The extracellular matrix of lip wounds in fetal, neonatal and adult mice

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Summary

Wound healing in the fetus occurs rapidly, by a regenerative process and without an inflammatory response, resulting in complete restitution of normal tissue function. By contrast, in the adult, wounds heal with scar formation, which may impair function and inhibit further growth. The cellular mechanisms underlying these differing forms of wound healing are unknown but the extracellular matrix (ECM), through its effects on cell function, may play a key role. We have studied the ECM in upper lip wounds of adult, neonatal and fetal mice at days 14, 16 and 18 of gestation. The spatial and temporal distribution of collagen types I, III, IV, V and VI, fibronectin, tenascin, laminin, chondroitin and heparan sulphates were examined immunohistochemically. Results from the fetal groups were essentially similar whilst there were distinct differences between fetus, neonate and adult. Fibronectin was present at the surface of the wound in all groups at 1 h post-wounding. Tenascin was also present at the wound surface but the time at which it was first present differed between fetus (1 h), neonate (12 h) and adult (24 h). The time of first appearance paralleled the rate of wound healing which was most rapid in the fetus and slowest in the adult. Tenascin inhibits the cell adhesion effect of fibronectin and during development the appearance of tenascin correlates with the initiation of cell migration. During wound healing the appearance of tenascin preceded cell migration and the rapid closure of fetal wounds may be due to the early appearance of tenascin in the wound. Collagen types I, III, IV, V and VI were present in all three wound groups but the timing and pattern of collagen deposition differed, with restoration of the normal collagen pattern in the fetus and a scar pattern in the adult. This confirms that lack of scarring in fetal wounds is due to the organisation of collagen within the wound and not simply lack of collagen formation. The distribution of chondroitin sulphate differed between normal fetal and adult tissues and between fetal and adult wounds. Its presence in the fetal wound may alter collagen fibril formation. No inflammatory response was seen in the fetal wounds. The differences in the ECM of fetal and adult wounds suggests that it may be possible to alter the adult wound so that it heals by a fetal-like process without scar formation, loss of tissue function or restriction of growth.

Key words: extracellular matrix, fetal wound healing, fibronectin, tenascin, collagen, chondroitin sulphate.

Introduction

Wound healing is a complex process involving cellular migration, proliferation and differentiation with extracellular matrix (ECM) synthesis and matrix remodelling (for review see Clark, 1988a). Many of these mechanisms are common to development and wound healing. Recent studies have demonstrated significant differences between adult and fetal wound healing (Adzick et al. 1985; Dixon, 1960; Hallock, 1985; Robinson and Goss, 1981; Rowsell, 1984). In the fetus, healing occurs rapidly, by a regenerative process without scar formation, while in the adult it leads to scar formation, often limiting tissue function and restricting further growth. The fetal response to a wound may be related to its developmental stage, the sterile aqueous fetal environment or a combination of both factors.

These observations and the advent of surgery in utero for lethal congenital anomalies (Harrison et al. 1990a,b) have stimulated interest in the possibility of in utero correction of craniofacial anomalies such as cleft lip and cleft palate (Christ, 1986; Dado et al. 1990). The anticipated benefits of fetal repair are scarless healing with normal midfacial growth and normal palatal function but further understanding of the mechanisms underlying fetal wound healing is essential. Furthermore, knowledge of fetal wound healing may also allow manipulation of the adult wound so that it heals in a fetal-like manner without scar formation, with enormous benefits for all types of reconstructive surgery,
where scar formation restricts tissue function and growth.

Collagen is the principal component of the ECM and a scar is loosely defined as an abnormal collection of collagen following wound repair. Recent studies of fetal wound healing have concentrated on collagen formation (DePalma et al. 1989; Longaker et al. 1990; Merkel et al. 1988; Siebert et al. 1990), utilizing different species, at varying gestational ages and several different wound models. The multiplicity of models has produced conflicting results, although there is agreement that, in incised fetal skin, wound healing takes place rapidly, in a regenerative manner, without scar formation and without an inflammatory response. Some studies using wound implants have shown no collagen deposition, whilst others have found collagen within the fetal wound. However, because the implant alters the healing process, it is difficult to relate these results to the scarless healing seen in an incisional wound and the role of collagen in fetal wounds is still disputed.

The dermal ECM is a complex aggregate of glycoproteins and glycosaminoglycans which in conjunction with collagen determine the physiological properties of the tissue (Uitto et al. 1989). The role played by these other components of the ECM in fetal wound healing is unknown but is likely to be significant.

The nature and time during development when the transition from fetal to adult wound healing occurs is unknown, but it may relate to the developmental stage of the fetus, maturity of the fetal immune system (as an inflammatory response is not seen in the fetal wound) or the change from fetal to neonatal environment.

To address these problems, in the least artificial model, we have carried out a detailed study of the ECM of incisional upper lip wounds in fetal mice at three gestational ages, in neonatal mice and in adult mice. Using indirect immunostaining, the spatial and temporal localisation of collagen types I, III, IV, V and VI, fibronectin, tenascin, laminin, chondroitin and heparan sulphates during wound healing has been established. Direct immunostaining of endogenous immunoglobulins was used as a marker of an inflammatory response at the wound.

Materials and methods

Five groups of wounds were studied: fetal wounds created at days 14, 16 or 18 of gestation, neonatal wounds created between 6 and 12 h post-parturition and adult wounds made in young mice of 6–8 weeks age. An upper lip wound was used in this study for two reasons. (1) A wound of reproducible size can be made under direct vision after exteriorising the fetal snout from the uterus, without significant fetal loss. (2) The wound allows study of the healing of two epithelial surfaces (skin and oral mucosa) and mesenchyme including skeletal muscle.

Day 16 and day 18 fetal wounds

Time-mated, female, MF1 mice were anaesthetised with halothane/oxygen/nitrous oxide on day 16 or day 18 of gestation. A midline laparotomy exposed the pregnant uterus. Using an operating microscope (Wild), one horn of the uterus was exteriorised and a purse-string suture of 10/0 nylon placed through all layers of the uterine wall on its anti-mesenteric surface. A cruciate incision through the myometrium and membranes allowed the snout of the fetus to be manipulated out of the uterus (Fig. 1A–C,G). Placement of the suture superior to the visible position of the snout and extension of the fetal neck to exteriorise the snout prevented escape of the placental vessels or upper limbs, which are impossible to return to the uterus. Use of the purse string suture restricts amniotic fluid loss and prevents expulsion of the fetus. A 2 mm, full thickness, vertical incision in the left upper lip of the fetus was made with microsurgical scissors (Fig. 1D and H). The edges of the wound lie in apposition and were left unsutured. The fetus was returned to the uterus, the purse string suture closed (Fig. 1E and I) and the abdominal wound was repaired. Three fetuses were wounded in each animal. The pregnancies then continued until the fetuses were harvested or birth occurred, on day 19 of gestation. For the fetal study, sham operations, where the above procedure was carried out except for the lip incision, were used as controls.

Day 14 fetal wounds

In day 14 fetuses, this technique required modification as it produced a high abortion rate and, in the remaining viable fetuses, there were marked deformities of the hind limbs and lower trunk. These appeared to be due to amniotic fluid loss and subsequent compression of the fetus by the myometrium. Day 16 and day 18 fetuses were more resistant to compression and did not show these deformities. Mouse embryos develop normally and survive surgical manipulation if released from the myometrium, as long as the uterine membranes remain intact and the fetus is attached to the placenta (Muneoka et al. 1986). In the day 14 fetus, following exposure of the uterus, the myometrium was incised longitudinally along its anti-mesenteric border, releasing the developing fetuses from the compressive forces of the myometrium. A purse string suture was placed through the uterine membranes only, which were then incised to allow exteriorisation of the fetal snout. The procedure continued as described for the day 16 and day 18 fetuses, with closure of the uterine membranes but without repair of the myometrium.

Neonatal and adult wounds

Under anaesthesia 3–4 mm, vertical, full thickness, upper lip wounds were made in neonatal mice between 6 and 12 h after birth and in adult mice at age 6–8 weeks. A single 6/0 nylon suture was placed through the lower margin of the wound to appose the wound surfaces and the animals were allowed to recover from anaesthesia.

Wound harvest and sectioning

Animals were killed by an overdose of chloroform at multiple time points post-wounding (pw). In the fetal wounds, these time points were at 1, 6, 12, 16, 20, 24, 48, 72 h and 5 days pw and, in both neonate and adult at 1, 6, 12, 24, 48, 72 h, 5, 7, and 12 days pw. With the 16 and 18 day fetal wounds, the latter time points were during the neonatal period. It was possible to recognise those neonatal animals wounded as a fetus by the presence of a narrow furrow visible on the upper lip at the site of the original wound (Fig. 1J).

For the fetal and neonatal wounds, the head was snap frozen in precooled isopentane, embedded in OCT compound (Miles Inc., Elkart, IN) and 7 μm transverse sections cut at −20°C in a Leitz cryostat. For the adult wounds, the upper lip...
Fig. 1. Operative procedure. (A) Midline abdominal incision. (B) Part of one uterine horn lifted from abdomen and suture placed in antimesenteric surface of uterus. (C) Uterus incised and fetal snout manipulated out of uterus. (D) Upper lip incision. (E and F) Uterine and abdominal incisions closed. (G) Fetus visible through uterine wall. (H) Upper lip incision. (I) Uterine suture closed. (J) SEM of 16 day fetal head 24 h post-wounding. The wound (arrow) is re-epithelialised. e, ear; s, snout; u, upper limb. Bar: 1 mm.

Table 1. Primary antibodies

<table>
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<th>Primary Ab raised against</th>
<th>Primary Ab raised in</th>
<th>Source</th>
<th>Dilution (in PBS)</th>
<th>Secondary Ab (see Table 2)</th>
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<td>Serotec</td>
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<tr>
<td>Tenascin</td>
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<tr>
<td>Heparan sulphate</td>
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<td>Dr P. Bletchley and Dr J. Anderson, Manchester</td>
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<td>Mouse (IgG) monoclonal</td>
<td>Developmental Biology Hybridoma Bank, Iowa</td>
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was excised, snap frozen and embedded as above and 7 µm transverse sections cut.

**Immunohistochemistry**

Primary and secondary antibodies used for indirect immunostaining are shown in Tables 1 and 2. Incubation with primary antisera was for 1 h followed by three rinses in phosphate-buffered saline (PBS). Incubation with FITC-conjugated secondary antisera was for 1 h followed by a further three rinses with PBS. The sections were mounted in a non-fading medium, DABCO (1,4-diazabicyclo-(2,2,2)-octane), and
photographed using a Leitz Dialux microscope on Kodak Ektachrome 160 ASA film corrected for tungsten light. Black and white prints were made from these colour slides. All of the antibodies used have previously published characterisation and specificities. None the less, in this study, for each antibody, specificity was tested by pre-absorption of the primary antibody with biochemically purified antigen prior to immunostaining by the above protocol. This abolished all staining by the antibody. For each primary antibody and each time point, control sections were stained, substituting PBS for the primary antibody.

Results

Overall 81.4% of the fetuses operated on survived (149/183). The majority of the failures were due to placental disruption at the time of surgery or accidental occlusion of the placental vessels whilst closing the uterus. 5 or 6 wounds were available for study at each of the time points in each fetal group. All neonatal (27) and adult (27) animals survived providing 3 wounds for study at each time point. All control staining procedures were negative.

The results from the three fetal groups were essentially similar and will be presented as one group. The same elements of the ECM were present in each wound group but there were distinct spatial and temporal differences between the fetal and adult wounds. The neonatal wound had features in common with fetal and adult wounds, suggesting that a gradual shift occurs from fetal type to adult type wound healing, rather than an abrupt transition.

Tables 3 and 4 summarise the results.

Epithelialisation, tenascin and fibronectin deposition
The rate of re-epithelialisation and closure of the mesenchymal defect varied between the three groups. In the fetal wounds epithelial migration was apparent at 12 h pw and by 20 h pw re-epithelialisation of the skin

| Table 3. Epithelialisation, fibronectin (FN) and tenasin (TN) deposition in wound |
|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                  | 1 hour | 6 hours | 12 hours | 24 hours | 48 hours | 72 hours | 5 days | 7 days | 12 days |
| Fetus            |         |         |         |         |         |         |       |       |        |
| Epithelialisation | Complete in fetus | Complete in neonate | Complete in adult | Complete in adult | Complete in adult | Complete in adult | Complete in adult | Complete in adult | Complete in adult |

Relative intensity of staining at wound site in comparison to normal tissue: 0, absent. 1, < normal; 2, normal (not normal pattern); 3, > normal; 4, •• normal.
Table 4. Collagen (C) and chondroitin sulphate (CS) deposition at site of wound

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<td></td>
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<td>All collagen types present</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Fetus</td>
<td>0</td>
<td>1</td>
<td>2 Normal pattern</td>
<td>2</td>
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<tr>
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<td>Present in epithelial basement membrane</td>
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<td>Present in epithelial basement membrane</td>
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<td>Adult</td>
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Relative intensity of staining at wound site in comparison to normal tissue: 0, absent; 1, <normal; 2, normal (not normal pattern), 3, >normal; 4, •normal

wound was complete. Re-epithelialisation of the oral mucosal wound was virtually complete by 16 h and in all of the wounds re-epithelialisation of the oral mucosa proceeded more rapidly than closure of the skin wound. Re-epithelialisation preceded closure of the mesenchymal wound which was complete by 48 h pw. In the neonatal wounds, re-epithelialisation of both skin and oral mucosa was complete by 48 h pw and the mesenchymal wound closed by 72 h. Re-epithelialisation was complete in the adult wound by 72 h and, as in the fetal and neonatal wounds, healing of the oral mucosa was quicker than healing of the skin wound. The connective tissue defect had closed by 5 d pw.

Fibronectin (Fn) was diffusely present in the mesenchymal ECM of the normal tissues adjacent to the wound with a linear staining pattern at the level of the basement membrane. In the fetal wounds at 1 h pw, a layer of Fn was present at the wound surface (Fig. 2A, D and F). Increased staining for Fn in the region of the wound was present at all time points up to 72 h pw in the 16 and 18 d fetal wounds. At 48 h the enhanced staining for Fn within the wound was patchy (Fig. 2B). In the 14 d fetal wounds at 48 h, the staining pattern for Fn in the region of the wound was indistinguishable from the adjacent normal tissue (Fig. 2E). Similarly in the 16 and 18 d wounds by 72 h a normal staining pattern for Fn was present and the original site of the wound was not detectable (Fig. 2C). At 1 h pw in both neonatal and adult wounds, a layer of Fn was present at the surface of the wound (Fig. 2G and H) and increased staining for Fn within the wound was present in both wound groups up to 7 d pw.

By contrast to Fn, tenascin (Tn) was very limited in its distribution. In the undamaged fetal tissues Tn was present in and below the basement membranes of the oral mucosa and the dermal–epidermal junction, particularly around the developing hair follicles (Fig. 3D). This pattern was also present in the normal neonatal and adult tissues although the staining was discontinuous and less intense at the dermal–epidermal junction.

At 1 h pw in the fetal wounds, tenascin was present on the surface of the wound (Fig. 3A, E and F). By 24 h pw the staining for Tn was more prominent and Tn was present within the mesenchyme adjacent to the wound (Fig. 3B). The most intense staining at this time was near the basement membranes of the epidermis and oral mucosa adjacent to the wound. Tn persisted within the wound at 48 h pw (Fig. 3C) and at 72 h pw was patchily present within the mesenchyme although still prominent at the dermal–epidermal junction. By 5 d pw a normal pattern of staining for Tn had been restored (Fig. 3D) and the site of the wound was only distinguishable by a narrow epithelial furrow.

Tn was first present at the surface of the neonatal wounds 12 h pw (Fig. 3G). At the outer margin of the wound, where the dermis was exposed, Tn staining was present within the mesenchyme but not directly at the surface of the wound (Fig. 3G – indicated by double arrow). The margin of this staining precisely mirrored
Fig. 2. Transverse lip sections, skin uppermost, oral mucosa facing downwards. Stained for fibronectin (Fn). (A) 16d fetus 1 h post-wounding (pw). Fn widely distributed in mesenchymal ECM, with linear staining at basement membrane of epidermis and oral mucosa. Layer of Fn seen at wound surface and within clot. (B) 16d fetus 48h pw. Wound re-epithelialized, mesenchymal defect closed. Patchy increased staining for Fn in wound area still visible. (C) 16d fetus 5 d pw. Normal Fn pattern, no scar formation. (D) 14d fetus 1 h pw. Fn at wound surface. (E) 14d fetus 48h pw. Normal Fn pattern, no scar pattern. (F) 18d fetus 1h pw. Fn at wound surface and within clot. (G) Neonate 1h pw. Fn at wound surface and within clot. (H) Adult 1h pw. One edge of wound shown, with Fn at wound surface. h, hair follicle; m, oral mucosa; o, oral mucosal salivary gland; s, skin; t, tongue. Arrow: site of wound. Bar: 100 μm.

the pattern of staining for endogenous immunoglobulins in the same wound (Fig. 7J). This precise congruity of staining patterns for Tn and endogenous immunoglobulins was only seen in the neonatal wounds at this time point. In a similar manner to the fetal wounds, the staining for Tn was more prominent within the mesenchyme at 24 h and 48h pw (Fig. 3H) with the most intense staining at the dermal–epidermal junction. By 5 d pw an essentially normal pattern of staining had been restored although the site of the wound was obvious because of the loss of hair follicles (Fig. 3I).

In the adult wounds, Tn was first, faintly, visible at the surface of the wound at 24 h pw (Fig. 3J). Staining was more intense, adjacent to the original wound, at 48 h pw and by 5 d pw Tn was diffusely present at the site of the wound (Fig. 3K). This staining was less marked at 7 d pw and at 12 d pw there was limited patchy staining within the healing connective tissue (Fig. 3L). At this time, intense staining for Tn was present at the dermal–epidermal junction where epithelial projections into the dermis (enhanced rete pegs) were prominent.

Collagen type IV, laminin and heparan sulphate
In undamaged fetal, neonatal and adult tissues, collagen type IV (CIV) localised in the epithelial basement membranes of the skin and oral mucosa and in endothelial basement membranes (Fig. 4G). No new
staining for CIV was demonstrated in relation to the wound surface in any of the wound groups.

In the fetal wounds, by 5 d pw a normal pattern of CIV staining was restored and the pattern at the site of the wound was indistinguishable from the surrounding tissue (Fig. 4G and H). Re-epithelialisation was complete before CIV was present in the adult at the site of the reforming basement membrane beneath the new epithelium. In the neonate, by 5 d pw on the oral side of the wound a normal pattern of CIV was restored. On the skin side of the wound staining for CIV highlighted the loss of hair follicles although the pattern of staining was otherwise essentially normal (Fig. 4I). In the adult wound at 72 h pw re-epithelialisation was complete and CIV staining was present in the reformed basement membranes.

In the adult wound, at 5 d (Fig. 4J) pw a band of granulation tissue containing Fn, CI, CIII, CV and CVI (see below) is present in the wound separating the normal tissues on either side of the wound. A few capillaries are present within this tissue demonstrated by the staining of CIV within endothelial basement membranes. By 7 d pw, new capillary formation within the wound is more obvious (Fig. 4K) and, by 12 d pw, profuse neovascularisation with multiple capillary loops is apparent (Fig. 4L). New capillary formation is an integral part of adult wound healing and is well demonstrated by staining of endothelial basement membranes. Exuberant new vessel formation was not seen in the healing fetal or neonatal wounds.

Staining for laminin (Ln) showed a similar pattern to the staining seen with CIV, as Ln co-localises in epithelial and endothelial basement membranes. No staining for Ln was seen in relation to the wound surface and re-epithelialisation was complete before staining for Ln was visible in the reformed basement membrane (Fig. 4A and B). Neovascularisation of the adult wound and its absence in the fetal wound was also displayed by Ln staining.

Ln was present in the endomysium of myotubules which in fetal wounds could be seen crossing the site of the original wound (Fig. 4C). Muscle regeneration in the fetal wounds was also demonstrable by using an antibody against light meromyosin, MF 20, which showed myotubules crossing the original wounded area (Fig. 4D). Muscle regeneration was not seen in neonatal or adult wounds.

Heparan sulphate (HS) localised only at the basement membranes of the epidermis and oral mucosa. HS staining at the wound was restricted to the reforming epithelial basement membranes and, in a similar pattern to CIV and Ln, re-epithelialisation was complete before HS was present in the basement membrane (Fig. 4E and F).

Collagen types I, III, V and VI

Type III collagen (CIII) was widely localised in the mesenchymal and connective tissues of fetus, neonate and adult (Fig. 5). In the fetal wounds CIII was not present within the wound at 24 h pw (Fig. 5A). By 48 h pw, collagen was visible within the wound (Fig. 5B) and, at 72 h, the reticular pattern of CIII staining was restored at the site of the wound (Fig. 5C). This pattern was indistinguishable from the pattern of CIII staining in the tissues adjacent to the wound and the only indication of the site of the wound was an epithelial furrow at the skin surface. Muscle regeneration was also demonstrated by CIII staining (Fig. 5D). In the 14 d fetus, collagen deposition was seen earlier than in 16 and 18 d fetuses (Fig. 5E) and regeneration of a normal reticular pattern was present at 48 h pw (Fig. 5F).

CIII was first deposited in the neonatal wounds at 48 h pw (Fig. 5G), with more intense staining at 72 h pw (Fig. 5H) and, at 5 d pw on the oral side of the wound, a normal reticular pattern of CIII had been restored (Fig. 5I). On the skin side, loss of hair follicles was apparent but the pattern of CIII deposition still shows a reticular configuration (Fig. 5I), unlike the parallel bundles of collagen fibres seen in the adult wound (Fig. 5L). In the adult wound, CIII deposition is not seen until after 72 h pw (Fig. 5J). At 5 d, CIII is present in the wound (Fig. 5K) and, at 12 d pw, bundles of CIII lying parallel to the wound surface are seen disrupting the normal tissue structure (Fig. 5L).

The other interstitial collagens studied, types I, V and VI, showed essentially similar patterns to CIII with regeneration of a normal pattern in the fetal wounds, scar formation in the adult and a mixed pattern in the neonate. In each wound group these collagens were all present from the same time point that CIII was first seen.

Type VI collagen (CVI) was distributed diffusely throughout the ECM in an identical pattern to CIII. It was first seen in the fetal wound at 48 h pw (Fig. 6A and B) and a normal pattern was present at 72 h pw (Fig. 6C – compare with Fig. 5C). In the neonate, CVI was present at 48 h pw and a similar pattern to CIII was seen at 5 d pw with loss of hair follicles but an otherwise essentially normal, reticular pattern of CVI. In the adult, CVI was present at 5 d and at 12 d pw fibrils of CVI are seen within the wound parallel to the wound surface (Fig. 6D).

Type V collagen (CV) is diffusely present throughout the ECM but also localises in the epithelial and endothelial basement membranes. Staining for CV was faint but discernable in all wound groups. As with CIII and CVI, staining for these collagens showed regeneration of a normal pattern in the fetal wounds (Fig. 6E – compare with Figs 5C and 6C), a virtually normal pattern in the neonatal wounds, apart from the loss of hair follicles, (Fig. 6F and H – compare with Fig. 5I) and scar formation in the adult (Fig. 6G). As CV localises in the endothelial basement membranes, the neovascularisation of the adult wound was also apparent.

Chondroitin sulphate

Chondroitin sulphate (CS) was diffusely present in the ECM of the fetal mesenchyme. The homogenous staining pattern of CS was distinctly different from the fibrillar pattern seen with the interstitial collagens and Fn. CS was not initially present within the fetal wound...
Fig. 3. For legend see p. 660
Fig. 4. For legend see p. 660
Fig. 3. Transverse lip sections, skin uppermost, oral mucosa facing downwards. Stained for tenascin (Tn). (A) 16 d fetus 1 h pw. Tn present in the basement membranes of the dermal—epidermal junction and oral mucosa. Tn staining present at surface of wound. (B) 16 d fetus 24 h pw. Tn staining present in mesenchyme adjacent to wound, particularly at the interface with the dermal—epidermal junction. (C) 16 d fetus 48 h pw. Wound re-epithelialised and mesenchymal defect closed. Staining for tenascin present at site of wound. (D) 16 d fetus 5 d pw. Near normal pattern of Tn — no staining within wound. (E) 14 d fetus 1 h pw. Tn present at surface of wound. (F) 18 d fetus 1 h pw. Tn present at surface of wound. (G) Neonate 12 h pw. Tn present at surface of wound. Where the dermis to the left of the wound is exposed, Tn is present within the dermis but not at the wound surface (indicated by double arrow). The limits of the staining for Tn mirror the extent of the inflammatory response at the surface of the wound demonstrated by staining for endogenous immunoglobulins (see Fig. 7J). (H) Neonate 48 h pw. Intense staining for Tn within healing wound. (I) Neonate 5 d pw. Tn staining restricted to epithelial basement membranes — note absence of hair follicles in skin side of wound but normal pattern of staining beneath oral mucosa. (J) Adult 24 h pw. Faint staining for Tn at basement membrane of epidermis and in hair follicles but no staining at wound surface. (K) Adult 5 d pw. Intense staining for Tn within healing wound. (L) Adult 12 d pw. Increased staining for Tn adjacent to basement membrane of epithelial ingrowths. Patchy staining for Tn still present within healing wound. b, basement membrane; h, hair follicle; m, oral mucosa; n, nasal cavity; o, oral mucosal salivary glands; s, skin; t, tongue. Arrow: site of wound. Bar: 100 μm.

Fig. 4. Transverse lip sections, skin uppermost, oral mucosa facing downwards. (A–C) stained for laminin (Ln). (A) 18 d fetus 24 h pw. Ln present in basement membranes of epidermis and hair follicles, oral mucosa and endothelium. Epithelialisation of wound complete, Ln absent beneath healed epithelium. No staining for Ln related to wound. (B) 18 d fetus 24 h pw. Higher magnification of oral mucosa from Fig. 4A. Note staining of Ln in mucosal basement membrane and absence of staining beneath re-epithelialised wound. (C) 16 d fetus 5 d pw. Ln staining outlines myotubules regenerating at the site of the wound. (D) Staining pattern with MF 20 (meromyosin). 16 d fetus 5 d pw. Staining of MF 20 highlights myotubule regeneration. (E–F) Stained for heparan sulphate (HS). (E) 16 d fetus 20 h pw. HS present in basement membrane of undamaged oral mucosa, but absent beneath migrating epithelium of the wound. (F) 16 d fetus 24 h pw. Basement membrane beneath reepithelialised wound has regenerated, showing presence of HS. (G–L) Stained for type IV collagen (CIV). (G) 16 d fetus 5 d pw. CIV present in mesenchyme of wound site, (H) 14 d fetus 72 h pw. CIV shows normal vascular pattern in skin aspect of wound but loss of hair follicles. (J) Adult 5 d pw. Band of scar tissue, containing CIV, CIII, CV, and CVI (see Fig. 5), separating normal surrounding tissues — no scar formation and no neovascularisation. (K) Adult 7 d pw. Further neovascularisation present within site of wound. (L) Adult 12 d pw. Profuse neovascularisation with multiple capillary loops present in wound site. b, basement membrane; m, oral mucosa; mt, myotubules; s, skin; t, tongue. Arrow: site of wound. Bar: 100 μm.

Fig. 7A) but from 20 h pw CS was present in the wound (Fig. 7B–D).

In the neonate, the distribution of CS within the mesenchyme is less diffuse and more marked on the oral side of the wound (Fig. 7E). CS was not present within the wound at any of the time points studied (Fig. 7F) and by 5 d pw CS is largely localised around the hair follicles and oral mucosal glands (Fig. 7G). This is similar to the pattern seen in the adult tissues where CS was only localised around the hair follicles and was not present within the wound except for a very small area immediately below the regenerated basement membrane (Fig. 7H).

Inflammatory response

Staining for endogenous immunoglobulins was used as an indicator of an inflammatory response in the wounds. The fetal wounds did not show any staining specific to the wound using anti-mouse IgG or IgM antibodies (Fig. 7I). Both neonatal and adult wounds showed an inflammatory response mainly around the skin wound and within the clot filling the wound. This
persisted in the outer part of the wound after epithelialisation was complete. In the neonate, this was present from 12 h pw (Fig. 7J) to 48 h pw and in the adult from 12 h pw to 72 h pw (Fig. 7K–L).

Discussion

Fibronectins are dimeric glycoproteins, which undergo post-transcriptional splicing to form at least 20 isotypes.
Fig. 6. Transverse lip sections, skin uppermost, oral mucosa facing downwards. (A–D) Stained for collagen type VI (CVI).
(A) 16d fetus 48 h pw. CVI diffusely present in ECM of mesenchyme and present within wound. (B) 16d fetus 48 h pw. High power view showing faint staining for CVI within wound. (C) 16d fetus 5d pw. Regeneration of normal pattern for CVI without scar formation. (D) Adult 12d pw. Fibres of CVI parallel to wound surfaces forming scar tissue. (E–G) Stained for collagen type V (CV). (E) 16d fetus 5d pw. CV diffusely present within ECM and also present as linear staining of basement membranes. Normal pattern of staining for CV at site of wound without scar formation. (F) Neonate 5d pw. Normal pattern of CV staining except for loss of hair follicles on skin side of wound. Regeneration of normal pattern beneath oral mucosa. (G) Adult 12d pw. Band of scar tissue containing numerous new capillaries with CV staining of endothelial basement membrane (cf. Fig. 4L). (H) stained for collagen type I (CI). Neonate 5d pw. Faint staining for CI. Similar pattern to CIII (Fig. 5 I). m, oral mucosa; s, skin. Arrow: site of wound. Bar: 100 µm.

(Hynes, 1985), present in many connective tissues and, as a soluble form, in plasma. Fibronectin is part of the ECM early in embryonic development (for review see Duband et al. 1987) where its primary role is mediating cell adhesion and cell migration (Duband et al. 1988). During wound healing, fibronectin has many potential roles (for review see Clark, 1988b): it acts as a substratum for cell migration (Donaldson and Mahan, 1983; Clark et al. 1982; Knox et al. 1986), as an opsonin (Martin et al. 1988) and as a provisional matrix for ECM assembly (Grinnell et al. 1981; Kurkinen et al. 1980; McDonald, 1988). The pattern of fibronectin splicing in some areas of a wound resembles an embryonic pattern (ffrench-Constant et al. 1989), which raises the possibility that different isoforms may have varying functions within the wound. In this study the timing and pattern of deposition of fibronectin within the wound was similar in the three wound groups, suggesting that its function may be similar in adult and fetal wounds.

Tenascin is a glycoprotein with a very limited distribution in adult connective tissues (Chiquet-Ehrismann, 1990; Erickson and Bourdon, 1989). It is present in the mesenchyme during development of peripheral nerves (Wehrle and Chiquet, 1990), mamm-
mary gland (Chiquet-Ehrismann et al. 1986), tooth (Thesleff et al. 1987; Vainio et al. 1989), kidney (Auferheide et al. 1987), cartilage and bone (Mackie et al. 1987), and is cyclically regulated in the stroma of normal human breast as a function of the menstrual cycle (Ferguson et al. 1990). It has also been found in the stroma of mammary carcinomas (Chiquet-Ehrismann et al. 1986; Ferguson et al. 1990), basal cell carcinomas (Stamp, 1989) and endometrial carcinomas (Vollmer et al. 1990).

In this study, the distribution of tenascin in unwounded skin and oral mucosa was similar in adult and fetus and concurred with the recent studies of Lightner et al. (1989) and Sloan et al. (1990). The spatial distribution of tenascin in the wound was similar in fetus and adult, but the time post-wounding at which tenascin was first present varied from fetus to neonate to adult, as did the period of time during which tenascin was present in the wound. Tenascin was first detected at the wound margins in the fetus at 1 h, in the neonate at 12 h and in the adult, the first faint staining for tenascin was at 24 h. In the fetus and the neonate, the pattern for tenascin staining had reverted to normal by 5 d pw, while in the adult tenascin was still present within the mesenchyme at 12 d pw. The time at which tenascin is first seen parallels the rate of epithelialisation and closure of the mesenchymal wound, which was most rapid in the fetus (complete by 24 h), slower in the neonate (complete by 48 h) and slowest in the adult (complete by 72 h).

Tenascin and fibronectin are both found in the migratory pathways of neural crest cells during development and the appearance of tenascin correlates with the initiation of cell migration (Mackie et al. 1988a). Fibronectin mediates cell–substratum adhesion through its interaction with cell surface receptors of the integrin family (Pytela et al. 1985; Buck and Horwitz, 1987). Tenascin interferes with integrin-mediated fibroblast attachment to fibronectin (Chiquet-Ehrismann et al. 1988) and addition of exogenous tenascin will inhibit mesodermal cell migration during gastrulation (Riou et al. 1990). Tenascin is present in adult wounds (Mackie et al. 1988b) and may have a critical role in initiating cell migration. In this study, the appearance of tenascin preceded keratinocyte and fibroblast migration and the early appearance of tenascin in fetal wounds, through its initiation of cell migration, may underlie the rapid epithelialisation seen in fetal wound healing. In relation to epithelialisation, it is of interest that the sequence for tenascin has recently been deduced and the molecule contains thirteen consecutive epidermal-growth-factor-like repeats (Pearson et al. 1988).

Re-epithelialisation of the wounds was complete before components of the basal lamina (Type IV collagen, laminin and heparan sulphate) reformed beneath the recently migrated epithelium. This pattern was seen in both fetal and adult wounds and is in agreement with previous studies showing that the basement membrane does not reform until after epithelial cells cease to migrate (Stanley et al. 1981).

All five types of collagen studied were present within the fetal, neonatal and adult wounds. The timing of collagen deposition in the wound varied from fetus to adult – in the fetal and neonatal wounds collagen was present from 48 h pw, whilst in the adult all five collagens were seen within the wound from 5 d pw. In each wound group the different collagen types appeared concurrently (e.g. in the fetus all collagen types were first apparent at 48 h).

For the interstitial collagens (types I, III, V and VI), the critical difference between fetus and adult was in the supramolecular organisation of the collagen fibrils deposited in the wound. In the fetus, a reticular collagen pattern was restored and this was indistinguishable from the surrounding normal tissue. In the adult, large, parallel bundles of collagen, typical of scar tissue, are seen running across the wound site approximately at right angles to the basement membrane, thus disrupting normal tissue architecture. One fundamental difference between fetal and adult wound healing is therefore the control and patterning of collagen fibrillogenesis.

Collagen fibril formation is a complex process which is only partially understood (Burgeson, 1988a,b; Fleischmajer, 1986). Collagen types I, III and V are present within the same fibrils (Birk et al. 1988; Burgeson, 1988; Keene et al. 1987) and interactions between different collagens will alter fibril size in vitro (Birk et al. 1990). Interactions with other macromolecules are also likely to influence collagen fibrillogenesis. Proteoglycans will alter fibril size (for review see Scott, 1988; Vogel and Trotter, 1987) as will fibronectin (Speranza et al. 1987). Finally the role of the cell has to be considered. Fibroblasts separate stages of fibril formation in distinct cellular compartments allowing the cell to control the final fibre orientation (Yang and Birk, 1988).

Several of the differences in the ECM of fetal and adult wounds, shown in this study, may potentially alter collagen fibril formation. Collagen types I, III, V and VI are present concurrently in both fetal and adult wounds but the relative proportions of these collagen types is unknown. Fetal tissues contain a higher proportion of type III collagen to type I collagen (Epstein, 1974; Merkel et al. 1988) and this may influence collagen fibril size. The role of glycosaminoglycans is intriguing as studies have shown high levels of hyaluronic acid in fetal wounds (Krummel et al. 1987; Longaker et al. 1989; De Palma et al. 1989) whilst addition of hyaluronic acid to an adult tympanic membrane wound decreased scar formation and altered the degree of organisation of collagen within the wound (Hellstrom and Laurent, 1987). The present study demonstrated the presence of chondroitin sulphate within fetal wounds at the time of collagen fibril formation and its absence from the adult wound. The interactions of sulphated GAGs with collagen may alter fibril formation within the fetal wound. Scott and Hughes (1986) demonstrated that during development, fetal bovine and chick collagen fibrils were small in diameter at a time when hyaluronic acid was abundant and a rapid increase in collagen fibril size during mid-
gestation coincided with a decrease in hyaluronic acid and chondroitin sulphate content.

The different patterning of collagen fibrils in healing fetal (regeneration of normal dermal pattern) and adult (scar consisting of parallel, densely packed collagen fibrils at right angles to the epithelial surface) wounds may therefore relate to differences in the cellular and extracellular milieu influencing the process of fibrillo-
Fig. 7. Transverse lip sections, skin uppermost, oral mucosa facing downwards. (A–H) Stained for chondroitin sulphate (CS). (A) 16 d fetus 12 h pw. Diffuse staining for CS in mesenchymal ECM. No staining for CS within wound. Note the blotchy pattern of staining contrasts with the fibrillar pattern seen with collagen and fibronectin. (B) 16 d fetus 20 h pw. CS is now present within wound. (C) 16 d fetus 20 h pw. Higher power view of (B). (D) 16 d fetus 48 h pw. (E) Neonate 6 h pw. Patchy staining for CS within connective tissue of lip, more obviously beneath oral mucosa. No staining for CS within wound. (F) Neonate 48 h pw. Unlike fetal wound CS not present within wound at later time point. (G) Neonate 5 d pw. Wound healed, staining for CS largely confined to dermis surrounding hair follicles and oral mucosal glands and connective tissue beneath oral mucosa. (H) Adult 7 d pw. Staining for CS localised to dermis surrounding hair follicles. CS not present within wound except for a very small area immediately adjacent to the regenerated basement membrane. (I–L) Stained for immunoglobulin IgG and IgM. (I) 16 d fetus 12 h pw. No endogenous immunoglobulins demonstrated in fetal wound. (J) Neonate 12 h pw. Immunoglobulins present at wound surface. (Compare tenascin staining in Fig. 3G.) (K) Adult 24 h pw. Immunoglobulins present at wound surface and within clot. (L) Adult 48 h pw. Immunoglobulin staining within healing wound. h, hair follicle; m, oral mucosa; o, oral mucosal salivary glands; s, skin. Arrow: site of wound. Bar: 100 μm.

...retain characteristics associated with embryonic cell lines, such as cell aggregation in tissue culture (Jahoda and Oliver, 1984a). In the present study, wound healing was always faster and more fetal like, on the oral mucosal side of the incision than on the skin side, even in the adult. The adult oral mucosa possesses many embryonic characteristics (Sloan et al. 1990) and its structure is relatively simple by comparison with the complex, heavily keratinized, structure of adult epidermis. The epithelium of the adult oral mucosa is more fetal-like in its state of differentiation (as reflected in the expression of cytokeratins), human adult oral mucosal fibroblasts exhibit numerous embryonic characteristics and additionally the oral mucosa is bathed in fluid (saliva) rich in growth factors and antimicrobial agents – like fetal skin and amniotic fluid. This study has concentrated on the ECM, as the functional properties (and problems) of a scar are largely due to its collagenous structure. However the state of epithelial differentiation and potential epithelial–mesenchymal interactions during wound healing requires further investigation and a study of cytokeratins in fetal and adult wounds has recently been completed (Whitby and Ferguson, in preparation).

Localisation of type IV collagen and laminin demonstrates the profuse neovascularisation present in the adult wound. The absence of neovascularisation in the fetal wounds suggests either a reduction in the level of angiogenic factors in such wounds or an inability of the cells to respond to factors in both. A lack of stimuli correlates with the absence of a marked inflammatory response in the fetal wound, demonstrated in this study by the lack of endogenous immunoglobulins. The absence of a cellular inflammatory response in fetal wounds is noted in several studies (Rowsell, 1984; Hallock, 1985; Berrington, 1971; Sopher, 1975) and the role of the macrophage, which plays a key role in adult wound healing (Liebovich and Ross, 1975), in fetal wound healing is unknown.

A number of differences between the ECM of the fetal and adult wound were demonstrated in this study, but the mechanisms controlling these differing processes are undefined. The differences in the ECM, the absence of neovascularisation in the fetal wound and the rapid healing of oral mucosal wounds may all relate to the growth factors present in the fetal wound. For example TGFβ will increase the synthesis of collagens, fibronectin and tenasin (Ignotz and Massague, 1986; Roberts et al. 1986; Pearson et al. 1988) and Krummel et al. (1988) have shown that addition of TGF to a fetal wound induces fibrosis. A separate study (Whitby and Ferguson, in preparation) analysing the distribution of various growth factors in fetal and adult wound healing has demonstrated variations in TGFβ and bFGF distribution between fetal and adult wounds, which may be causative of some of the differences observed in the present study.

Finally, the present study has demonstrated that, in the mouse, there is no sudden switch from a fetal to an adult wound healing pattern; rather there is a slow
transition in and around the neonatal period. This slow transition is also present in long gestation animals, such as sheep, where it occurs in the third trimester (Longaker et al. 1990). Such a slow transition is indicative of the multifactorial differences between fetal and adult wound healing: rapidity of wound closure, degree of inflammatory response, maturity and competence of the immune system, degree of organisation and differentiation of the wounded tissues, composition and organisation of the extracellular matrix, presence or absence of an aqueous sterile environment (amniotic fluid) rich in growth (and other) factors, differences in tissue oxygenation, wound metabolism and pH, degree of tissue tension from function, presence or absence of acute inflammation. Moreover, whether or not fetal wounds scar also depends upon: the type and size of the wound (large, deep excisional wounds scar), the tissue wounded (internal tissues e.g. diaphragm, scar) (Longaker et al. 1991) and the species used – rabbit fetuses do not heal their wounds properly (Krummel et al. 1987) probably due to the large amounts of maternal immunoglobulin in rabbit amniotic fluid as a result of the size of the zona chorion during placentation (Wild 1965). Therefore investigations searching for a magic transition point (and differences at that point) (Inara et al. 1990) are unlikely to be productive.

The differences between fetal and adult wound healing are multiple and subtle. This study suggests a number of ways in which the healing adult wound might be experimentally manipulated to reduce scarring: enhance cell migration by addition of exogenous tenascin, develop a more open early wound scaffold to facilitate fibroblast migration and regeneration of the normal reticular collagen fibril orientation by addition of exogenous glycosaminoglycans and proteoglycans, clonally expand or transplant (e.g. from the oral mucosa) fibroblasts with embryonic characteristics at the wound site. It may be possible to manipulate adult wound healing to show a more fetal-like, scarcely, pattern by altering only one or two of the (major) components that are different between fetal and adult healing.

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