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Wound closure in foetal rat skin

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Summary

Foetal rat skin rapidly closes an open wound in organ culture and *in vivo*, this possibly being unique to organs still in the morphogenetic stage. In the present study, examination was made of morphological changes in foetal rat skin during closure of open wounds inflicted at day 16 of gestation. Phase-contrast microscopy of open-wounded skin cultured *in vitro* indicated inward spreading of the peripheral skin to be responsible for wound closure. Wound closure *in vitro* was inhibited by cytochalasin B (10 μg/ml), not by hydroxyurea (2 mM), indicating prenatal wound closure to be mediated by regulation of the microfilament system rather than cell proliferation. During wound closure *in vitro* and *in vivo*, light and scanning electron microscopy of the peripheral skin showed cells in the periderm, the outermost layer of the foetal epidermis, to elongate centripetally and *en masse*, whereas the shape of underlying epidermal cells not to change. Numerous spindle-shaped cells and fibrous matrices in the mesenchyme were redistributed, becoming oriented along the wound edge. Following isolation of the mesenchyme and epidermis by treatment with Dispase and separate culturing, the capacity for wound closure *in vitro* was found to be retained only by the mesenchyme. Cellular activity within the mesenchyme, rather than in the epidermis, would thus appear essential to wound closure in foetal rat.

Key words: rat foetus, wound healing, wound closure, tissue spreading, skin, organ culture.

Introduction

The process of wound healing involves cytological events, such as cell division, migration and differentiation, all fundamental to the process of development. Certain mechanisms for cell-to-cell and cell-to-extracellular matrix interactions may also be common to both these processes. However, the dynamics of healing in organs wounded at mid-development remains unclear. For example, whether open wounds in foetuses heal through the recruitment of new cells by enhanced mitotic activity or by covering with tissue already available about the wound site is a point remaining to be clarified. Both mechanisms are possible since developing organs are generally mitotic and malleable. It is also unclear as to how each constituent tissue, such as epithelium and mesenchyme, behaves during wound closure.

The main reason why foetal wound healing is not well understood is that most studies on this process have been conducted *in utero* (Goss, 1977; Hallock, 1985; Krummel et al. 1987; Siebert et al. 1990; earlier studies reviewed in Sopher, 1975). An organ-culture system that permits the simulation of the *in vivo* healing as much as possible is essential for such study. A model of foetal wound healing was previously devised by the authors in which open-wounded skin from rat foetuses was cultured *in vitro* free of artificial interactions associated with interference or contact with the surface of culture dishes (Ihara et al. 1990). By this model, an open wound in day-16 foetal skin closed within 3 days of culture, whereas, in day-18 foetal skin, a fibrin network in the open space was formed without wound closure. The transition of wound healing patterns was essentially the same as that noted for intrauterine healing. Prompted by these findings and data obtained by organ-culture techniques, attempt was made in the present study to clarify the mechanisms of wound closure and determine the sources of materials that fill the lesions in day-16 foetal rats. Morphological changes at the periphery of a wound were found to involve spreading, not shrinkage, of surrounding skin. Foetal wound closure may possibly be mediated by cellular movement within the mesenchymal layer, independent of cell proliferation.

Materials and methods

Animals

Sprague-Dawley rats (Japan SLC Incorporation, Hamamatsu, Japan) were used. The day on which sperm could be

detected in the vagina was taken as day 0 of gestation. Dams were killled by etherization on day 16 of gestation; the foetuses were removed aseptically from the uteri and used for skin cultures.

Skin culture

Wounds (1 mm in diameter) were made by excision in the dorsal skin of day-16 foetuses. The skin (approximately 1 × 1 cm^2) surrounding the wound was cut out and cultured as described previously (Ihara et al. 1990). The culture medium was supplemented with either 10% foetal calf serum (FCS) or serum prepared from pregnant rats at day 16 of gestation (PRS). The results were consistent, regardless of the kind of serum used. Phase-contrast photomicrographs were taken at specified intervals during culture, and remaining wound area was estimated by tracing the circumference of the wound on photographs using an image analysing system (IBAS; Kontron Elektronik, München, Germany). Each skin-culture experiment was conducted in duplicate, using 10 to 14 foetuses from a single litter. The results of the experiments showed close agreement in all cases.

Inhibition study

In some skin-culture experiments (Figs. 3-5), the culture medium was supplemented with one of the following inhibitors: 2 mM hydroxyurea (Young and Hodas, 1964); 10 μg/ml cytochalasin B (Spooner and Wessells, 1970); 50 ng/ml colcemid (Kleinfeld and Sisken, 1966); 5 μg/ml cycloheximide (Ennis and Lubin, 1964); or 100 μg/ml L-azetidine-2-carboxylic acid (LACA, Stenn et al. 1979). Each culture was examined by phase-contrast microscopy (Ihara et al. 1990).

Separation of epidermis and mesenchyme

Immediately after the wounds had been inflicted (Fig. 9), pieces of skin from the wound area were treated with 1,000 units/ml Dispase (Godo Shusei, Tokyo, Japan) in Dulbecco's modified Eagle medium (DMEM) at 4°C for 3 h. The epidermis and mesenchyme were separated by forceps and each was rinsed twice with fresh DMEM and cultured separately from the other in the same manner as for full-thickness skin.

Immunohistochemical detection of proliferating cells

Four pieces of wounded skin from day-16 sibling foetuses were cultured for 18 h with 2 mM hydroxyurea in the culture medium (DMEM containing FCS) and for an additional 6 h in fresh medium with 2 mM hydroxyurea containing 10 μM bromodeoxyuridine (BrdU). For comparison, four other pieces of skin from the same litter were incubated with BrdU, but without hydroxyurea in the culture medium. The BrdU-treated pieces of skin were fixed and embedded by the AMeX method (Sato et al. 1986).

By indirect immunofluorescence, the AMeX preparations were examined for the presence of BrdU-positive nuclei (Fig. 4). Following deparaffinization with xylene and rinsing with acetone, the sections (3 μm thick) were pretreated with 0.3% hydrogen peroxide for 30 min, 1 N HCl for 20 min, 0.05% protease (type XXVII, Sigma, St. Louis, MO, USA) in phosphate-buffered saline (PBS) at 37°C for 10 min, and 1.5% normal equine serum in PBS for 20 min, in this order (Sugihara et al. 1986). The sections were then incubated for 30 min in 1:20 diluted monoclonal antibody against BrdU (Dakopatts, Glostrup, Denmark) and for 30 min in a 1:40 diluted fluorescein-conjugated sheep anti-mouse IgG (Cappel, West Chester, PA, USA). The sections were washed with PBS after each incubation. The buffer used for diluting the primary and secondary antibodies was PBS containing 1 mg/ml crystallised bovine serum albumin. The stained sections were mounted in carbonate-buffered glycerol (pH 9.3) and examined by an Olympus microscope (model BHT, Olympus, Tokyo, Japan) equipped with epifluorescence optics (model BH2-RFK).

In vivo *study*

On each of three foetuses per litter, a 1-mm excisional wound was inflicted by excision *in utero* on day 16 without termination of pregnancy and with minimal amniotic disturbance (Ihara et al. 1990). The dams were killed by etherization 38 h later. The wounded skin was removed from each foetus and examined by scanning electron microscopy (SEM). The reproducibility of results was confirmed by repeating the experiment on 30 foetuses representing 10 litters.

Other methods

The procedures for histology and SEM were the same as described elsewhere (Ihara et al. 1990).

Results

Spreading of peripheral skin

Even at full thickness, skin excised from foetal rats was so thin that the distribution of hair follicle rudiments and bloodstains, when present, could be easily seen under a phase-contrast microscope. With these constituents as markers, the spreading of skin at the zone peripheral to the edge of the wound could be repeatedly observed during wound closure *in vitro*. Phase-contrast micrographs from a representative experiment are shown in Fig. 1. The distance from each of several markers (points a through f) to the nearest point on the edge of a wound was measured and plotted at 0, 16, 24, and 48 h of culture (Fig. 2A). This parameter was found to progressively increase with time, particularly during the period of 16 to 48 h of culture. The amount of skin increased, as was evident from the net area of the region of skin enclosed by the pentagon abcde (Fig. 2B). The centripetal spreading of peripheral skin is thus shown to be the means (mechanism) for wound closure. It is also evident that incidental shrinkage due to the experimental environment would make little contribution to this.

Proliferation of cells

Due to prolific mitosis, dorsal skin of the rat embryo shows profuse growth rate at day 16 (Stern et al. 1970). Increase in the area of skin along the periphery of a wound *in vitro* would thus prompt the consideration that cell proliferation may be the major factor for wound closure. To examine this point, 2 mM hydroxyurea was added to the skin-culture medium (Fig. 3). However, the rate of wound closure showed virtually no change. Immunohistochemical examination using BrdU-specific antibody showed DNA synthesis to be blocked by 2 mM hydroxyurea (Fig. 4). Consequently, wound closure is independent of any special stimulation directed to cell proliferation.

Inhibition of wound closure

To determine the roles of other endogenous factors in

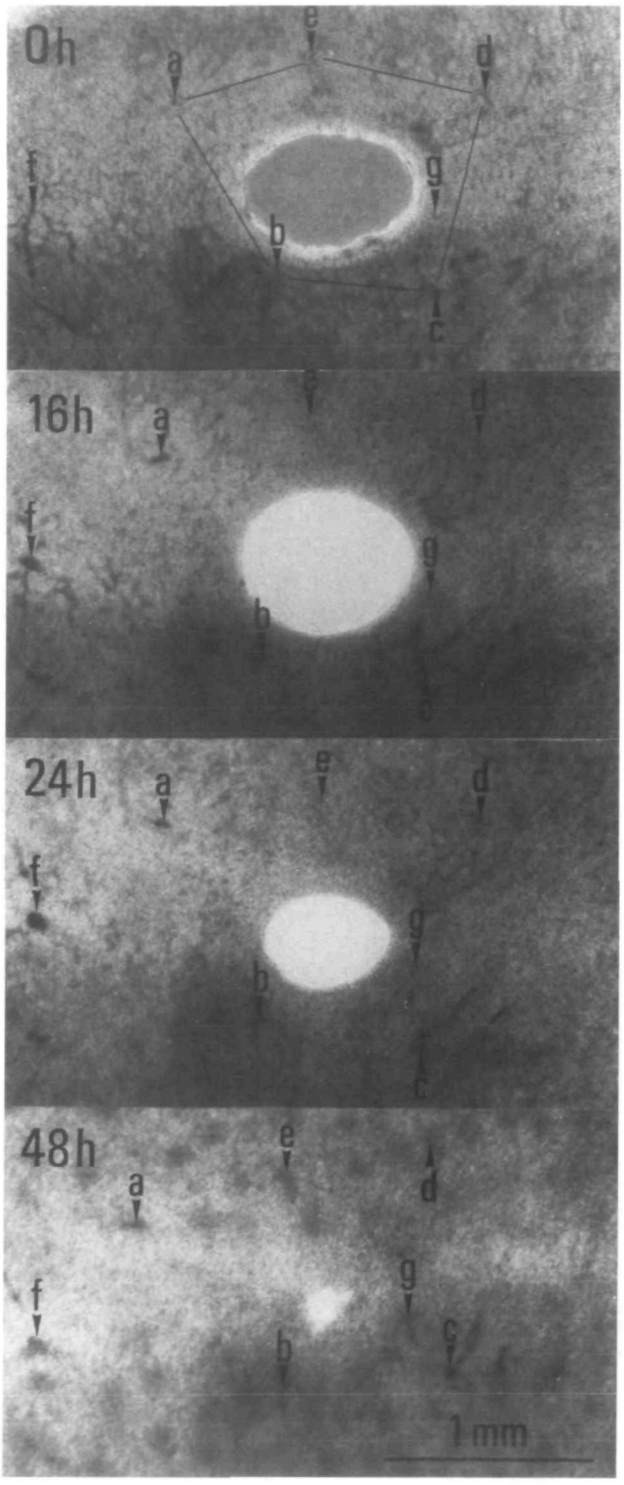

Fig. 1. Phase-contrast microscopic survey of wound closure *in vitro*. A foetal skin piece wounded at day 16 of gestation was cultured in DMEM containing PRS. Phase-contrast micrographs were taken at the times indicated. a-g: hair bulbs or bloodstains.

wound closure *in vitro*, examination was made of the influence of each of four inhibitors on this process (Fig. 5). Colcemid had no effect on the rate of wound closure, whereas reversible inhibition was noted in the

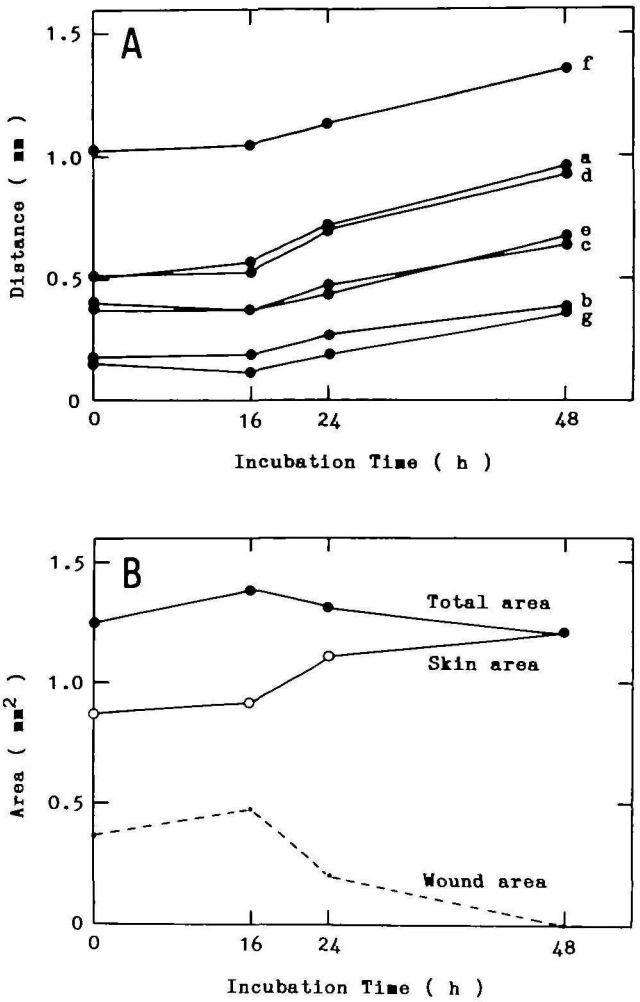

Fig. 2. Spreading of surrounding skin during wound closure *in vitro*. (A) Time course of distances from specified points to wound edge. The distances from points a-g in Fig. 1 to wound edge were plotted against the times indicated. (B) Time course of wound area and that of the specified skin region surrounding the wound. ●——● area of pentagon abcde; ○——○ skin area enclosed by the pentagon; ---, wound area itself.

case of cytochalasin B. In the presence of cycloheximide, total protein synthesis was blocked, thus retarding healing and wound area increased considerably. LACA, an inhibitor of collagen deposition (Takeuchi and Prockop, 1969), had little effect on wound closure.

Morphological changes during wound closure

Wound closure in progress is shown by photomicrographs of histological sections of full-thickness skin cultured *in vitro* (Fig. 6). The cells along the margin of the wound were still round in shape just after inflicting the wound (Fig. 6A), but at 23 h of culture, skin pieces showed multiple rows of fully elongated cells as bundles in the margin of the edge of the wound (Fig. 6B). Most other cells at this time were elongated without any definite orientation.

In the epidermis, no reshaping of cells associated with the progress of wound closure could be detected

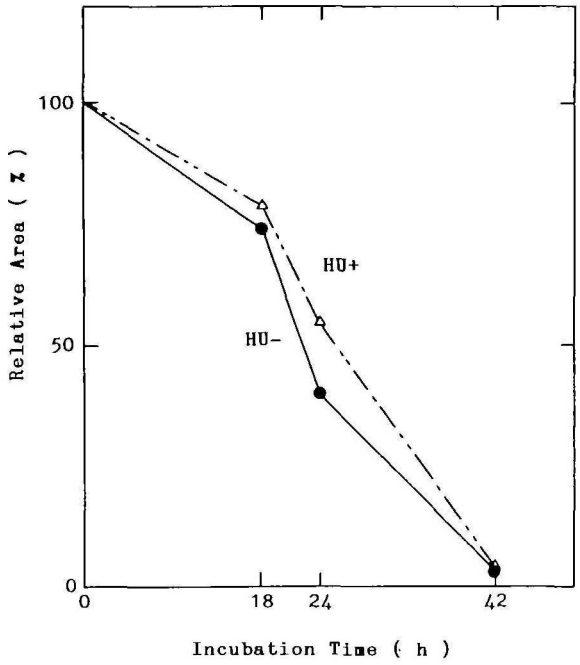

Fig. 3. Effects of hydroxyurea (HU) on wound closure *in vitro*. Foetal skin pieces wounded at day 16 of gestation were cultured in DMEM containing PRS with (△) or without (●) 2 mM hydroxyurea. Mean wound area in duplicate assay was plotted against the times indicated. The deviation of area in each assay was less than 21%.

(Fig. 4), this being consistent with earlier observations by light microscopy (Ihara et al. 1990). The morphological stability of the epidermis indicated the spatial increase in area of the peripheral skin (Figs. 1 and 2) to possibly be maintained continuously and in a manner that would enable individual epidermal cells to retain their shape. In the monolayer of cells covering the foetal epidermis (periderm), the cells became elongated perpendicular to the edge of the wound (Fig. 7, left panels) whereas, in the mesenchyme, cells abundantly present around the wound edge became elongated and aligned in a concentric pattern along the wound edge (Fig. 7, lower right panel), as seen by SEM. The mesenchymal cells clinging to the edge were considered the same as those noted at the edge of the wound in the light micrographs at 23 h (Fig. 6B).

The long cytoplasmic axes of the elongating peridermal cells were at right angles to those of the mesenchymal cells *in vitro* as well as *in vivo*, as indicated by SEM (Fig. 8). Skin specimens from embryos on which open wounds had been made at day 16 and maintained for an additional 38 h *in utero* were shown by SEM to exhibit regular alignment of elongating mesenchymal cells covered by a fine extracellular matrix and oriented along the edge of the wound. The alignment *in vivo* was even more clearly evident than *in vitro* (Fig. 8, lower panels).

Wound closure in isolated mesenchyme

The epidermis and mesenchyme were separated by treating skin fragments with Dispase immediately after

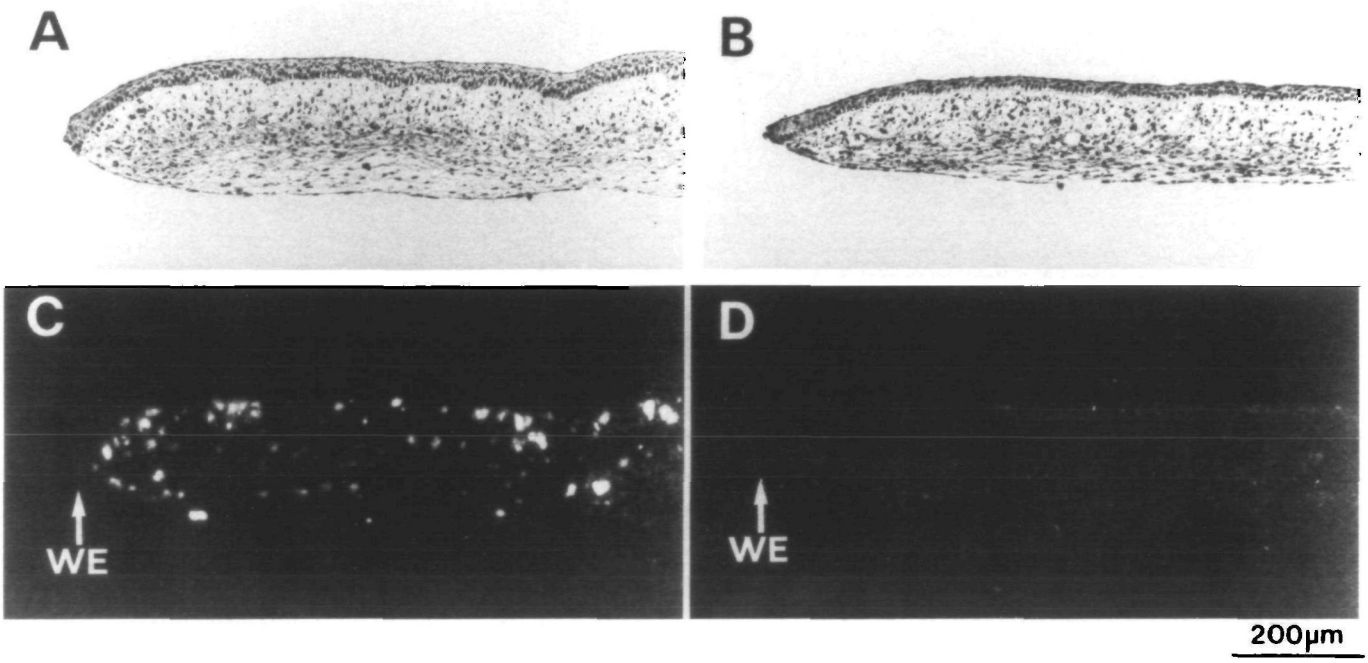

Fig. 4. Anti-bromodeoxyuridine staining of foetal wound closure *in vitro*. Foetal skin pieces wounded at day 16 of gestation were cultured for 24 h in DMEM containing FCS with or without 2 mM hydroxyurea. BrdU was included for the last 6 h of culture. Adjacent sections from skin pieces were stained either with hematoxylin and eosin (A,B) or with the BrdU-specific antibody (C,D). (A),(C) Control specimen without hydroxyurea, whose wound decreased to 60.6% and 31.0% the original area at 18 and 24 h of culture, respectively. (B),(D) Hydroxyurea-treated specimen, whose wound area decreased to 61.1% and 29.3% at 18 and 24 h of culture, respectively. Microscopic fields of sections A and B correspond to those of sections C and D, respectively. WE: wound edge.

wounding. The two isolated tissue strata were cultured separately but in the same manner as for full-thickness skin. At the start of culture, wound size was slightly smaller than at the time it had been made, since by treatment with Dispase, the entire piece of skin unavoidably shrank. During culture, the fates of open wounds in the two types of isolated tissue differed greatly. The mesenchymal wound closed at essentially the same rate as that in full-thickness skin (Fig. 9B), while the epidermal wound increased in size within 12 hours (Fig. 9A).

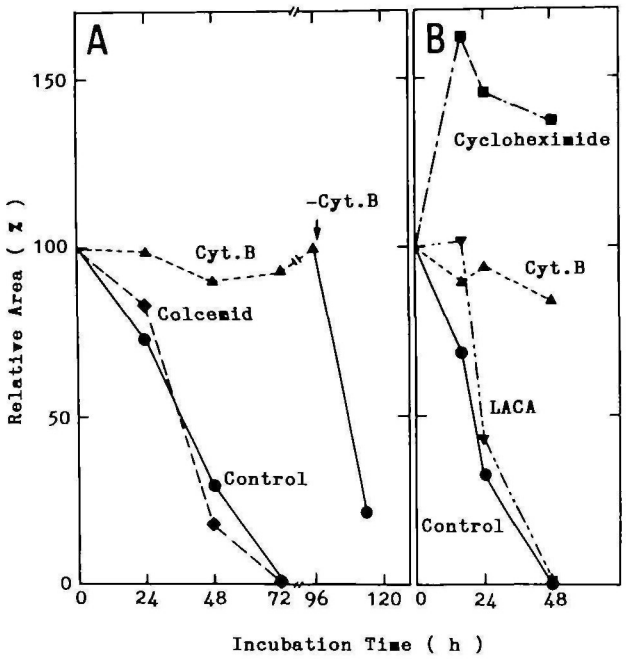

Fig. 5. Influence of various inhibitors on wound closure *in vitro*. Foetal skin pieces wounded at day 16 of gestation were cultured in DMEM containing FCS (A) or PRS (B) with cytochalasin B (▲ 10 μg/ml), colcemid (◆ 50 ng/ml), cycloheximide (■ 5 μg/ml), LACA (▼ 100 μg/ml) or without any inhibitor (●). Mean wound area in duplicate assay was plotted against the times indicated. The deviation of area in each assay was less than 20%.

Discussion

Our previous study *in vitro*, in which the present culture system was used, indicated foetal rat skin at day 16 of gestation to be capable of closing an open wound and that, on that particular day, conditions were best for wound closure (Ihara et al. 1990). In regard to the rapid healing of open wounds, our previous and present findings indicate the following. (a) During replenishment of skin destroyed by injury, the covering over the wound derives from full-thickness skin surrounding the wound without the need for any additional structures. (b) The skin surrounding the wound does not decrease in size but rather spreads out toward the wound during closure (Figs. 1 and 2). Distortion of the peripheral skin, i.e., 'spreading,' involves contraction of the tangential coordinate and elongation of the centripetal coordinate (Fig. 10A). (c) The spreading, independent of cell proliferation, depends on the regulatory action of the microfilament system within cells of the peripheral skin (Figs. 3-5).

The gradual but progressive reduction in size of open wounds in rat skin is a physiological process that may be considered in a broad sense as wound contraction (Abercrombie et al. 1954). As presently understood by prenatal healing in mammals, rapid wound closure in

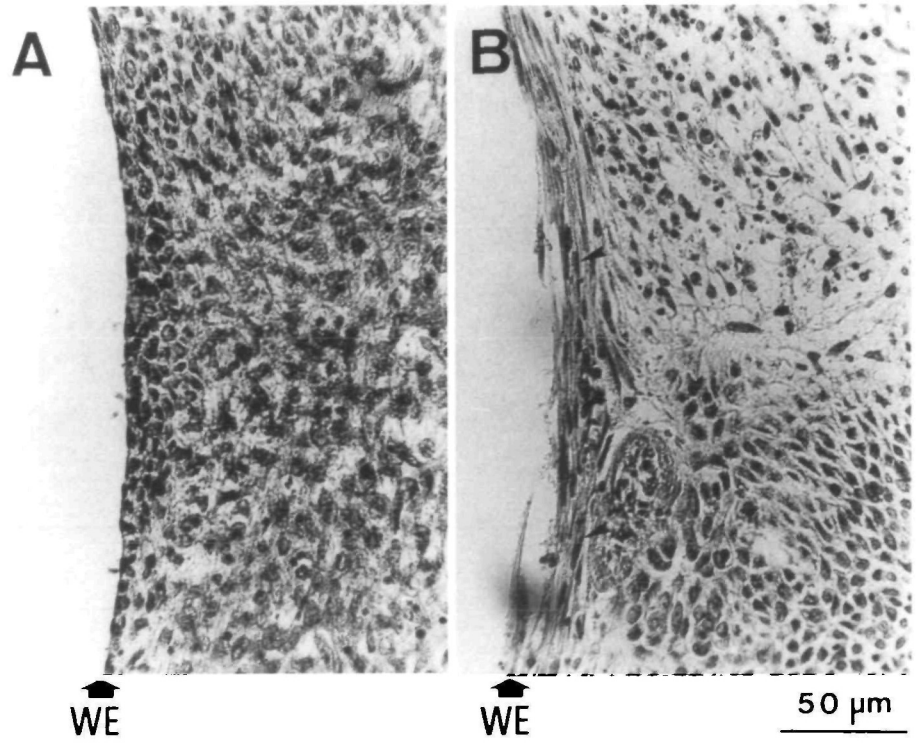

Fig. 6. Photomicrographs of foetal wound closing *in vitro*. Foetal skin pieces wounded at day 16 of gestation were cultured in DMEM containing PRS. Just after wounding (A) or at 23 h of culture (B), when wound area had decreased to 75% the original area, the piece was fixed in 10% formalin, sectioned parallel to the apical surface of the skin and stained with hematoxylin and eosin. Arrowheads indicate cells elongating along wound edge. WE: wound edge.

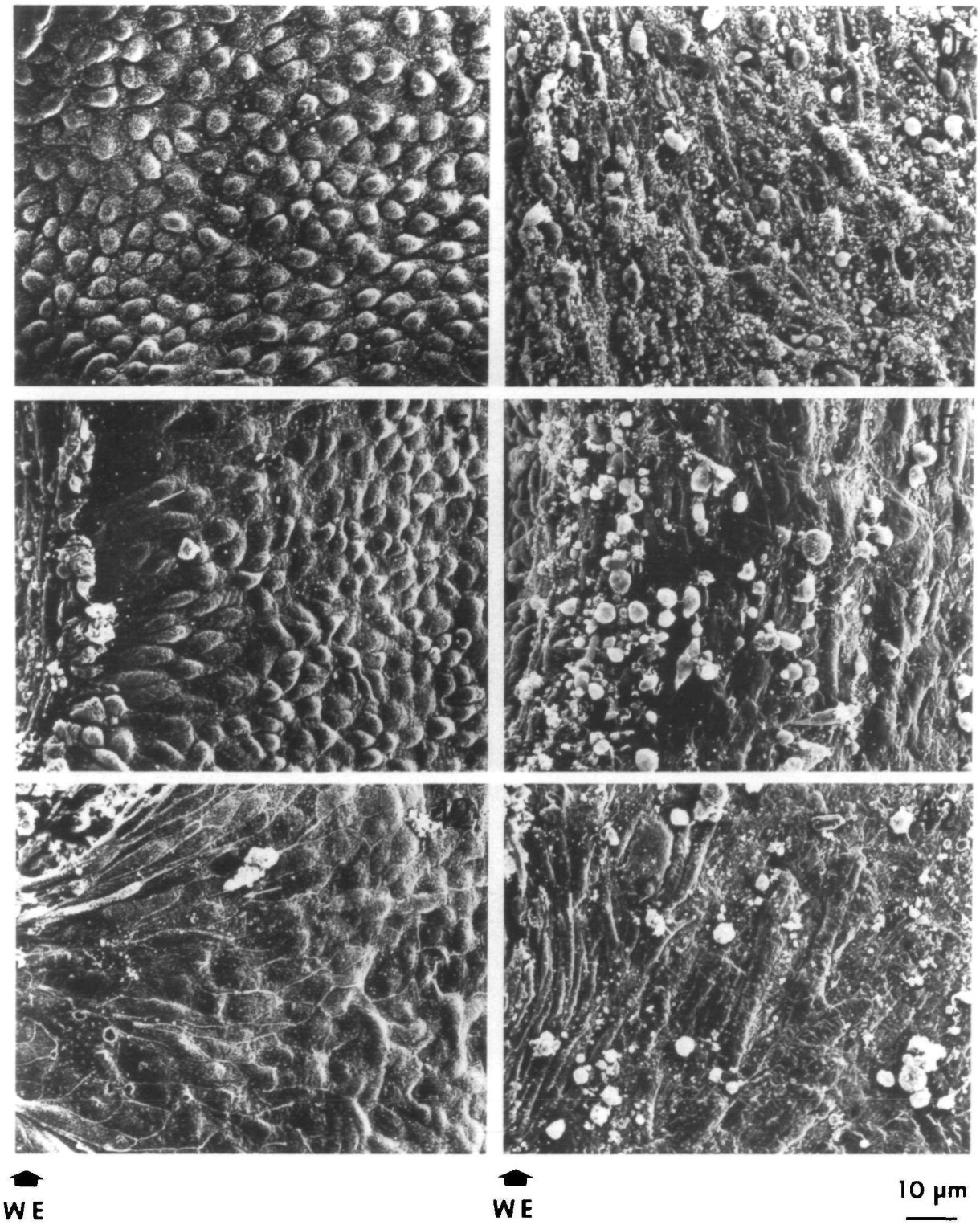

Fig. 7. Scanning electron microscopic survey of wound closure *in vitro*. Foetal skin pieces wounded at day 16 of gestation were cultured in DMEM containing PRS. At 0 h (top), 15 h (middle) and 42 h (bottom) of culture, the skin pieces were examined by SEM. Left: views of apical surfaces. Right: views of basal surfaces of the same specimens as on the left. In the specimen shown in the middle, wound area has not changed, whereas the wound shown at the bottom has closed almost completely (1.0% the original area). WE: wound edge.

this study may also be defined as wound contraction, since there is no apparent distinction between the periphery of a foetal wound and that of an adult wound, and the mechanism for contraction in either case has yet to be determined. The results of the present study indicate a contractile force to possibly be involved in

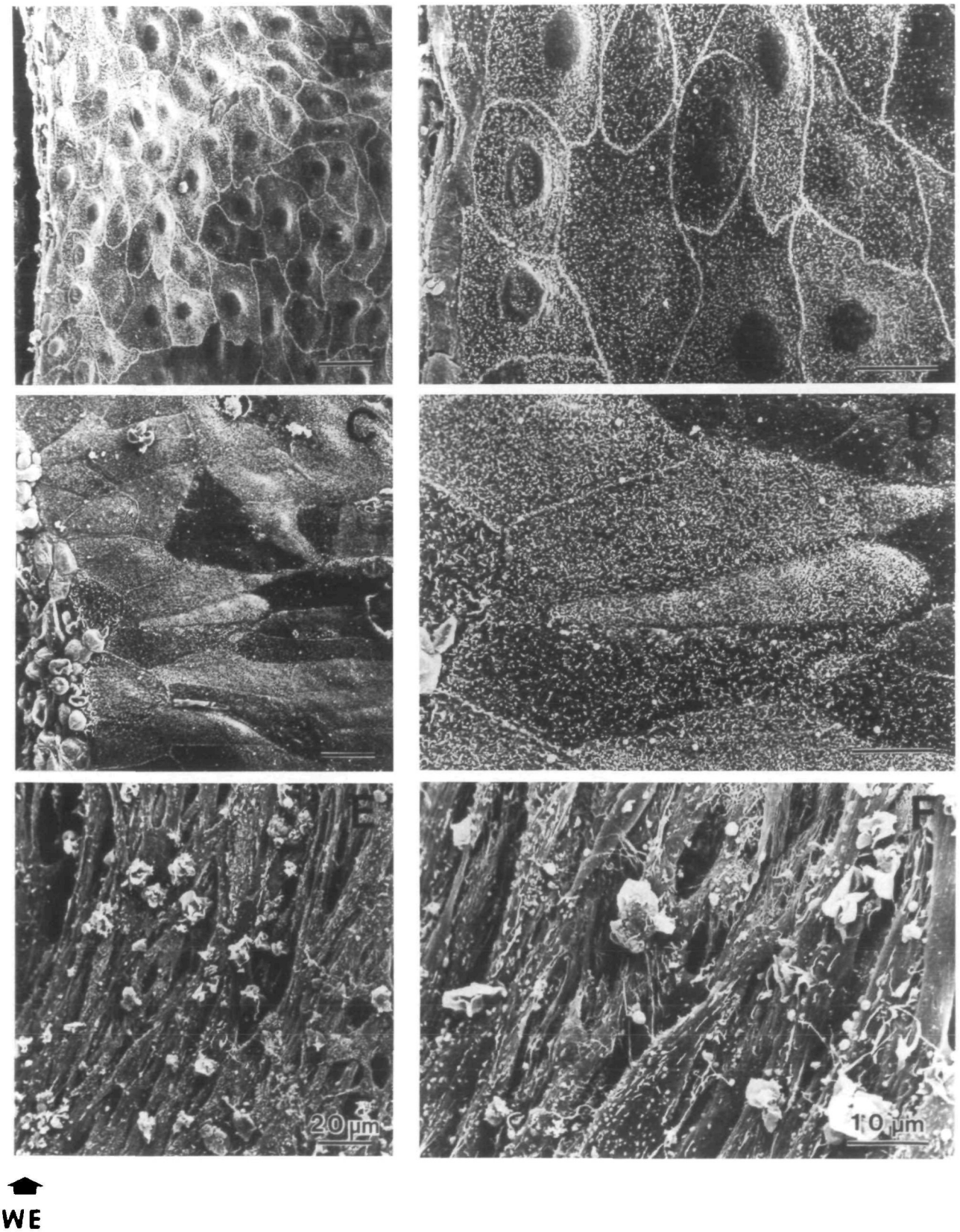

Fig. 8. Scanning electron micrographs of foetal wound healing *in vivo*. Wounds were made by excision in day-16 foetuses. The wounded skin pieces were excised immediately thereafter (A, B) or at 38 h after the operation (C-F) and examined by SEM. At the top and middle, apical surfaces, and at the bottom, basal surfaces are shown. Left (A, C and E): low magnification. Right (B, D and F): high magnification. WE: wound edge.

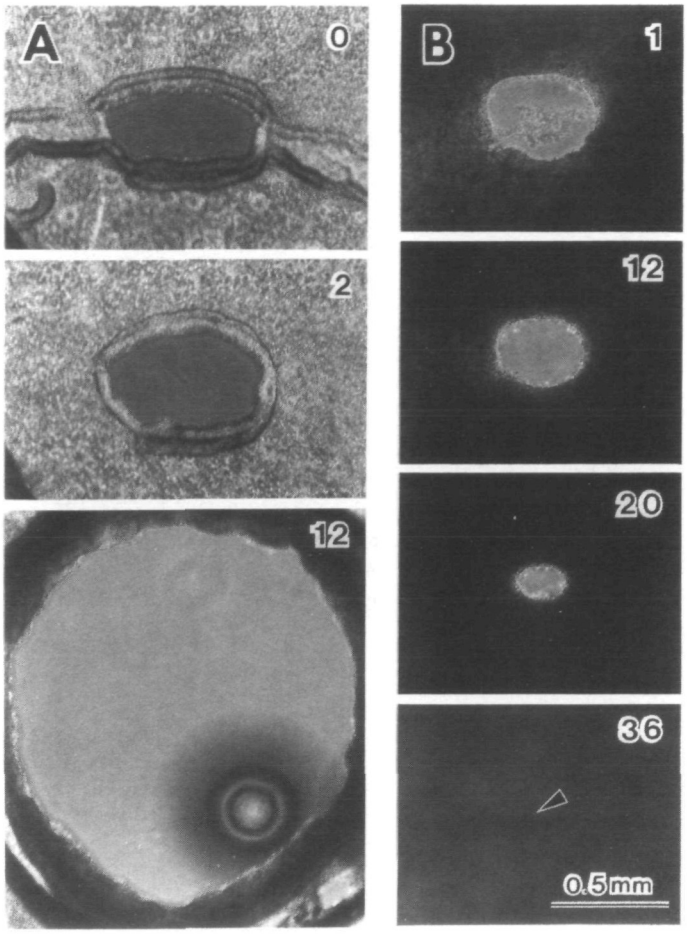

Fig. 9. Wound closure *in vitro* in isolated mesenchyme. Foetal skin pieces were wounded at day 16 of gestation, and epidermal (A) and mesenchymal (B) pieces isolated by Dispase treatment were cultured in DMEM containing PRS. Phase-contrast micrographs were taken at times (hours) indicated. Arrowhead: wound trace.

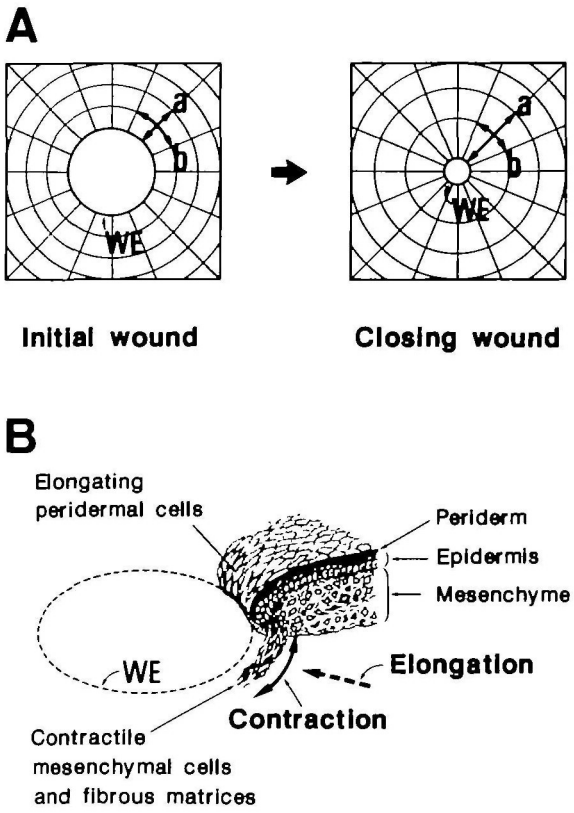

Fig. 10. Schematic view of foetal wound closure. (A) Two-dimensional view of open wound and peripheral skin. The overall morphological change is inward spreading of peripheral skin. A fraction of skin nearest the wound edge has elongated centripetally (a) and contracted tangentially (b) with time. (B) Movements of individual cells in the periderm, epidermis and mesenchyme. Contractile mesenchymal cells elongating along the wound edge may be initially essential for closure. Epidermal spreading is inconspicuous and peridermal cells elongate centripetally to cover underlying epidermis. The total process results in increased skin area. WE: wound edge.

wound closure. However, a foetal wound as the site for this process is at variance with conventionally held views.

The presence of granulation tissue would be essential for wound contraction in adult rats. Granulation tissue contains numerous myofibroblasts rich in bundles of microfilaments (Gabbiani et al. 1971, 1972, 1979). Moreover, the contraction of collagen lattices by fibroblasts maintained *in vitro* has been noted to be basically the same as that of granulation tissue *in vivo* (Bell et al. 1979; Bellows et al. 1982; Gillery et al. 1986; Montesano and Orci, 1988). The closure of foetal wounds even without granulation tissue as noted here may be explained as follows. Foetal skin may be the counterpart of granulation tissue *ab origine* since, as in the case of granulation tissue, it is rich in type III collagen (Merkel et al. 1988; Clore et al. 1979) and hyaluronic acid (Loewi and Meyer, 1958; Bentley, 1967). Type III collagen forms thinner fibrils than type I collagen (Fleischmajer et al. 1980), this possibly enhancing the malleability of the skin, and a hyaluronate meshwork may facilitate cell migration, a possibility also pointed out by Toole (1981). In addition to such an extracellular matrix possessed by both foetal skin and granulation tissue, foetal skin may contain cells analogous to myofibroblasts in granulation tissue. Thus, the foetal skin would be able to bypass the time-consuming formation of granulation tissue.

The present study has indicated wound closure to be possible even without granulation tissue and raises the issue as to whether epidermis or mesenchyme is most important for foetal wound closure. Malleability, essential for the closure of open skin wounds, may be a property possessed by both the epidermis and mesenchyme at day 16 of gestation. This would particularly appear so since the epidermis is not fully differentiated at this stage of incomplete stratification and the mesenchyme shows some resemblance to granulation tissue, as explained above. Regarding the alignment of cells and direction of cell elongation, layer specificity was noted in peridermal, epidermal and mesenchymal

layers (Figs. 6-8). To detect contractile action possibly contributing to wound closure, epidermal and mesenchymal layers with open wounds were isolated and cultured separately. In the mesenchyme, the wounds closed completely and at a rate essentially the same as that in full-thickness skin. In the epidermis, wound size increased in half a day. Wound gaping in the epidermal layer may be an indication of tissue break-down and deterioration due to deorganization and degeneration of cells retrogressing from the wound edge (Fig. 9). Wound closure in the isolated mesenchymal layer required the presence of serum in the culture medium (not shown), a condition also noted for full-thickness skin (Ihara et al. 1990). Mesenchymal wound closure *in vitro* would thus be merely a non-physiological artifact.

Based on the present results, a schema was constructed to show cellular events accompanying foetal wound closure (Fig. 10B diagram), as follows. The primary event may be the regular alignment of elongating mesenchymal cells and fine fibrous matrices densely arrayed along the wound edge. A mechanical force may be generated within the circular alignment to promote a counter-struggle between diametrically opposite tangents, possibly resulting in reduction in wound circumference. Tautness in the reduced circumference may in turn cause an inward spreading of malleable foetal skin in the region peripheral to the wound. Due to the proximity of the mesenchyme and epidermis, the spreading, driven by mesenchymal machinery, may cause passive spreading of the epidermis along with that of the mesenchyme. Cells in the epidermis would not have to undergo modification of shape during this process. Consequently, factors inducing foetal wound closure are considered inseparably allied with cellular alignment specifically present in the mesenchyme.

Foetal wound closure as observed *in vitro* indicates foetal rat skin to be capable of filling in the lesion by entering the site of the lesion itself. However, the present culture model does not have a wound bed that functions as a substratum for cell ingrowth and thus examination of cytological behaviour allied with a network of substrata or scaffolding is not possible. Further study should be conducted to clarify the role of cell migration in foetal wound healing, using an appropriate wound model which adequately considers the wound bed. Total effect of LACA should also be studied by such an improved model. The rate of wound closure was not influenced by LACA, indicating the deposition of collagen in the mesenchyme not to be essential for wound closure (Fig. 5). However, in preliminary studies using a conventional explant culture system (unpublished results), epidermal outgrowth from foetal rat skin was found to be inhibited by LACA as efficiently as that from adult skin, (Stenn et al. 1979).

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