), and the formation of the neural crest (Vermeij-Keers & Poelmann, 1980).

Cell kinetics in different parts of the ectoderm

Colchicine is a drug known to disrupt microtubules. It was expected that dividing cells could be arrested in metaphase and that conclusions could be drawn from the number of cells entering metaphase per time unit. This did not prove to be the case, because a low dose of colchicine did not lead to a change in the percentage of mitosis, whereas a higher dose or prolonged treatment killed the embryos. This was in accordance with the results obtained by Kerr (1947) in a study on the breakdown of embryos and their resorption by the uterus. In
cell biology colchicine seems to be a useful technique for the study of e.g. nuclear-cytoplasmic interactions (Shay & Clark, 1977), but seems to have no value for the investigation of cell kinetics in tissues or organs, that must retain their natural spatial coherence. The occurrence of micronuclei after colchicine treatment was first reported by Phillips & Phillips (1969) in cell cultures.

The mitotic index differs significantly between the frontal and lateral ectoderm, being highest for the latter. The MI of pre-primitive streak embryos is the same as that of the frontal ectoderm of primitive streak stages. Further investigation of the cell cycle revealed that the time required for the S phase (6-4 h) and the total cell cycle (7-6 h) is the same for both stages and all regions considered. At first it seems surprising that regions with different mitotic indices show the same cell cycle characteristics. However, the mitotic index is estimated as the number of metaphase-anaphase figures compared with the whole cell population. It is incorrect to compare the mitotic index with cell cycle time, except under the following conditions: (a) cells enter mitosis randomly, (b) all cells divide, and (c) daughter cells are capable of dividing too (see also Snow, 1977). In view of the loss of cells by degeneration, it is doubtful whether conditions (b) and (c) pertain. It is also unlikely that cells ‘preparing’ for degeneration (but not showing it yet) are involved in the incorporation of tritiated thymidine, although a small number of degenerated cells appear to be labelled. One must also take into consideration the finding that even after prolonged treatment with thymidine, a considerable number of so-called interphase cells are not labelled, thus indicating that a subpopulation of non-dividing cells exists. This is in conflict with the report of Solter, Skreb & Damjanov (1971), who found labelling of all cells in autoradiograms of thick sections. On purely theoretical grounds, however, their findings must be regarded with great reserve (see Snow, 1976). It is therefore concluded that a relatively large number of cells do not enter mitosis within the interval investigated. Some of these cells may degenerate. Interpretation of the labelling index of interphase nuclei seems to be very difficult. It requires not only knowledge about cell cycle parameters, but also the knowing whether labelled interphase nuclei are still in the S phase or have divided already. Therefore, the interphase labelling index may not be used quantitatively.

The percentages of non-dividing cells in the frontal ectoderm (35–40 %) and lateral ectoderm (0–15 %) correspond nicely with the DI in both regions, being 13-5 % and 4-9 %, respectively. The autoradiographically determined cycle time must be considered as an average of the dividing population, and therefore does not represent the doubling time of the whole population.

Goedbloed (1977) using the same strain, determined volumetrically a doubling time of 8 h for the ectoderm as a whole, and Solter et al. (1971) found autoradiographically a cycle time of approximately 6-5 h, which is slightly less than that reported in this study. In the other two studies however, the ectoderm was considered as a unit and was not subdivided into regions.
Differential mitosis and degeneration pattern in mouse embryos

Fig. 12. These sketches express the proposed form changes of the ectoderm related to local differences in mitosis and degeneration. The lateral ectoderm exhibits a faster growth compared with the neur ectoderm. The directions of growth are perpendicular to each other. The third dimension is omitted, because both regions grow approximately at the same rate in that direction.

The cell numbers reported by Snow (1976, 1977) for mouse embryos in consecutive stages would require a considerably shorter doubling time than those mentioned by Goedbloed, Solter et al. (1971) and ourselves. Snow (1977, 1978) postulated a small number of rapidly dividing cells located in the axial ectoderm immediately anterior to the primitive streak, which is supported by the finding of a high MI in the same region. This so-called proliferation zone would account for the massive mesoderm formation through the primitive streak. In the present study this part of the ectoderm was not taken into consideration for the reasons mentioned above, and therefore we cannot provide support for the hypothesis concerning a proliferation zone, or evidence contradicting this concept. Snow, however, did not take into account the changes in shape occurring in the embryonic ectoderm, i.e. the subject of the present study. The model presented here is based not only on cell numbers and MI, but also on autoradiographically obtained cell cycle parameters. Hence, the increase in cell number in the various regions can serve as a control for the described alterations in shape. The dimensions of lateral ectoderm and axial ectoderm (taking the frontal and the primitive streak ectoderm together) in consecutive stages (Table 1), make it clear that growth of the axial ectoderm is essentially one-dimensional in a rostro-caudal direction, whereas the lateral ectoderm shows two-dimensional growth. Fig. 12 gives a schematic model of the postulated growth pattern of the ectodermal parts in transverse sections, incorporating the finding that the lateral ectoderm grows faster and in a different direction than the axial ectoderm. It is assumed in this model that cells retain their original position and do not shift, at least not to any considerable extent, to another region, i.e. from the frontal to the lateral ectoderm, or vice versa. It is also very unlikely that the differences in MI described above are evoked by a shift of cells from one region to another, because in that case the cell shift would be very selective, involving only either dividing or non-dividing cells.

In general it may be concluded that a change in shape of a tissue is adequately
explained by differential cell kinetics, apart from ultrastructural differentiation. In the present study the analysis was simplified by the fact that the tissue is a unilayered epithelium that does not undergo three-dimensional growth, but rather two-dimensional growth in the case of the lateral ectoderm and a one-dimensional growth in the case of the frontal ectoderm.

The author wishes to express his gratitude to Liesbeth Hillmann for her skilful assistance, to J. Tinkelenberg for reconstructing some of the embryos, and to L. D. C. Verschragen and C. J. v.d. Sijp for photographic assistance. Drs N. M. Gerrits and J. J. L. v.d. Want helped me through the difficulties of autoradiography, and Dr E. A. v.d. Velde (Department of Medical Statistics) provided statistical advice. Thanks are also due to Professors J. M. F. Landsmeer and W. T. Daems, and particularly to Dr W. de Priester, for their criticism during the preparation of the manuscript. The manuscript was typed by Jeannette de Lange and the English text was read by Mrs I. Seeger. The Laboratory for Electron Microscopy provided assistance and facilities whenever needed.

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(Received 29 March, revised 2 August 1979)