MORPHOGENETIC ASPECTS OF MULTILAYERING IN PETRI DISH CULTURES OF HUMAN FETAL LUNG FIBROBLASTS

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ABSTRACT

Randomly seeded Petri dish cultures of embryonic human lung fibroblasts generate, in the course of their growth, highly ordered cellular arrangements. Thick, bilaterally symmetrical ridges with an axial polarity and an orthogonal, multilayered internal organization are observed within stationary cultures. The generation of these structures has been investigated. Ridges result from the spontaneous aggregation of cells in postconfluent cultures brought about by directed cell movements. These movements are promoted by the localized production of extracellular matrix sheets containing collagen, which provide new substrates for cellular colonization. Cells that have colonized one matrix substrate may secrete another above themselves, which will in turn be colonized. By a continuation of this cycle, thick stacks consisting of alternate layers of cells and matrix are produced to yield the observed aggregations. The distribution and shape of ridges in a culture imply that matrix substrates are confined to specific locations. The suggested control hypothesis assumes that all the cells in fibroblast cultures are potential producers of a single species of matrix. The serviceability of this matrix as a substrate for cellular colonization, however, is destroyed if the producer cells are motile. Matrix substrates, therefore, are only made by nonmotile cells.

We have observed that fetal lung fibroblasts passaged in Petri dish cultures exhibit pronounced multilayering, growing to approximately six confluence equivalents before net growth ceases. This behaviour contrasts with that of lines of fetal gut fibroblasts which attain their maximum cell density only slightly after confluence and show little or no multilayering. This paper considers morphogenetic aspects of multilayering and examines the form and generation of the dense cellular associations observed in stationary cultures of fetal lung fibroblasts.

MATERIALS AND METHODS

Cell Lines

Lines of fibroblasts were established from the lungs of human fetuses 10–16 wk after conception, by using primary cultures of chopped fragments or cell suspensions prepared with trypsin (Difco Laboratories, Inc., Detroit, Mich.; 1:300) and collagenase (Worthington C.L.S.; Worthington Biochemical Corp., Freehold, N.J.). To eliminate nonfibroblastic cells, lines were subcultured at least twice before use.

Cultures were seeded, after trypsin dispersal of parent cultures, usually in Falcon disposable plastic
tissue culture dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) but occasionally in Nunclon disposable plastic tissue culture dishes or other dishes more appropriate to particular experiments, as indicated. Routine medium consisted of Ham's F 10 (13) supplemented with 8% Tryptose phosphate broth, 10% partially deglobulinized bovine serum, 50 µg/ml ascorbic acid, penicillin, and streptomycin.

Cell counts were made with a Coulter model A cell counter.

Collagenase

Stock solutions of collagenase containing 6 mg/ml in Dulbecco saline were sterilized by filtration and kept not longer than 4 days at 4°C.

Rat tail collagen was prepared by acid extraction and was used to prepare collagen surfaces in Petri dishes (1 mg of collagen per 2 in. dish) by the method of Ehrmann and Gey (6).

Collagen Estimation

Procedure 2 of Prockop and Udenfriend (22) was used to measure hydroxyproline as an index of collagen present in cultures. The culture medium was drained from the dishes which were then washed three times with 10-ml volumes of physiological saline during 5 min. Concentrated hydrochloric acid was added directly to the dishes, the whole cultures were suspended in the acid by scraping, and the mixture was transferred to glass ampoules. The dishes were washed with an equal volume of distilled water, and the washings were added to the mixture in the ampoules. For hydrolysis the ampoules were sealed and maintained at 130°C for 16 hr.

Time-lapse films were made in collaboration with Mr. E. C. A. Lucey of the Edinburgh University Film Unit.

Still photography was performed on methanol- and acetone-fixed cultures stained with hematoxylin.

EXPERIMENTS AND RESULTS

Simple Culture for the Observation of Multilayering and Collagen Estimation

Petri dish cultures for the observation of pattern formation, were initiated at preconfluent densities using cell suspensions obtained by the trypsinization of stock lines. Cultures were maintained without subculturing on thrice weekly medium changes; more frequent medium changing was sometimes necessary to prevent the cells from

Figure 1  Confluent culture, EHL fibroblasts. Even distribution, cells becoming organized into a rough patchwork of small independent parallel arrays. X 70.

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detaching from the dish floor. The cultures were observed in all cases until net growth had ceased and, in some cases, for up to 120 days after initiation. Time-lapse films were made during representative periods of each phase of selected cultures. Except where stated otherwise, these conditions of maintenance and observation were the same in all experiments. The hydroxyproline content of the cell mass was estimated at intervals of 2–3 days.

At seeding the cells are arranged randomly, but by the time that confluence is attained a tendency to parallel arrangement is evident. This tendency exhibited in numerous small locations imparts a “patchwork” appearance to the confluent culture (Fig. 1). The cells are arranged in independent parallel groupings containing from 20 or 30 to several hundreds of cells; such an arrangement we shall term a group. Each group is orientated at random with respect to every other group and is separated from its neighbors by narrow packing interstices, which we shall call frontiers. Multilayer growth of a newly confluent monolayer commences, at numerous independent sites, to produce, initially, two-layered overlaps which invariably arise at or beside the frontiers (Fig. 2); they are not observed over the centers of the groups. Primary overlaps are long and narrow, with the overlapping cells orientated perpendicular to the

![Image](image1.png)

**FIGURE 2** Confluent EHL fibroblast culture with primary overlap. Portions of three groups of parallel-arranged cells are shown. Overlapping above the frontier between two of the groups is just commencing. X 100.

![Image](image2.png)

**FIGURE 3** Stationary culture, EHL fibroblasts. Low-power view, showing uneven distribution of the cells. Thick ridges and thin intervening regions. X 6.7.
longer axis; they may extend in length, but their lateral dimension does not increase. Some of the primary overlaps become foci of aggregation, attracting cells in their vicinity, and they develop into long narrow ridges up to approximately 30 cells thick (Figs. 3 and 4). A stationary culture, therefore, consists of a monolayer of cells, on the dish, overlain by numerous elongated and irregularly arranged ridges up to 30 cells thick. Seen from above, the ridges appear to consist of an orthogonal arrangement of discrete horizontal monolayers of cells arranged in parallel, the cells in adjacent layers being oriented at right angles (Fig. 5).

Upon prolonged maintenance of stationary cultures, there is a tendency, of varying degree, to dissolution of the ridges by dispersal of their component cells (Fig. 6a, b). This dispersal, together with accompanying changes in the bottom layer of the culture, results in the gradual reorganization of the culture to form large, uniformly thick, parallel arrays of cells (7). The pace of this reorganization may be very slow, in some cases being incomplete after 4 months. The formations observed in cultures in which reorganization is advanced will be referred to as extended parallel arrays.

Hydroxyproline measurements show that, during trypsin preparation of cell suspensions, collagen previously associated with the cells is largely removed. Newly initiated cultures, therefore, contain negligible amounts of collagen; accumulation commences shortly after initiation and is appreciable at confluence. A representative result is shown in Fig. 10a. Cultures in extended parallel array also contain an abundance of collagen.

Culture in Collagenase Modified Medium

Cultures were initiated as for simple culture, but after attachment of the cells to the dishes,
the medium was replaced by a medium modified by addition of varying amounts of collagenase. Modification of the normal medium by addition of collagenase at concentrations of 200 µg/ml or more leads to rounding up and detachment of the cells. It was shown, however, that collagenase added to the medium at a concentration of 60 µg/ml had no such effect; moreover, no change in the appearance of individual cells or in the rate of growth of the cultures could be demonstrated. Several fibroblast cell lines have been maintained for periods of up to a month in such a collagenase modified medium without signs of deterioration.

These cultures establish a normal group and frontier pattern at confluence, but no aggregations develop during subsequent growth, the ensuing increase in cell numbers being accommodated within the existing group and frontier pattern (Fig. 7). This results in the frontiers achieving a striking prominence (Fig. 7, inset).

Substitution of normal medium for the collagenase modified medium leads to a complete reversal of the collagenase-imposed restraint; overlaps and multilayered aggregations are rapidly generated.

**Substitution of Collagenase Modified Medium for Normal Medium in Stationary Cultures**

Cultures were initiated and maintained up to the stationary phase, as for simple cultures; thereafter, collagenase modified (60 µg/ml) medium was used. Rapid spoiling of the orthogonal patterns seen in the ridges of simple cultures is brought about when the normal medium is replaced by collagenase modified medium. The cells round up and either aggregate into irregular peaks or form detached flaps. The cells of the monolayer on the dish are unaffected (Fig. 8). Formed collagen largely disappears from cultures after transfer to collagenase modified medium, and further collagen accumulation is prevented. Accumulation recommences 1–2 days after removal of the collagenase. The lag may be due to retention of collagenase in the cell mass after it had been washed and the medium changed (Fig. 10b).
Substitution of Collagenase Modified Medium for Normal Medium in Cultures Grown on a Known Collagen Substrate, in Tissue Culture, and Nontissue Culture Grade Dishes

Rat tail collagen substrates were deposited in both “tissue culture” and “nontissue culture” grades of Petri dishes. Cultures were initiated at a confluent density in both types of dishes. After 24 hr, collagenase modified medium was substituted for normal medium. Concentrations of collagenase and periods of exposure were varied to make the conditions of the experiment more rigorous (see legend to Fig. 9).

Fibroblasts grow equally well on rat tail collagen substrates whether these be contained in tissue culture or nontissue culture grade dishes. Replacement of the normal medium by collagenase modified medium, however, results in the detachment of the cells grown in the latter, while no change is apparent in those grown in the former (Fig. 9). Complete destruction of the rat tail collagen substrate by the collagenase is thereby demonstrated.

Cell Motility

Time-lapse films show that the cells within the groups of a confluent or postconfluent culture, whether in the presence or absence of collagenase, retain considerable motility, moving up and down the parallel arrangements but never across their neighbors to disrupt that arrangement. This motility appears to be unaffected by dense packing of the cells within the groups. The cells lining the frontiers, in contrast, are immobile, as also are the cells in the ridges subsequently generated over the frontiers. The frontiers thus serve as barriers preventing movement of cells from one group to another. The effectiveness of this restraint is strikingly demonstrated in the presence of collagenase, the frontiers continuing to ob-
FIGURE 8 Stationary culture, EHL fibroblasts, grown in routine medium and then transferred to collagenase supplemented medium (60 µg/ml) for 24 hr and fixed. Cells forming the bottom layer of the culture and attached to the plastic substrate are unaffected by the enzyme. Cells above the bottom layer are rounded up, suggesting that they were deployed on collagenase-sensitive substrates. X 63.

FIGURE 9 Destruction of rat tail collagen substrates by low concentrations of collagenase. All cultures were initiated at a confluent density and fixed on the second day. X 45. a, Nontissue culture plastic dish. Poor attachment. b, Nontissue culture plastic dish covered by a rat tail collagen layer (c. 1 mg of collagen per 2 in. diameter dish). Good attachment. c, Parallel culture to b after exposure to collagenase supplemented medium (8 µg/ml) for 5 min. Rapid detachment of the cells due to destruction of the collagen substrate. d, Tissue culture dish. After exposure to collagenase supplemented medium (120 µg/ml) for 60 min. No detachment; the cells in these dishes transfer their attachments directly to the plastic following the destruction of their collagen substrate.
FIGURE 10 a Accumulation of bound hydroxyproline in EHL fibroblast cultures. Replicate cultures were initiated at $5 \times 10^5$ cells per 4 in. dish. Each point is derived from assays on several dishes. Two replicates were counted individually in a cell counter to derive an estimate of cells per dish for each point. The data have been plotted twice; the curve drawn through the squares represents hydroxyproline ($Hyp$) per culture; the curve through the circles represents $Hyp$ per $1 \times 10^6$ cells. $Hyp$ was undetectable in cultures assayed within 1 hr of setting up. The cultures were confluent at 4 days, by which time an appreciable accumulation of $Hyp$ is evident. At the final harvest shown here, the cultures were about one doubling from their stationary density. The data are consistent with the conclusion that the rate of $Hyp$ accumulation is proportionate to the number of cells.

FIGURE 10 b The destruction of formed collagen and the prevention of collagen accumulation in fibroblast cultures exposed to collagenase supplemented medium. Replicate confluent cultures were divided into two groups. One group received routine medium throughout; the other group was transferred to collagenase supplemented medium (60 µg/ml) and returned to routine medium 2 days later. The collagenase removed the greater part of bound hydroxyproline ($Hyp$) and, following withdrawal of the enzyme, $Hyp$ accumulation recommenced after a lag probably due to residual enzyme activity.
struct cell movement even when the cells within the groups are densely packed. Time-lapse films of frontiers show that the confronting cells of the two adjacent groups are continually breaking and remaking their leading-edge contacts and, on projection, give a subjective impression of a repulsion between the leading edges of the two groups of cells. It appears that the immobility of the frontier cells makes it difficult for their immediate neighbors to move also; the immobilizing effect of the frontiers is thus transmitted with diminishing strength several cell lengths into the groups. This is supported by the observation that there is little movement in very small or oddly shaped groups.

Culture under Agarose Gels Designed to Restrain Cell Movement

Cultures were initiated as for simple culture but, before confluence was attained, the medium was replaced with medium plus 1% Agarose (Seravac, Capetown, South Africa).

Cultures under Agarose gels grow at the same rate as simple cultures, the expected stationary density of six monolayer equivalents (8-10 × 10^6 cells per 2 in. dish) being attained. Cell motility, however, is greatly diminished, and an imperfect group and frontier pattern is often established in the confluent cultures. The five-fold increase in cell numbers thenceforth produces a uniform distribution of cells throughout the culture. Ridge formation as seen in simple culture is abolished.

Cutting Experiment Designed to Induce the Formation of Frontiers in Extended Parallel Arrays

Simple cultures were maintained until they became organized in extended parallel array. Cuts of two types were made in cell sheets. One type was made with a sharp instrument, leaving a single scratch on the dish; the other type was made with a less sharp instrument, not scratching the dish. In each case the cut was 1 cm or more long and was perpendicular to the long axis of the cells.

After it was cut, whether with a sharp or a blunt instrument, the tensed cell sheet retracts from the line of the cut, leaving a gap of up to several millimeters wide. During the next few days, cells move out from the edges of the wound to recolonize the space. When a sharp instrument has been employed and a scratch made on the dish surface, the first cells to encounter the scratch turn through 90° to lie parallel to it (Fig. 11 a). Cells advancing from behind encounter these first cells at right angles, resulting in the establishment of frontiers along the full length of the scratch and on either side of it. After a few days, two ranks of equally spaced ridges develop beside...
each frontier on the side away from the scratch (Fig. 11 b), producing a pattern reminiscent of somites flanking the notochord in the early vertebrate embryo. If a blunt instrument is used to make the cut, without scratching the dish, no frontiers are formed by the recolonizing cells which interdigitate to restore the original order of the culture.

**Cutting Experiment in Presence of Collagenase**

This experiment was identical with the foregoing, except that, after cutting, the medium was replaced with collagenase (60 μg/ml) modified medium.

The effect of maintaining the cut cultures in collagenase modified medium is to prevent the formation of ridges in the scratched dish. The initial frontier formation is observed, but no aggregation occurs, and the cells lying parallel to the scratch are jostled back into line in the course of a few days. The original order is restored (Fig. 11 c).

**DISCUSSION**

The results presented are consistent with the following hypothesis: fibroblasts in culture make an extracellular collagenase-sensitive material which may be utilized by fibroblasts as a substrate for attachment and locomotion. A primary overlap represents a site, in a newly confluent culture, at which such a substrate has been deposited and colonized by cells moving in from outside its boundaries. The colonizing cells themselves are capable of producing substrate which is, in its turn, available for colonization. By continuation of this process a ridge is built up (Fig. 12).

**Localization of Substrates**

If this hypothesis is valid, it is obvious that substrates cannot be formed everywhere in a confluent culture; were substrates formed everywhere, the observed pattern of discrete primary overlaps and ridges would not be expected. Substrate formation must be confined to specific locations in a newly confluent culture and must be limited to these same locations subsequently. It has been reported that cells at frontiers and in ridges are nonmotile. The cutting experiment in which the dish surface was scratched resulted in the artificial induction of frontiers, lined by nonmotile cells. Frontiers are sites of ridge initiation. Cells within groups and in extended parallel arrays have been reported to be motile. No ridge formation occurs in these areas. It is reasonable, therefore, to suggest that substrate formation is confined to areas containing nonmotile cells. Further support for this view can be obtained from the Agarose gel experiment, in which motility was greatly reduced throughout the culture and ridge formation was not evident, possibly due to a tendency to uniform substrate production.

**Nature of Substrates**

The data on collagen accumulation and the evidence from the effects of collagenase point to the probability that the substrates contain collagen. The results do not preclude the possibility that the substrates contain other materials or that the collagenase may degrade substances other than collagen. However, two lines of evidence are advanced to demonstrate that the collagenase as employed here is capable of degrading collagen (Figs. 9, 10). It is suggested that these substrates are made up of materials which are normal constituents of the extracellular matrix which fibroblasts are specialized to make.
Nonsubstrate Collagen

The finding that cultures organized in extended parallel array contain abundant collagen suggests that cell motility does not inhibit production of substrate components. The possibility, therefore, arises that movement of the producer cells creates sufficient disturbance to prevent assembly of those components into a form utilizable as substrate. Our results to date provide no assistance in visualizing how this might come about, but the following speculation is made: a serviceable substrate for colonization would be provided by a continuous sheet of matrix; holes in the matrix would decrease colonizable area and permit contact inhibition between colonizing cells and those below. The to-and-fro motions of cells arranged in parallel would be likely to prevent assembly of a continuous sheet of matrix and would thereby prevent the type of aggregation necessary for ridge building.

Directed Migration Into Ridges

The behavior of cultures grown under agarose and the reversibility of inhibition of ridge formation in stationary cultures grown in collagenase point to the conclusion that ridges are formed as a result of directed cell migration rather than differential cell division. A sufficient explanation of the directed cell migration observed during focal aggregation in fibroblast cultures is provided in terms of the local provision of new substrates (vide infra). This mechanism could operate to promote morphogenetic movements in embryos, and it is perhaps relevant that Low (20) has recently provided an electron microscope demonstration of extracellular fibrous protein in early chick embryos. The disappearance of many of the ridges during prolonged maintenance for periods of up to 4 months probably reflects imperfect maintenance of substrates under the culture conditions employed.

Aggregation of Cells Liable to Contact Inhibition

The cells employed in this investigation exhibit orthodox contact inhibition, which is demonstrated in two ways. First, there is a tendency to form stable parallel arrays; contact inhibition provides a mechanism to prevent the disruption of these arrays by random crossing of the cells. Secondly, the immobility of the frontier cells clearly demonstrates contact inhibition. The observation of movement among cells arranged in parallel does not exclude the operation of contact inhibition; there is obviously movement among the cells arranged in parallel which comprise the expanding radial outgrowths of fibroblasts from fragments of chick embryo heart muscle, the material on which many studies of contact inhibition have been made (1, 2, 4). It is assumed that cells moving on a matrix substrate are not in contact with the cells beneath them and, therefore, suffer no inhibition of movement. On this basis, it is possible to envisage the spontaneous aggregation of fibroblasts which, individually, retain liability to contact inhibition.

Aggregation and Dispersion of Fibroblasts as Similar Processes

A generally observed feature of fibroblast behavior in culture is the tendency to colonize all available substrate. If our fibroblast cultures are initiated with an imperfect cell suspension containing clumps, the initial tendency is for the cells to leave the clumps and disperse over the dish floor. It is only after confluence that the contrary behavior is exhibited, and ridge formation commences.

It has generally been assumed that cells aggregating and cells dispersing from a tissue explant are acting differently. This is a reasonable assumption when these phenomena are induced by relatively drastic chemical changes (4). In our cultures no such change in culture conditions accounts for the apparent reversal of cell behavior at confluence. The present proposal, therefore, is that dispersing fibroblasts, such as those emigrating from a tissue explant, and fibroblasts spontaneously aggregating in mass cultures are acting in essentially the same way. We propose that in both cases the activities of the cells are directed towards colonization of available substrates and that it is solely the location of the substrates relative to the cells which determines whether this colonization is manifested as aggregation or dispersion (Fig. 13).

Although we have investigated cultures of only one type of fibroblast in detail, it is probable that the multilayering mechanism suggested here applies to other fibroblast lines which grow appreciably beyond confluence. Many lines of normal fibroblasts accumulate collagen and other matrix components in culture (5, 8–11). We have
seen ridges, apparently similar to those we have reported here, in cultures of chick embryo carcinoma fibroblasts and BHK 21 cells. Yardley has published electron micrographs of vertical sections through multilayered regions of chick embryo heart fibroblast cultures which show the cells arranged in horizontal layers interleaved with bands of collagen fibers (30). If the substrates we refer to are made of collagen, this configuration is obligatory under our hypothesis.

The concept that ground substance plays an important role in morphogenesis is generally accepted largely due to the cogent advocacy and experimental support of Weiss and his collaborators (25-28). They have demonstrated the alignment of fibroblasts on ordered collagen substrates and have considered in detail the implications of the existence of periodic fibers in the extracellular matrix for the transition from "thread to fabric." Rosenberg has drawn attention to cellular microexudates and has demonstrated further ways in which cells respond to substrate characteristics (23, 24). Additional evidence of the suitability of collagen as a substrate for cells is provided by the practical utility of reconstituted collagen surfaces in culturing a variety of fastidious cells (3, 6, 14, 16). It is also apparent that extracellular matrix, including its collagen component, is important in the epithelio-mesenchymal interactions essential to morphogenesis of many soft tissues (17, 18, 29).

Failure of most freshly isolated cells to grow in suspension suggests the existence of substrate requirements (15). The unsuitability of agar as a substrate implies that these requirements are more specific than simply for semirigid support (21). Further, the observation frequently made by our colleague Dr. Harnden, that normal human fibroblasts transferred from a plastic substrate into agarose suspension will usually undergo two or three divisions and form small clusters before growth ceases, indicates that these cells cannot satisfy their substrate requirements by mutual contacts only. If this is generally true, it suggests that the ubiquitous extracellular connective tissue matrix within the body may

FIGURE 13 Diagram to show how the colonization of a substrate by fibroblasts can be observed as either dispersion or aggregation. Cross-hatching represents sheets of cells; dots represent substrates. The upper three figures represent stages in the formation of an outgrowth of fibroblasts derived from a piece of tissue explanted onto a bare plastic substrate. The lower figures represent the local accumulation and subsequent colonization of a matrix substrate produced within a confluent culture of fibroblasts (plastic substrate not shown).
provide an extracellular substrate phase upon which tissue cells are dependent for their growth and well being. If this is the case, then the provision of such a substrate phase will be a primary task in embryogenesis, and the specific location and colonization of new substrates in the embryo could provide a mechanism for the observed morphogenetic movements therein, by analogy with the directed cell movements taking place in the model fibroblast system described.

One of the prices we have to pay for the ease

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TERMINATION

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