COLLAGEN SUBSTRATA FOR
STUDIES ON CELL BEHAVIOR

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ABSTRACT

A simple technique is described for the preparation of collagen substrata containing 0 1% of collagen by weight, in the form of native bundles with a 640 A period, the substrata are similar in these respects to soft-tissue matrices These substrate are hydrated collagen lattices (HCLs) in which the watery milieu is held within a fibrous collagen net mainly by capillary forces. HCLs have been characterized in terms of the course of collagen precipitation and aggregation, ultrastructure, and their stability under various conditions. The ways in which HCLs can be employed as both two- and three-dimensional substrata in cell behavioral studies are illustrated with some preliminary observations on the form, motility, adhesion, and growth of human diploid cells and two lines of malignant cells.

INTRODUCTION

Snatched from a life of obscurity and installed in contemporary glass and plastic palaces, cells are in danger of becoming Pygmalion's protégés Housed in more traditional residences constructed of water and collagen instead of plastic or glass, do cells lead primitive, less cultured lives?

Collagen preparations have been used from time to time in tissue culture, usually for the immediate purpose of growing particularly fastidious cells (2, 3, 6, 7). A technique is described for preparing collagen substrata for use in cell behavioral investigations These substrata consist of a hydrated collagen lattice (HCL), containing around 0 1% by weight of collagen in the form of native bundles varying in diameter from 500 to 5000 A and possessing a 640 A lateral period. These characteristics approximate to the concentration and form of collagen in extracellular connective tissue matrices After a description of the ultrastructure of HCLs, various methods of employment are outlined and illustrated by some preliminary observations.

The methods described are simple, cheap, and require no special skills.

ANCILLARY MATERIALS AND METHODS

Cell Cultures

Normal fibroblasts were early subcultures of human, fetal, diploid lung fibroblasts, established in this laboratory from hospital abortions. Simian virus-transformed rat cells (SVTR) were rat fibroblasts established from embryo carcasses, transformed by SV40 virus. This uncloned line of transformed cells was developed by Dr. David Young of this laboratory

All cells were grown routinely in Eagle's minimal essential medium (MEM), (Grand Island Biological Co., Grand Island, N. Y.) with 10% tryptose broth, 10% fetal calf serum and penicillin, streptomycin, and Fungizone (E. R. Squibb & Son, New York), as described previously (4).

Collection and Solubilization Procedures for Counting Petri Dish Cultures Labeled with Thymidine-H^3

Dishes were washed with saline, 4 mg/ml collagenase (CLS, Worthington Biochemical Corp.,

Freehold, N J.), was added, and incubation was carried out at 37°C for 1 hr. 5 ml of 0.25% trypsin was added to each dish, and incubation was continued for a further 10 min at 37°C. The cells were pelleted by centrifugation, resuspended in saline, and pelleted again. The cells were fixed in cold, 5% trichloroacetic acid, three changes in 24 hr. The pellets were dissolved in 1 ml of Hyamine (37°C for 1 hr), and the volume was made up to 16 ml with a standard 2,5-diphenyloxazole/1,4-bis[2-(5-phenyloxazolyl)]benzene (PPO/POPOP) mixture.

Electron Microscopy[1]

NEGATIVE STAINING TECHNIQUE. One drop of collagen mix (see below) was transferred to each of several Formvar-coated grids Excess material was removed by touching with a piece of filter paper. A drop of phosphotungstic acid solution (pH 7.0) was removed after 30 sec contact, and the preparations were allowed to dry.

REPLICA TECHNIQUE A two-stage replica technique was developed to examine the surface of HCLs HCLs prepared in 50-mm plastic dishes were fixed in 2% unbuffered osmium tetroxide for 15 min, washed in three changes of 0 1 M cacodylate buffer, and allowed to dry slowly at room temperature When completely dry, the surface of an HCL was moistened with acetone, and a piece of cellulose acetate 0 5 mm thick was layered on top and left undisturbed until the acetone had completely evaporated The cellulose acetate was carefully removed, leaving the HCL intact, and placed replica-face-up on a microscope slide, and secured with Sellotape. The preparation was carbon-coated along two perpendicular axes at an angle of 45°. Electron microscope grids were placed on a layer of filter paper immersed in chloroform such that the upper surface of the paper was moist, small squares of the replica were placed on the grids and left for 3 days in order to remove all celluose acetate.

SECTIONING TECHNIQUES · Collagen drop lattices containing cells (for preparation, see below) of about 0 1 ml vol were fixed in 5% glutaraldehyde in cacodylate buffer They were processed and embedded in Araldite, then sectioned, and stained with uranyl acetate-lead citrate.

All specimens were examined and photographed in an EM6B electron microscope.

PREPARATION AND PROPERTIES OF HYDRATED COLLAGEN LATTICES

Preparation of Collagen Solutions

AIM: The aim is to prepare clean and stable collagen solutions in a solvent that itself introduces no alteration within a tissue culture medium.

[1] In collaboration with Mr. Alan Ross.

PRINCIPLE · Collagen solutions are stable at low ionic strength and low pH.

METHOD. Rat tail tendons are extracted in 0.5 M acetic acid at 4°C for 1–2 days (12). The solution is passed through muslin to remove gross insoluble material. 100–300 ml of solution are dialyzed for periods of 24 hr against two 4-liter lots of one-tenth strength Eagle's medium. The second lot of diluted Eagle's medium is adjusted to pH 4 0 with HCl. The dialyzed solution is centrifuged for 24 hr at 17,000 rpm in a refrigerated ultracentrifuge. The almost clear solution so obtained is nearly always sterile, and keeps indefinitely at 4°C Final solutions contain 0.1% ± 0 01 by weight of collagen as estimated from hydroxyproline assay (4)

Preparation of Hydrated Collagen Lattices

AIM: The aim is to precipitate the collagen and have it aggregate into dispersed native bundles within a fluid having the same composition as the tissue culture medium employed (Eagle's medium and 10% fetal calf serum).

PRINCIPLE: The collagen is caused to precipitate and aggregate into native bundles by raising the ionic strength and pH to physiological levels.

METHOD: To avoid a useless flocculation it is necessary to adjust the pH and ionic strength almost simultaneously The following procedure has been found convenient. A sample of collagen solution is placed on ice Three disposable syringes are prepared containing appropriate quantities of serum and 10× Eagle's medium to reconstitute the standard medium, and a predetermined volume of 0.142 M NaOH to bring the pH of the mixture to 7.6. Working quickly, the three ingredients are added to the collagen solution and the mixture ("collagen mix") is dispersed into containers The containers are left undisturbed for several minutes while the mixture sets (freshly prepared collagen solutions set quickly; stored solutions set, conveniently, more slowly). Glycosaminoglycans may be conveniently introduced, taking advantage of their solubility at high ionic strength, in the 10× Eagle's medium.

Superficial Characteristics of HCLs

The mix described above sets with a small increase in turbidity to a consistency that allows the containers to be inverted. Superficially, HCLs have the consistency of a soft agar gel However, whereas gels can be cut with a knife without loss of water, such an interference causes collapse of the collagen lattice and loss of water, the collagen collecting as a tenuous membrane For practical purposes, including timelapse filming, HCLs are transparent
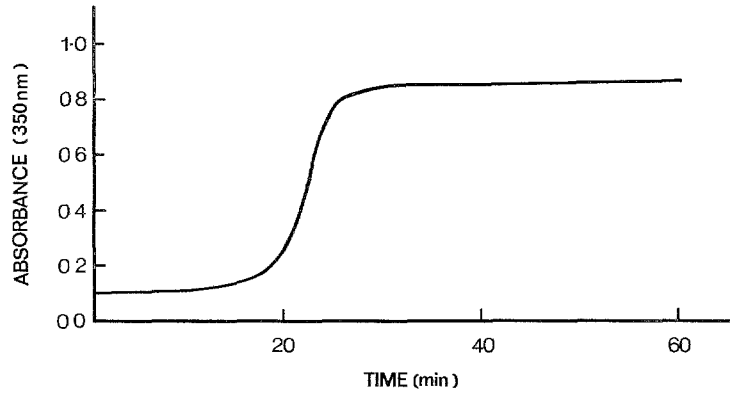
FIGURE 1 The precipitation of collagen, OD curve. One part of collagen solution (0.9 mg/ml collagen) was mixed with two parts of 1.5× medium F10 and the pH was adjusted to 7.6 with 0.142 M NaOH. After mixing, the solution was dispensed into a cuvette and the absorbance of light of wavelength 350 μm was monitored in a Unicam SP800 (Unicam Instruments Ltd., Cambridge, England). The reason for diluting the collagen instead of using a 10× medium concentrate was to slow down the rate of precipitation (which is concentration dependent) to better display the sigmoid rise in the OD curve after the initial lag (see text).
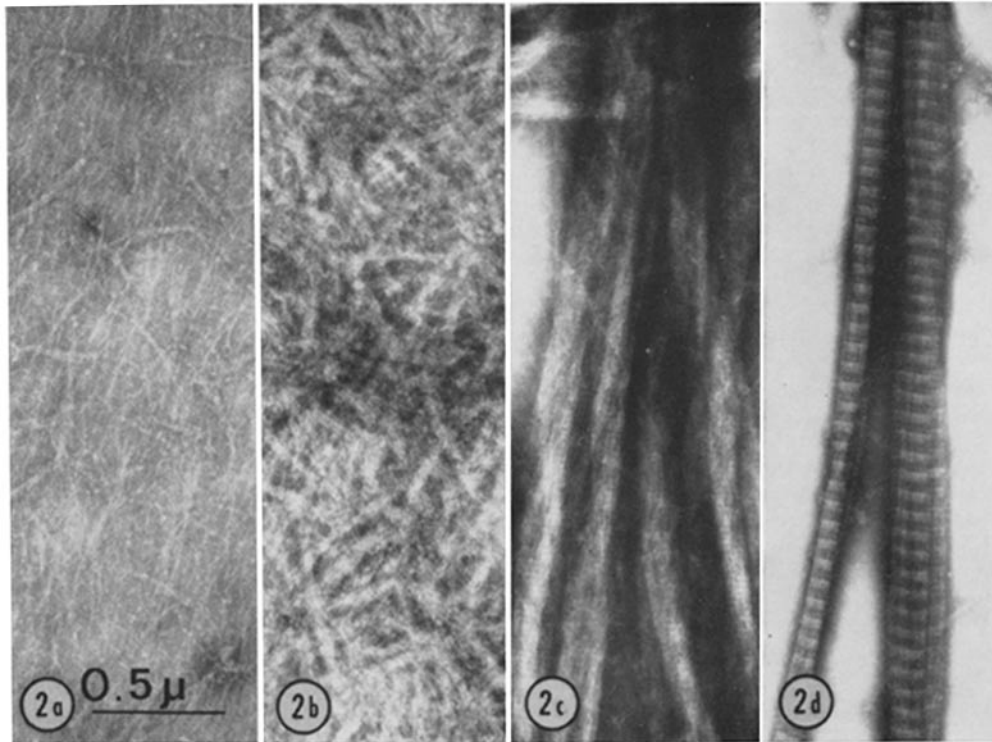

FIGURE 2 Electron micrographs, negatively stained preparations. Stages in the assembly of collagen bundles during the maturation of HCLs. Cuvette sampled at 15, 30, 90 min, and after 24 hr, (see text). (a) shows the state of the mix after the completion of the sigmoid rise in OD. (b) and (c) correlate with a slow linear rise in OD. (d) shows the final form of the collagen bundles, not lateral periodicity. For collagen structure, see Hodge (8). × 35,000.

Collagen Precipitation and Ultrastructure

Collagen precipitation has been followed by dispensing fresh mix into cuvettes and continuously monitoring the optical dispersion Fig. 1 shows an optical density (OD) curve. After a short lag there is a rapid rise in OD Thereafter, there is a slower linear rise that continues for several hr at room temperature

By sampling cuvettes at various times and examining negatively stained images of the precipitated collagen, the OD curve can be correlated with the state of aggregation of the collagen The sigmoid region of the OD curve reflects the precipitation of the collagen as very fine fibrils The linear portion reflects the subsequent aggregation of these fibrils, culminating in bundles of up to 5000 A diameter showing a 640 A period (Fig 2).

Sections through HCLs viewed in the electron microscope show occasional bundles throughout the interior (Fig. 3) and a somewhat greater congregation in the superficial 2μ Shadowed replicas of the surface of HCLs show mats of long bundles usually straight and often branching, occasionally with a helical twist (Fig. 4 a) It is usual to observe the larger

bundles in a single field roughly oriented in the same direction. By a trivial modification in technique, this tendency can be accentuated to prepare aligned HCLs. Mix is spread over the surface of a dish and left to set leaning against a vertical support. Excess mix drains across the dish to collect at the lowest point. Replicas show all the larger bundles roughly aligned along the axis of draining (Fig. 4 b).

Stability of HCLs

Instability to mechanical disturbance has been noted. HCLs poured into plastic dishes have the very desirable property (unlike agar gels) of not usually floating free under a fluid medium. HCLs are best prepared within a day of use and kept in airtight bags at room temperature, they are unstable at low temperatures, losing water.

Trypsin has no effect on HCLs; traces of bacterial collagenase lead to rapid dissolution.

CULTURE METHODS WITH HCLs

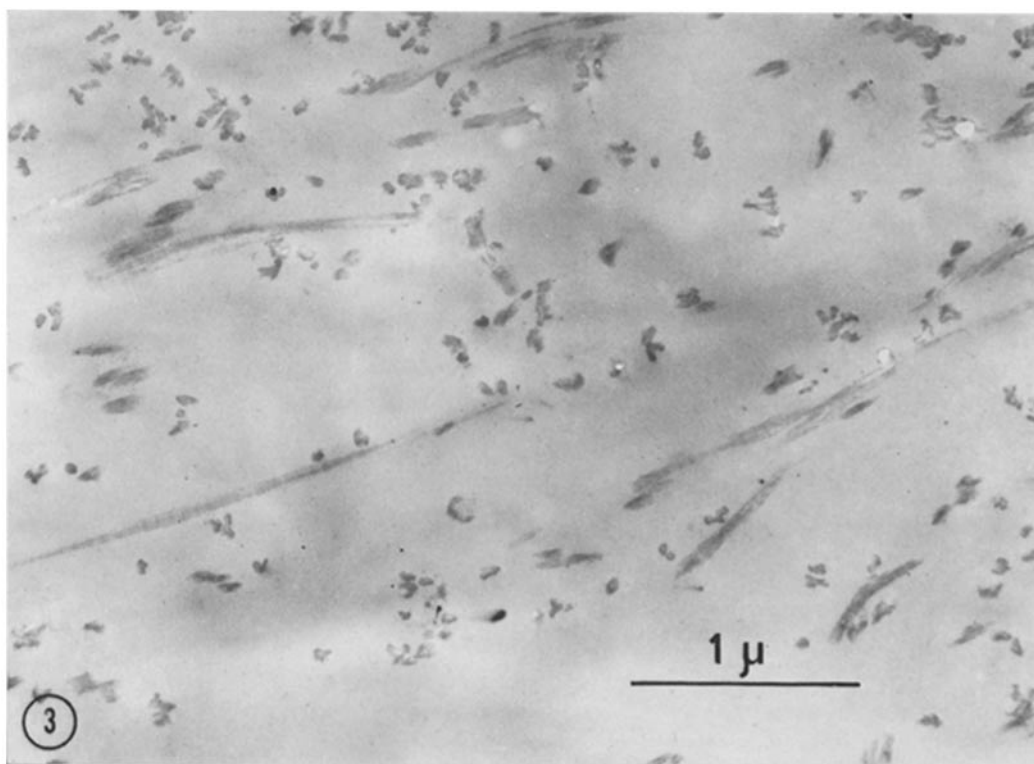HCLs have been employed in three ways. to provide a two-dimensional substratum, to overlay cells already

FIGURE 3 Electron micrograph of a section through a matured HCL. Collagen bundles are dispersed within the watery milieu Bundles oriented in the same plane as the page show the native transverse periodicity × 30,000.

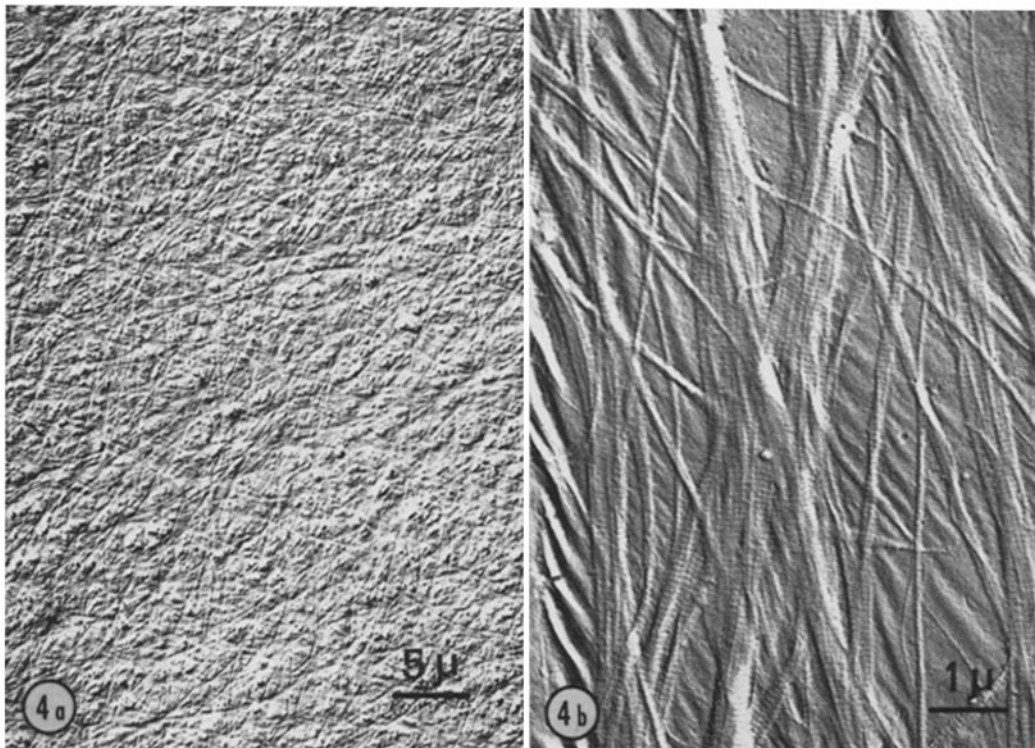FIGURE 4 Electron micrographs. Surface replicas of HCL. (a), low power, unaligned HCL. × 2000
(b), higher power of an aligned HCL × 10,000. The larger bundles share the same approximate orienta-
tion from which only the smaller bundles depart.

attached to a substratum, and to provide a three-di-
mensional substratum incorporating cells

Mix can be poured or spread over the whole or a
part of the surface of a dish to provide substrata of a
desired area and thickness. The draining technique
can be used to align the collagen bundles if desired.

Pouring mix directly onto well-drained cultures
provides an overlay containing around 0.1% by
weight of collagen. Here again, the draining tech-
nique can be employed to align the collagen bundles.
Cells respond to much lower concentrations of colla-
gen in their environment, however, dilute collagen
overlays are simply made by mixing a small quantity
of collagen into the existing fluid medium. Dishes so
treated should be returned to the incubator and left
undisturbed for several hours, for the precipitation of
the diluted collagen, being concentration dependent,
is slow.

A cell suspension may be incorporated with the
mix, thus trapping cells within the lattice itself. The
mix containing cells may be poured into a dish, onto a
foundation HCL if no contact between cells and
plastic is desired Buoyant and floating drop lattices
are another useful procedure. The mix is set out as

rounded drops in nontissue culture plastic dishes, and
fluid medium is applied after setting. Drop lattices
detach on shaking; having virtually the same density
as the fluid medium, they are buoyant. By incorpo-
rating an air bubble in each drop, it can be arranged
for drop lattices to float at the surface of the medium,
thus creating favorable conditions for exchange with
the medium and the atmosphere. Drop lattices with-
out incorporated cells can be prepared and subse-
quently seeded with cells on their surface. Although
of the same order of size, the collagen bundles within
drop lattices tend to be coarser than those in natural
matrices (Fig. 6).

The following preliminary observations are pro-
vided to illustrate the various employments described
above.

OBSERVATIONS ON HUMAN DIPLOID
FIBROBLASTS

Cell Form and Alignment

Cells plated onto the surface of an HCL attach
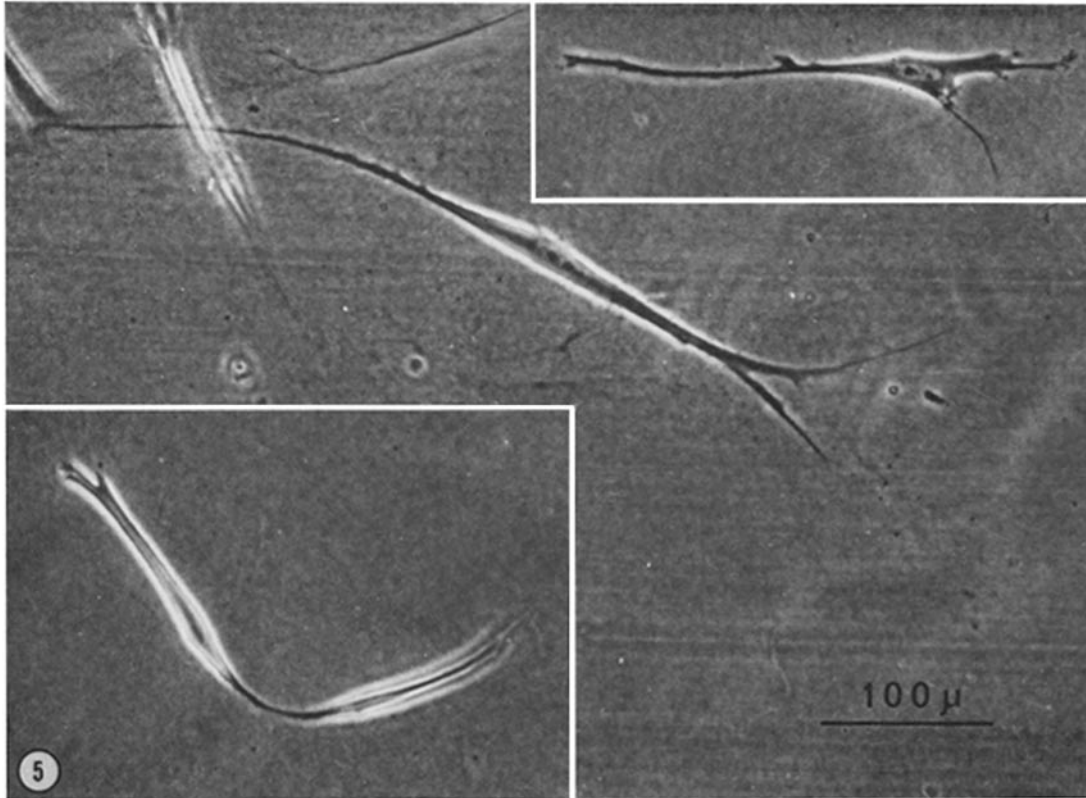and extend themselves, proceeding to colonize the

FIGURE 5 Phase contrast photomicrograph of human embryonic lung fibroblasts within an HCL. Bipolar spindle form predominates. Pseudopodia are not expanded into the fanlike ruffling membranes characteristic of cells plated onto plastic at subconfluent densities. × 280.

surface by their growth and locomotion and to behave superficially much as they would on plastic The form of the cells in sparse culture is, however, quite different (4)

Seeded sparsely onto plastic, normal fibroblasts adopt the *ruffling membrane form* The cell typically possesses one or more broad, flattened pseudopodia, the edges of which may show the so-called ruffling activity. There appears to be no stable differentiation between pseudopodial and nonpseudopodial surface; pseudopodia can be produced from any point on the cell surface, giving the cell a constantly changing outline in time lapse films.

Seeded sparsely onto HCLs, fibroblasts characteristically adopt the *bipolar spindle form* The cell is greatly extended with a clear differentiation between pseudopodial and nonpseudopodial surface, pseudopodia being confined to the two ends of the cell. Unlike ruffling membranes, these pseudopodia

are usually small and not expanded, and they often terminate in several fine prolongations (Fig. 5). There is no major invasion of the interior by cells plated onto the surface of HCLs. The few invaders are conspicuous by their random orientation and pronounced spindle form

It was of interest to observe whether cells incorporated within a lattice, growing therefore in a three-dimensional substratum, exhibited a predominance of the multipolar forms observed by Weiss and Gerber (11), among chick fibrocytes within unstressed fibrin lattices Although occasional multipolar forms are observed among human fibroblasts within HCLs, the predominant form remains the bipolar spindle

After the original observation of Weiss, that fibrocytes orient themselves along the ordered collagen arrays exposed by the cleavage of fish scales, it has been accepted that connective tissue cells on collagen display at least the first four of the five
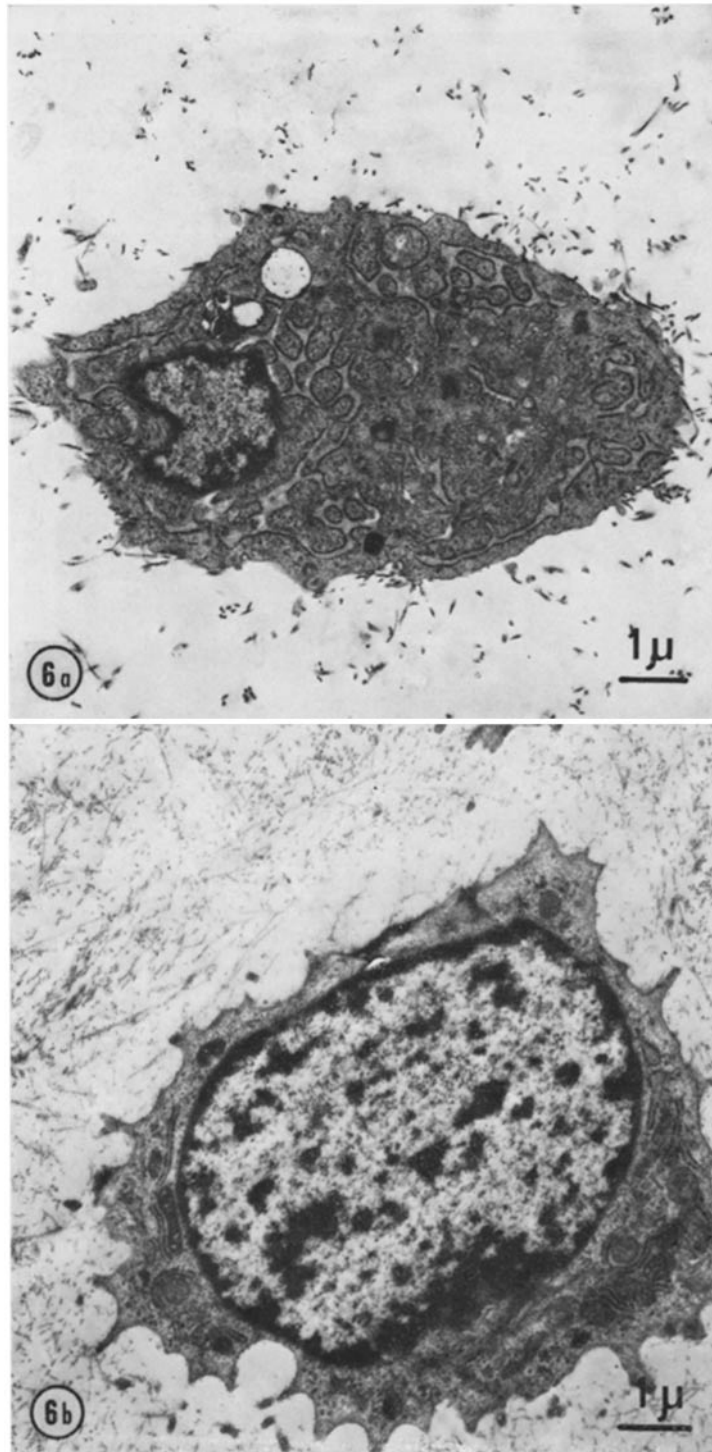
FIGURE 6   Electron micrographs of sections. (a), an HCL with incorporated human lung fibroblasts. (b), connective tissue within the tow of a 14 wk human fetus. The illustrations are similar. Collagen bundles within the HCL are somewhat coarser than those in the toe matrix. × 9,000.

phenomena that Weiss predicates the contact guidance (10) This is amply confirmed by the behavior of cells on HCLs, and the present techniques allow the ready contrivance of the situations necessary for this demonstration Fig 7 illustrates the way in which fibroblasts plated onto aligned HCLs extend along the axes of draining. The bipolar spindle form reflects the extension of the cells along the major collagen bundles in the substratum. This is also reflected by the appearance of strings of cells in close file within HCLs Time-lapse films show sparse cells on aligned HCLs moving inde pendently to and fro along the axis of draining, other motions not being observed

Cell Adhesion

Normal fibroblasts adhere strongly to HCLs. Confluent membranes of fibroblasts investing drop lattices cannot be removed by shear in a Whirlmix (Beardsley & Piper Div , Chicago, Ill) A standard 0 25% trypsin solution used for subculturing causes cells to detach from plastic in less than 15 min at

37°C This treatment has no effect on cells attached to an HCL After 1 or 2 hr there is a general shortening of the cells, some of which are no longer firmly attached After 12 hr all cells are detached and moribund

This observation is interesting in relation to the finding that it is impossible to prepare good single cell suspensions, by using trypsin alone, from dense fibroblast cultures grown on plastic in the presence of 15 μg/ml added ascorbic acid This supplement provides for about a fivefold increase in collagen synthesis and accumulation. It appears that the attachments which cells make to collagen-contain int substrate are different from those made to plastic

Cells overlaid by an HCL tend to release their attachments to the plastic and move a little way into the collagen substratum, taking on a pronounced spindle form Cells have been observed to respond in this way to concentrations of collagen in the medium as low as 0 005% by weight (0 2 m of collagen dispersed in 5 ml of medium) In ex
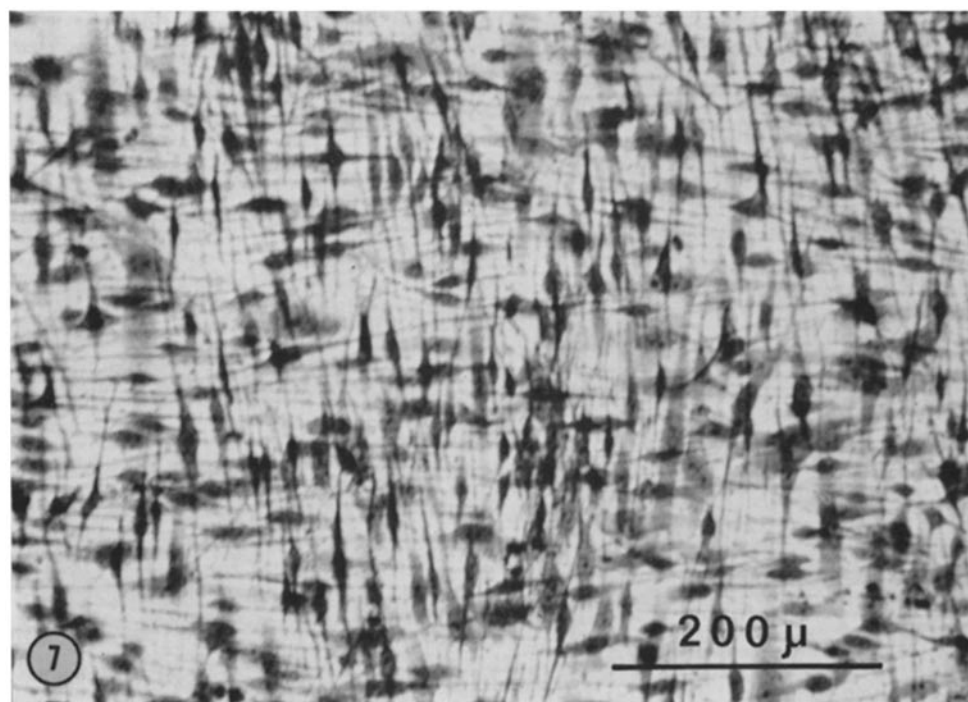

FIGURE 7 Photomicrograph of a Giemsa-stained preparation, to illustrate the ordered alignment of normal fibroblasts on drained HCLs (see text) The cells aligned across the page were plated onto an HCL drained in the same direction. 1 day after plating, the medium was removed and a second HCL was drained perpendicular to the first across the cells The cells of a second inoculum falling onto this thin HCL extend up and down the page. × 170 Refer to Fig. 4 b for the appearance of an aligned HCL

periments where the cells did not so respond, there is the possibility that a thin layer of fluid medium insulated the cells from the diluted collagen overlay. It is likely that with improved technique cells will be shown to respond to even lower concentrations of collagen in their environment.

Cell Motility

Time-lapse films show that cells on and within HCLs indulge in bursts of rapid movement. Motion may be accompanied by a pear-shaped deformation of the cell, the blunt end trailing, or the cells may move, with no apparent deformation, by a gliding motion.

By observing the behavior of drop lattices, a rough comparison can be made between the motilities of two strains of cells This comparison makes use of the fact that HCLs are susceptible to mechanical disturbance. The activity of motile cells within a lattice makes a disturbance, causing a

gradual collapse of the lattice to a dense, opaque body less than one-tenth of the original size After preparing drop lattices each containing equal concentrations of cells from different lines, the rates of collapse can be compared In this way it has been demonstrated that HeLa cells and SVTR cells cause a slower collapse than normal human fibroblasts. Whether this difference is entirely due to a lower motility among the former cells is a matter for further investigation

Cell Growth

Normal fibroblasts seeded at low densities may grow at a slower rate on HCLs than on plastic. Seeded at confluent densities, there is no difference The density at which a difference becomes significant varies with the cell strain and, perhaps, with the batch of collagen. The rate of growth of normal human fibroblasts on plastic is strongly density dependent. The maximum growth rate is achieved

TABLE I

Growth of Human Fibroblasts in the Presence of Collagen-Containing Media

Substratum	Medium	dpm/dish $\times$ 10^{-3}	Mean count
On collagen	Routine medium	16 2, 16.0, 15.2, 16.2	15 9
In collagen	Routine medium	14 8, 14.6, 16.3, 16 2	15 4
On plastic	Routine medium	38 2, 36.7	37 5
" "	Collagenase-solubilized collagen medium	15.8, 17.9	16 9
" "	Control · routine medium and collagenase	36.9, 36.3	36 6
" "	Gelatinized collagen medium	18 0, 16 7	17.4
" "	Control routine medium to 56°C, 1 hr	34.6, 34 3	34 4

During the preparation of the collagen solution used in this experiment, some of the second 4 liter lot of 0 1 Eagle's medium, against which the collagen solution had been dialyzed, was sterilized by filtration and used to prepare all the fluid media used in the experiment. A single batch of 10x Eagle's concentrate with added glutamine was used throughout. Fetal calf serum was used at 10%. Thymidine-H^3 was incorporated into all media at 0.1 μCi/ml. All dishes received replicate inocula, of 1.5 $\times$ 10^6 cells/50 mm plate, of human fetal lung fibroblasts, the final volume in all dishes being 5 ml Cultures were counted individually after 48 hr.

On collagen· Two dishes containing 1 ml HCL and 4 ml fluid medium (FM). Two dishes containing 4 ml HCL and 1 ml FM. Both classes gave similar counts and have been included together in the table

In collagen· Four dishes. 1 ml foundation HCL. Cells incorporated into 4 ml HCL poured on top, no cells in contact with plastic

On plastic: Collagenase-solubilized collagen and control. Two dishes, each class 9 ml HCL containing 100 μg collagenase, and 9 ml FM and collagenase incubated at 37°C for 1 hr to completely solubilize the collagen. Serum and thymidine-H^3 were added before use.

Gelatinized collagen medium and control. Two dishes, each class. 9 ml HCL and 9 ml FM heated to 56°C for 1 hr, collagen completely solubilized. Serum and thymidine-H^3 were added before use.

at densities around one-seventh confluent, the rate declining steeply with increasing density (1).

It appears that there is no general slowing down of growth on and within HCLs, but rather a change in the way growth rate varies with cell density. The maximum rate of growth on and within HCLs is slower for a given medium than on plastic.

Table I shows an experiment designed to investigate the apparent growth reduction within HCLs, using thymidine labeling. Replicate cultures on and within HCLs incorporated only half the counts of control cultures on plastic. The same lowered counts were obtained from cultures on plastic fed a fluid medium containing collagen, solubilized, either by collagenase treatment or by heat-induced gelatinization. It appears, therefore, that the altered growth characteristics in the presence of collagen depend not upon the physical nature of the collagen-containing substratum, but upon the chemical constituents in the preparations

Normal fibroblasts have not been cloned within HCLs.

Biopsies

The explantation of pieces from adult human skin biopsies within HCLs provides a favorable milieu for the outgrowth of fibroblasts, subsequently recovered by the use of collangease. We believe this procedure to be an improvement over existing methods for handling this material.

OBSERVATIONS ON THREE LINES OF ABNORMAL CELLS

Seeded onto plastic at high densities (3×10^6 cells/50 mm plate = ca 2 monolayer equivalents), SVTR cells, like normal fibroblasts, attach with high efficiency to provide uniform cell sheets. Normal fibroblasts behave similarly on HCLs. SVTR cells, however, behave quite differently. Most of the SVTR cells plated onto HCLs fail to attach, and can be removed at the first medium change, leaving numerous, small, dense clusters of cells that together occupy only a small proportion of the HCL surface (Fig. 8). There is subsequently some increase in the number of cells, accompanied by
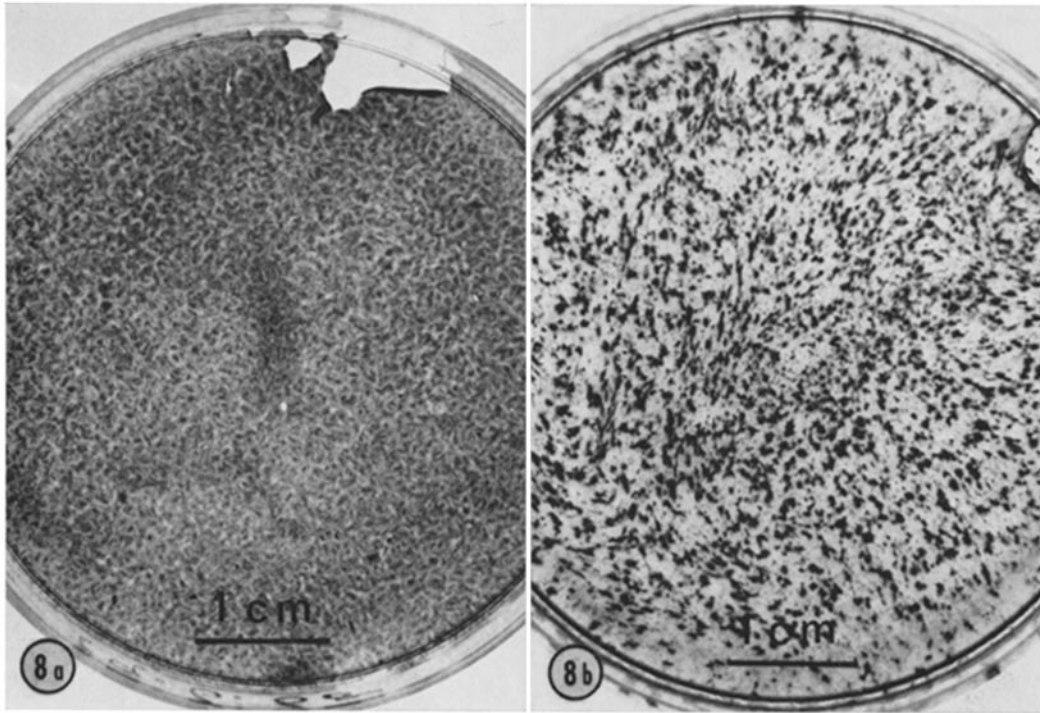

FIGURE 8   Stained Petri dish cultures of SV40 transformed rat fibroblasts. 3×10^6 cells/50 mm plate. 2-day cultures fixed after removing unattached cells (a) plated onto plastic. Virtually 100% attachment to give a supraconfluent cell sheet. (b), plated onto an HCL. The majority of cells were unattached and have been removed; the remaining cells are in clusters. Most of the HCL surface is uncolonized by cells.

expansion of the clusters, but even after 3 wk most of the HCL substratum is still free of cells Progressively smaller inocula yield a higher proportion of attached cells· attachment is virtually 100% when sparse inocula (one-tenth confluent) are used. These latter inocula usually grow to form confluent sheets Very sparse inocula that will still form confluent sheets on plastic tend to provide discrete colonies on HCLs Clustering behavior at high densities is also shown by BHK21 cells but not by HeLa cells.

SVTR cells have been seeded sparsely onto the surfaces of drop lattices. While they remain sparse, the cells adhere firmly to the surface and cannot be dislodged by shear. The thick investing membranes eventually formed by the growth of the cells are only weakly adherent, however, and their spontaneous detachment has been observed, providing orange-peel-like pieces on the floor of the dish and leaving the drop lattice intact and unchanged

SVTR cells and HeLa cells clone readily both on and within HCLs (Fig. 9). Cloned within HCLs, the SVTR line yields a range of colony morphol-

ogies from compact to diffuse. HeLa cells similarly cloned yield uniform, compact, spherical colonies. It appears that cloning within a three-dimensional substratum is especially appropriate for revealing differences in colony morphologies.

We have never observed the solubilization of HCLs in the presence of cells that is suggestive of the secretion of collagenolytic substances.

DISCUSSION

Our preliminary observations raise many interesting questions concerning the form, growth, and behavior of cells. The use of HCLs reveals novel cellular behaviors, and it is reasonable to assume that these novelties properly explored could provide for a better understanding of how cells function in the body.

The ability of SVTR cells to clone within HCLs, and the failure to grow colonies of normal cells under the same conditions, appears superficially to mirror the results of cloning cells in soft agar (9). There is, however, an important difference Cells in soft agar do not attach and extend; in HCLs
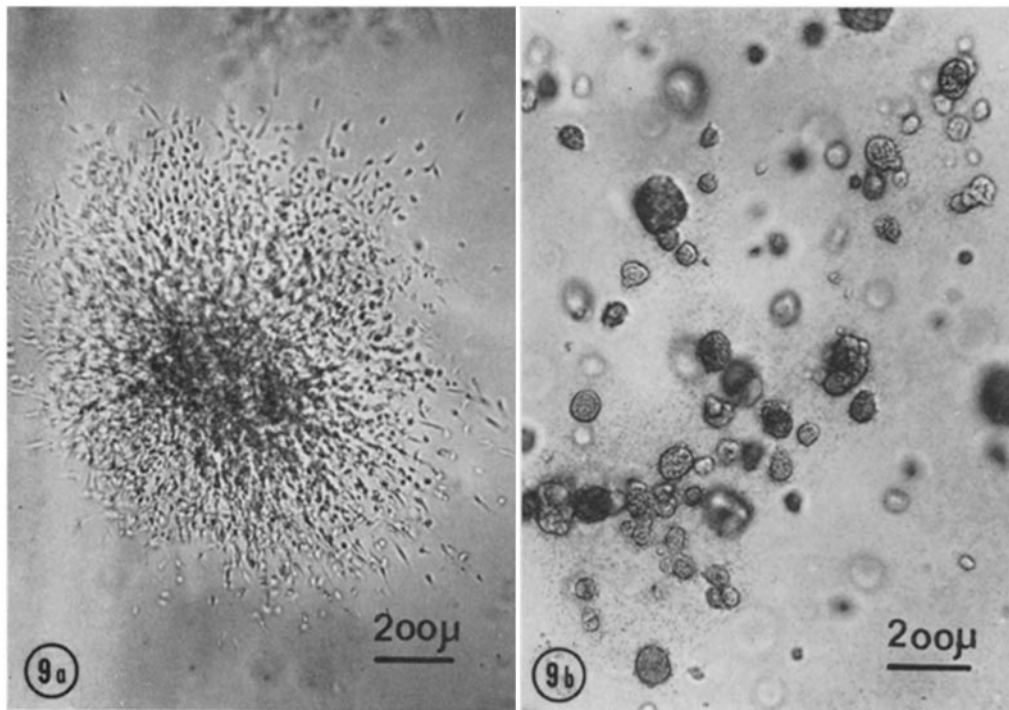

FIGURE 9 Photomicrographs of fixed and cleared whole mounts of drop lattices, showing clonal growth within HCLs (see text). (a), SV40 transferred rat fibroblast colony; (b), dense spherical HeLa cell colonies. × 50.

they do. The agar cloning results may be explained by invoking unsatisfied substratum requirements in the case of normal cells, and the absence of such requirements in the case of transformed cells. The failure to clone normal cells in HCLs may merely reflect a coincidence of circumstances unfavorable to the undertaking: the slow growth of the cells and their high motility may lead simultaneously to the dispersion of the young colonies and the undermining of the lattice.

Clustering behavior at high densities and the spontaneous detachment of membranes investing drop lattices represent departures from normal behavior and suggest that SVTR cells preferentially transfer their attachments from HCLs to one another. A clustering tendency is masked on plastic, probably because the attachments to this substratum are overriding. This illustrates how the use of a different substratum can reveal new aspects of cell behavior. It would be interesting to know whether other malignant cells of mesenchymal origin also show abnormal clustering behavior.

It is probably safe to assume that most tissue cells are permanent residents of the tissues to which they belong by virtue of their attachment to the extracellular connective tissue matrix. The behavior of SVTR cells in and on HCLs suggests the possibility that cells may undergo changes which lead to their uncoupling from the matrix. Uncoupling might affect their resident status, allowing for the cells to be passively transported via the circulation. Whether this possibility is relevant to the metastasis of tumors is a matter for further investigation. So, also, is the possible connection between the observed clustering and focal growth of SVTR cells on HCLs, and the focal growth often observed at the advancing fronts of tumors.

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