Surface ectodermal wound healing in the chick embryo

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ABSTRACT

Wound healing has been studied in the surface ectoderm overlying the midbrain region of stages 16–20 chick embryos by light microscopy, scanning and transmission electron microscopy, and immunofluorescent techniques. The embryos were divided into 6 groups, i.e. stages 16–17 for groups I, V and VI, and stages 19–20 for groups II, III and IV. For groups I and II embryos, a longitudinal incision about 0.6 mm was made close to the dorsal midline and the embryos incubated for varying periods of time up to 24 h. To determine the role of actin in the process of healing, selected groups I and II embryos were stained with FITC phalloidin and the wound margins examined using a confocal microscope. Wounds of all embryos in group I and about 20% in group II healed completely within 24 h of reincubation. The process of healing involved a change in the shapes of the ectodermal cells at the wound ends. This appeared as a zipping-up of the wound from both ends. In about 80% of group II embryos where healing did not occur, wound gaping was marked. Intense actin staining (actin cable) was observed at the wound margins of groups I and II embryos suggesting that the actin purse-string mechanism may play a role during wound healing in this epithelial model. The role of tension in wound healing was also determined by placing 2 secondary wounds about 0.5–0.7 mm long close to, and at right angles to the ends of the primary wound in groups III and V embryos. The procedure decreased the tension within the ectodermal cells at the wound ends. Groups IV and VI embryos served as controls for groups III and V embryos, respectively. Healing of both primary and secondary wounds after reduction of tension was rapid. Most primary wounds in group V embryos healed completely within 3 h of reincubation and the rate of reepithelialisation after the reduction of tension was about 160% more than that in group VI (control) embryos. Similarly, most primary wounds in group III embryos were almost closed within 6 h of reincubation. Here, the rate of reepithelialisation was 80% more than that in group IV (controls). Thus tension is an important factor in wound healing in this model.

Key words: Embryonic wound healing; ectoderm; actin.

INTRODUCTION

Embryonic wounding healing has attracted increasing interest in recent years for its potential usefulness as a model for studying some of the complicated and poorly understood mechanisms of normal morphogenesis (Smedley & Stanisstreet, 1984; Martin & Lewis, 1992a, b; Lawson & England, 1992). Wounds in embryos heal by cellular activities including cell proliferation, cell migration, and a change in cell shape, all of which are normally employed during morphogenesis (England & Cowper, 1977; Mareel & Vakaet, 1977; Stanisstreet & Panayi, 1980; Stanisstreet et al. 1980; Smedley & Stanisstreet, 1984; Clark & Scothorne, 1990; Lawson & England, 1992; Martin & Lewis, 1992a; Martin et al. 1994). Wounds made in the neuroepithelium of the chick embryo, for example, heal by apposition and fusion of the wound margins, a process that simulates normal neural fold fusion (Lawson & England, 1992, 1996). An absence of, or inadequate apposition of the wound margins, often results in a failure of the wounds to heal, just as the absence of apposition of the tips of the neural folds leads to a failure of neural fold fusion and the formation of neural tube defects. An analysis of the factors that effect, control and coordinate wound

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healing might therefore yield useful information about the mechanisms of morphogenesis (Smedley & Stanisstreet, 1984).

Embryonic wound healing also serves as a simplified model for studying adult wound healing which is characterised by an inflammatory response and often complicated by scarring and impairment of healing (Martin & Lewis, 1992a; Martin et al. 1994; McCallion & Ferguson, 1996; Martin, 1997). The reepithelialisation of slit ectodermal wounds is remarkably rapid and involves a combination of purse-string contraction and a zipping-up of the wound (Brock et al. 1996). It is suggested (Brock et al. 1996) that the actin cable present at the wound margin provides the contractile force that constricts the wound margin by a purse-string action and pulls on the zipper that draws the wound edges together. The tension within this layer may be increased. This might result in wound gaping and a failure of the ectodermal wounds to heal in the older embryos. The hypothesis is yet to be tested.

The present study examines the exact mechanism(s) by which wounds made in the surface ectoderm overlying the midbrain of stages 16/17 and 19/20 embryos heal. It also investigates the influence that tension might have on the healing process.

**MATERIALS AND METHODS**

Fertilised eggs from White Leghorn chickens were incubated at 38 °C to obtain 50 embryos each at stages 16/17 and 19/20 (Hamburger & Hamilton, 1951) of development. The eggs were windowed by standard techniques and the embryos were staged. They were then divided into 2 groups i.e. stages 16/17 for group I and stages 19/20 for group II (Table 1). A small slit was made in the vitelline membrane overlying the midbrain and a single longitudinal incision (about 0.6 mm) parallel to the midline was made in the ectoderm. Care was taken to avoid damage to the underlying neuroepithelium. The embryos were either fixed immediately in ovo to obtain 0 h wounds or reincubated for varying periods up to 24 h before fixation. A few group II embryos were reincubated for a period of 48 h to determine whether the wounds which failed to heal within 24 h had the ability to reepithelialise when incubated further or not.

**Light microscopy (LM) and scanning electron microscopy (SEM)**

Embryos were fixed in Karnovsky’s fixative (Karnovsky, 1956) overnight and washed in 0.2 M sodium cacodylate buffer (pH 7.4) for 3 h. Those for light microscopy had 1% tannic acid in the fixative to

<table>
<thead>
<tr>
<th>Grouping</th>
<th>Staging</th>
<th>Number of embryos</th>
<th>Wounding*</th>
<th>Incubation (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>16/17</td>
<td>50</td>
<td>P</td>
<td>0–24</td>
</tr>
<tr>
<td>II</td>
<td>19/20</td>
<td>50</td>
<td>P</td>
<td>0–24</td>
</tr>
<tr>
<td>III</td>
<td>19/20</td>
<td>20</td>
<td>P + S</td>
<td>0–6</td>
</tr>
<tr>
<td>IV</td>
<td>19/20</td>
<td>9</td>
<td>P</td>
<td>0–6</td>
</tr>
<tr>
<td>V</td>
<td>16/17</td>
<td>10</td>
<td>P + S</td>
<td>0–3</td>
</tr>
<tr>
<td>VI</td>
<td>16/17</td>
<td>10</td>
<td>P</td>
<td>0–3</td>
</tr>
</tbody>
</table>

* P. primary wound; S. secondary wound.
investigated by reducing tension first in wounds that prevent the leakage of glycosaminoglycans into solution. All embryos were then dehydrated through a graded ascending series of ethanol up to 100% ethanol. Those for light microscopy were embedded in Araldite and transverse sections 2 µm thick were obtained. Specimens for SEM were transferred from 100% ethanol to 100% acetone for about 1 h. They were critical point dried, mounted on aluminium stubs and coated with 20 nm of gold in a Polaron gold coater. They were finally examined with a DS130 scanning electron microscope.

**Immunocytochemistry:** FITC-phalloidin staining
Twenty embryos from group I and 25 from group II were wounded and reincubated for varying periods of time (0 min, 30 min, 6 h and 10 h). Five group II embryos whose wounds had not healed after 24 h of reincubation were selected. All the embryos were fixed overnight in a freshly prepared 4% paraformaldehyde in PBS solution followed by a rinse in PBS. They were then incubated at room temperature for 1 h as whole mounts in FITC-phalloidin (Sigma; 2.5 µg ml⁻¹ in PBS). This procedure was carried out in darkness. As controls, a similar number of embryos from groups I and II were incubated in PBS alone. All embryos were rinsed thoroughly for 30 min through several changes of PBS. They were mounted as whole mounts on glass slides using Slow Fade mountant (Molecular Probes). The embryos were examined with a Zeiss Axiovert 10 confocal laser scanning microscope.

**Tension and wound healing**
The role of tension in ectodermal wound healing was investigated by reducing tension first in wounds that would normally not heal (i.e. group II embryos) and secondly, in wounds that heal normally (i.e. group I embryos). For the first experiment, 20 additional group II embryos were selected and their ectoderms wounded as described above. After 1 h of reincubation, 2 new incisions (secondary wounds) about 0.5–0.7 mm long were made in the ectoderm perpendicular to and about 0.2 mm from the original (primary) wound ends (Fig. 1). These embryos were designated group III embryos (Tables 1, 2). Three group III embryos were fixed immediately and the others were reincubated for 6 more hours before fixation. Nine stages 19/20 embryos with healing primary wounds served as controls (designated group IV embryos). For the second experiment, 10 group I embryos (designated group V) were wounded as described above for group III embryos, but reincubated for 3 h only after making the secondary wounds (Tables 1, 3). Ten group I embryos served as controls (group VI).

### Table 2. Effect of reduction of tension on ectodermal wound healing (group III embryos)*

<table>
<thead>
<tr>
<th>Number</th>
<th>0 h length (mm)</th>
<th>6 h length (mm)</th>
<th>Length healed (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.7 (0.6)</td>
<td>0.07 (0.4)</td>
<td>0.63 (0.2)</td>
</tr>
<tr>
<td>2</td>
<td>0.7 (0.6)</td>
<td>0.21 (0.4)</td>
<td>0.49 (0.2)</td>
</tr>
<tr>
<td>3</td>
<td>0.7 (0.7)</td>
<td>0.04 (0.5)</td>
<td>0.66 (0.2)</td>
</tr>
<tr>
<td>4</td>
<td>0.7 (0.7)</td>
<td>0.0 (0.4)</td>
<td>0.7 (0.3)</td>
</tr>
<tr>
<td>5</td>
<td>0.7 (0.7)</td>
<td>0.07 (0.4)</td>
<td>0.7 (0.63)</td>
</tr>
<tr>
<td>6</td>
<td>0.7 (0.7)</td>
<td>0.6 (0.2)</td>
<td>0.11 (0.5)</td>
</tr>
<tr>
<td>7</td>
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<td>0.1 (0.5)</td>
<td>0.6 (0.2)</td>
</tr>
<tr>
<td>8</td>
<td>0.7 (0.7)</td>
<td>0.0 (0.6)</td>
<td>0.7 (0.1)</td>
</tr>
<tr>
<td>9</td>
<td>0.7 (0.7)</td>
<td>0.2 (0.5)</td>
<td>0.5 (0.2)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.7 (0.68)</td>
<td>0.14 (0.40)</td>
<td>0.56 (0.28)</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0 (0.01)</td>
<td>0.06 (0.06)</td>
<td>0.06 (0.06)</td>
</tr>
</tbody>
</table>

* Values for control (group IV) embryos with normal tension in parenthesis. Test of significance using Student’s *t* test: *t* = 3.2995; *P* = 0.005.

### Table 3. Effect of reduction of tension on ectodermal wound healing (group V embryos)*

<table>
<thead>
<tr>
<th>Number</th>
<th>0 h length (mm)</th>
<th>3 h length (mm)</th>
<th>Length healed (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.7 (0.7)</td>
<td>0.3 (0.4)</td>
<td>0.4 (0.3)</td>
</tr>
<tr>
<td>2</td>
<td>0.7 (0.7)</td>
<td>0.04 (0.5)</td>
<td>0.66 (0.2)</td>
</tr>
<tr>
<td>3</td>
<td>0.7 (0.7)</td>
<td>0.0 (0.5)</td>
<td>0.7 (0.2)</td>
</tr>
<tr>
<td>4</td>
<td>0.7 (0.7)</td>
<td>0.0 (0.5)</td>
<td>0.7 (0.2)</td>
</tr>
<tr>
<td>5</td>
<td>0.7 (0.7)</td>
<td>0.04 (0.6)</td>
<td>0.66 (0.1)</td>
</tr>
<tr>
<td>6</td>
<td>0.7 (0.7)</td>
<td>0 (0.2)</td>
<td>0.7 (0.5)</td>
</tr>
<tr>
<td>Mean</td>
<td>0.7 (0.7)</td>
<td>0.06 (0.45)</td>
<td>0.64 (0.25)</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0 (0)</td>
<td>0.05 (0.06)</td>
<td>0.05 (0.06)</td>
</tr>
</tbody>
</table>

* Figures for control (group VI) embryos with normal tension in parenthesis. Test of significance using Student’s *t* test: *t* = 5.2276; *P* < 0.001.
Statistical analysis was carried out to determine whether the reduction of tension in groups III and V embryos significantly affected the rate of healing. Nine embryos each from groups III and IV and 6 each from groups V and VI were randomly selected from the groups described above for the analysis. The mean rate of healing was calculated for each group for the different time points and Student’s *t* test used to determine whether the reduction of tension had a significant effect on the healing rate.

**RESULTS**

**Control embryos (stages 16/17 and 19/20)**

The midbrain surface ectoderm comprised a single layer of flattened cells whose dorsal surfaces were polygonal in shape. A few microvilli were scattered on the surfaces of the cells and along the cell perimeters, giving the epithelium a ‘pavement-like’ appearance. The underlying head mesenchyme typically consisted of cells with numerous processes enmeshed in an extracellular matrix.

**Wound healing**

*0 h.* Freshly made wounds of groups I and II embryos gaped widely in all the embryos exposing the underlying head mesenchyme (Fig. 2). The gaping wounds were bordered on their lateral margins by about 5–7 rows of cells longitudinally oriented and directed towards the ends of the wound. There was, however, very little change among the ectodermal cells bordering the ends of the wounds. Actin staining was localised only around the cell perimeters.

*30 min.* By 30 min of reincubation, differences between group I and a few group II wounds on one hand and the majority of group II wounds on the other hand were observable. The former were described as healing wounds and the latter as nonhealing wounds. The healing wounds had triangular shaped cells whose ends tapered towards the acute-angled ends of the wounds (Fig. 3), while the ends of most of the nonhealing wounds tended to be wider with the cells immediately bordering the wound edge elongated along the smooth margin (Fig. 4). There was an intense staining of actin (i.e. an actin cable) at the margins of both healing and nonhealing wounds (Figs 5, 6). Sometimes, the actin cable in the healing wounds was disrupted at a few points along the wound margins.

*6 h.* Following 6 h of reincubation, the differences between healing and nonhealing wounds became more obvious. In the former, the length of the wound had decreased by about a third, and cells with 3 different shapes namely triangular, fusiform and rounded could be identified at the ends of the wound. The triangular cells lay farthest from the wound end, followed by the fusiform cells and finally the rounded cells that filled the acute-angled ends of the wound (Fig. 7a). The rounded cells had numerous microvilli on their surfaces and some of them appeared paired. A few of the paired ones which looked like daughter cells were held together by cytoplasmic bridges (Fig. 7b). Light microscopic examination of transverse sections across the wound in the region of the rounded cells revealed a flattened layer of ectodermal cells deep to the rounded cells that was continuous with the unwounded ectodermal cells (Fig. 7c). The flattened cells had a basal lamina. The central portion of the wound where rounded cells were absent, contained mainly mesodermal cell debris with no evidence of reepithelialisation. Along the lateral wound margins, the ectodermal cells immediately bordering the wound appeared elongated parallel to the wound margin but occasionally, at 1 or 2 points along this margin, a cluster of 2 or 3 rounded cells was seen. The actin cable was still present at the wound margins. The nonhealing wounds were similar in appearance to those at 30 min of reincubation with mesenchymal cells forming a flattened layer in the wound.

*10 h.* By 10 h of reincubation, healing was well advanced and the length of the wound was markedly reduced. In a majority of embryos, the wound site was marked by a pile of rounded cells some of which appeared like cell debris (Fig. 8). The actin cable had also disappeared leaving a diffuse staining of actin in the region of the rounded cells (Fig. 9). In the nonhealing wounds, the wound ends continued to gape so that the wounds became oval in shape. The actin cable was observable at the margins of the nonhealing wounds.

*24 h.* Wounds of all group I and about 20% of group II embryos healed completely between 15 and 24 h of reincubation and it became difficult to identify their original positions. The healed wound area was represented by a few ectodermal cells with bulging surfaces (Fig. 10). The unhealed wounds of group II embryos were either rounded or oval in shape and often appeared much larger in size than the original wounds, occupying most of the lateral surface of the midbrain (Fig. 11). The actin cable was still present at the margins of these wounds (Fig. 12). Occasionally, the amnion was observed attached to or covering group II wounds that did not heal.

*48 h.* The wounds of group II embryos which have
Fig. 2. SEM of a gaping freshly made surface ectodermal wound in the midbrain of a stage 17 embryo. Note the several parallel rows of elongated ectodermal cells at the wound margins. Head mesenchyme (Hm). Bar, 200 µm.

Fig. 3. One end of a wound in a stage 17 embryo reincubated for 30 min. Triangular ectodermal cells (T) taper towards the wound. One ectodermal cell (small arrow) has numerous microvilli on its surface. Bar, 30 µm.

Fig. 4. One end of a wound in a stage 20 embryo reincubated for 30 min. The wound angle is gaping and elongated ectodermal cells (small arrows) run parallel to the margin. Bar, 44 µm.

Fig. 5. FITC-stained wound in a stage 17 embryo showing presence of an actin cable (open-ended arrows) at the wound margins. Reincubation for 30 min. Bar; 300 µm.

Fig. 6. FITC-stained wound in a stage 20 embryo reincubated for 30 min. An actin cable (open-ended arrows) is also present at the wound margins. Bar, 300 µm.
Fig. 7. (a) SEM of a wound in a stage 19 embryo reincubated for 6 h. Healing is in progress from the ends of the wound. The ectodermal cells at the ends of the wound are triangular (T) or fusiform (F). The rounded cells (R) fill the angles of the wound. The ectodermal cells that border the lateral margins of the wound (arrows) are stretched along the margins. S, mesenchymal cell debris. Bar, 190 µm. (b) Higher magnification of the left end of the wound. Rounded cells (R) have numerous microvilli on their surfaces. Two daughter cells (asterisks) are connected by a cytoplasmic bridge. Bar, 31 µm. (c) LM of a wound in a stage 16 embryo reincubated for 6 h showing a layer of flattened ectodermal cells (small arrows) deep to the rounded cells (R). A basal lamina (open-ended arrow) formed immediately deep to the flattened cells separates the head mesenchymal cells (H) which are clumped together from the flattened cells. Bar, 160 µm.

Fig. 8. SEM of a wound in a stage 16 embryo reincubated for 10 h. A mound of rounded ectodermal cells marks the position of the wound. I, cell debris. Bar, 44 µm.

Fig. 9. FITC-phalloidin stained stage 17 wound reincubated for 10 h showing a diffuse, localised actin staining (arrow). Bar, 160 µm.
failed to heal within 24 h did not reepithelialise after 48 h of reincubation.

**Tension and wound healing**

Creating 2 secondary wounds near the original wound ends of groups III and V embryos to reduce tension resulted in the formation of ‘H’-shaped wounds (Fig. 13). Healing was significantly faster in these embryos ($P = 0.005$ and $P < 0.001$, respectively) than in control embryos (groups IV and VI) reincubated for the same periods of time (Tables 2, 3). At 6 h of reincubation, the mean healing rate for group III embryos was 80% more than that for group IV. Indeed by this time, the primary wounds were almost completely healed in most group III embryos while healing of the secondary wounds was well advanced (Fig. 14). Likewise, the mean healing rate for group V embryos was 162.5% higher than that for group VI embryos following 3 h of reincubation.

**DISCUSSION**

The principal findings of the study are that (1) reepithelialisation of wounds made in the surface ectoderm overlying the midbrain region of chick embryos occurs mainly by a change in the shapes of the cells at the wound ends and results in a zipping-up of the wound, and (2) the healing process is influenced by the level of tension in the ectoderm, the process being inhibited or enhanced depending on whether the tension is high or low.

**Reepithelialisation**

The results of the present study suggest a method by which the zipping-up of slit surface ectodermal wounds occurs: through a change in cell shape. A progressive change in cell shape which is mostly restricted to the ectodermal cells at the ends of the wound involves 3 types of cell, i.e. triangular, fusiform and rounded. It is suggested that the triangular cells arise from the polygonal cells which normally characterise the unwounded surface ectoderm. They become fusiform and ultimately rounded. The observation of a layer of flattened cells with a definite basal lamina deep to the rounded cells suggests that the latter may provide a pool of cells that participates in reepithelialisation. The morphological differences observed between the healing and nonhealing wounds provide evidence in support of the proposed mechanism of healing, for while the ectodermal cells at the ends of the healing wounds altered their shapes and became rounded, those of the nonhealing wounds did not; they behaved typically like cells at the lateral wound margins which were only stretched along the margins. In the final stages of healing, rounded cells
are heaped in the centre of the ectodermal wound. The heap, according to Martin & Lewis (1992a, b), arises from an overrun of wound closure by the actin purse-string mechanism. Based on the results of the present study, we suggest that it may also represent redundant rounded cells that have not participated in re-epithelialisation.

The assembly of an actin cable along the margins of the healing wounds and its dissappearance at the end of healing, may suggest a role for the actin purse-string mechanism (Martin & Lewis, 1992b) of healing. It is possible that in the present study, the actin cable may provide the force that drives the rounded cells to rearrange their contacts and participate in re-epithelialisation as suggested by Martin et al. (1994) and Brock et al. (1996).

The role of tension
The results demonstrate that tension plays a key role in surface ectodermal wound healing. It is significant that the process described above (i.e. change in cell shape) was not observed at the lateral wound margins. This apparent lack of involvement of the cells at the lateral wound margins is probably due to the effect of
longitudinal tension along the lateral margin. As observed by other investigators (Martin & Lewis, 1992a; Sherratt & Lewis, 1993; Stanisstreet et al. 1985), the act of wounding abolishes the component of tension acting at right angles to the cut leaving the longitudinal tension to act along the lateral wound margins. The latter force must have been enormous in causing about 5–7 rows of ectodermal cells to stretch parallel to the lateral wound margins. Another effect of longitudinal tension at the lateral wound margins is in the suppression of the protrusive activity of the marginal cells as demonstrated by Kolega (1986); cell migration, therefore, was not a feature of healing. Our results did not confirm the suggestion by Stanisstreet et al. (1985) that an increase in tension at the ends of slit ectodermal wounds serves as a trigger for a change in cell shape during wound healing. We have demonstrated that decreasing tension at the ends of wounds that would normally not heal (group II embryos) results in a rapid wound closure. Furthermore, decreasing tension at the ends of wounds that normally heal (group I embryos) results in a faster rate of healing (162.5% increase) than normal. In another study (unpublished data), where a piece of egg shell was placed at one end of group I wounds between the ectoderm and neuroepithelium to increase tension, healing was abolished at that end. Collectively, the results show that the failure of the surface ectodermal wounds of group II embryos to heal may be due to an increase in tension within the ectodermal layer. This may be due to morphogenetic brain expansion and probably accounts for the difference in healing characteristics between groups I and II embryos reported in our previous study (Lawson & England, 1996).

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