THE LOSS OF PHENOTYPIC TRAITS BY DIFFERENTIATED CELLS

VI. Behavior of the Progeny of a Single Chondrocyte*

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When mitotically quiescent chick chondrocytes are liberated from their polysaccharide matrix and cultured on a fibrin clot in the presence of embryo extract, the following is observed: (*a*) Within 18 hr the rounded chondrocytes transform into fibroblastic cells and cease synthesizing chondroitin sulfate. (*b*) Within 24 hr over 50% of the cells incorporate thymidine, and by 35 hr over 95% have completed at least one cell division. (*c*) The amount of chondroitin sulfate synthesized per cell in the log phase of growth is less than 5% that of the nondividing progenitor cells. (*d*) If after 7–10 days such fibroblastic chondrocytes contact their siblings, many resume a rounded shape and synthesize chondroitin sulfate in quantities characteristic of their in vivo progenitors. (*e*) When cultured as fibroblastic cells for over 2 wk and then allowed at high density to contact siblings, they neither round up nor deposit chondroitin sulfate. (*f*) Fibroblastic chondrocytes obtained by protracted culturing do not display the surface affinities of normal chondrocytes. Chondrocyte progeny which do not deposit chondroitin sulfate or aggregate with one another or with normal chondrocytes have been termed "dedifferentiated" or "altered" chondrocytes (1–5). Many of these observations have been confirmed by Kuroda (6) and Shulman and Meyer (7) with chick chondrocytes, by Chiakulas (8) with frog chondrocytes and by Manning and Bonner (9) with human chondrocytes.

Coon's (10) report on cloned chondrocytes both supports and conflicts with the above analysis. Coon made the important observation that many dedifferentiated chondrocytes which do not synthesize chondroitin sulfate in high density cultures, do yield progeny which synthesize the polysaccharide if cultured at low density. In addition, Coon and Cahn (11) and Cahn, Coon, and Cahn (12) claim that a high molecular weight, heat-labile factor in embryo extract selectively inhibits the synthesis of chondroitin sulfate by chondrocytes and melanin by pigment cells (see however, 13, 14). Medium containing this dedifferentiation factor was termed "nonpermissive"; medium

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without this factor was termed "permissive". The perplexing behavior of cultured cells at different densities and in the presence of various factors has been the focus of many recent studies (15–21).

Coon (10) and Coon and Marzullo (22) further report that chondrocytes dedifferentiated by growth in nonpermissive medium readily revert to functional chondrocytes by culturing them in permissive medium at clonal densities. However, it is not clear from their analysis whether all, some, or only a selected minority of the dedifferentiated cells express their chondrogenic phenotype when cloned in permissive medium (5).

The experiments to be described suggest that: (*a*) Chondrocytes grown in permissive medium dedifferentiate at both low and high density; (*b*) Most dedifferentiated chondrocytes do not revert to functional chondrocytes when challenged by a cloning situation; (*c*) A clonal environment selects for functional chondrocytes and against dedifferentiated chondrocytes; (*d*) Suppressive cell-cell or cell-exudate interactions promote dedifferentiation; (*e*) Frequent subculturing promotes expression of the functional phenotype.

Materials and Methods

In general, culture procedures followed those recommended by Coon (10) and Cahn, Coon and Cahn (12).

10-day vertebral cartilages were stripped of their adhering connective tissues (1) and incubated 2½ hr in a trypsin-collagenase mixture (0.1% trypsin, 1.4 mg/ml collagenase, 10% chick serum in calcium-magnesium–free Simm's balanced salt). After flushing the cartilages through a small bore pipette, the freshly liberated chondrocytes were counted in a hemocytometer and placed into 100 mm Petri dishes (Falcon tissue culture grade) at a density of 5×10^5 cells/dish in 10 ml of medium. After 3–5 days, two distinct cell populations were apparent: a floating population and a population adherent to the substrate. The round, floating cells, referred to as "primary floaters," were found to be contaminated with less than 1% of nonchondrogenic cells.

Experiments were performed with cells derived from cultures initially begun with a single primary floater cell. This procedure eliminates unequivocally the possibility of overgrowth of chondrogenic cells by "true" fibroblasts inadvertently included in the culture (33). Procedures for establishing chondrogenic cultures with a single cell have been described (23). Cultures of mononucleated muscle cells were prepared as described elsewhere (24, 25).

Low density cultures were plated at 1×10^3 or 1×10^4 cells/60 mm plate (Falcon, tissue culture grade) in 4 ml medium. High density cultures were plated at 5×10^5 cells/60 mm plate. High density pellets were prepared by centrifuging 1×10^6 cells into a pellet. After 24 hr incubation in the bottom of a centrifuge tube, pellets were transferred to millipore rafts floating on medium in watch glasses (28). Fibrin clot substrates were prepared in the bottom of Falcon dishes by mixing young cockerel plasma and 11-day chick embryo extract in a 1:1 proportion. Cultures were fed either daily or on alternate days: 2 ml of medium was decanted and 2 ml of fresh medium added.

To avoid confusion we have adopted Coon's (10) designations of "permissive" and "nonpermissive" medium. The word permissive as used by Coon refers to the fact that medium without embryo extract permits chondrogenesis by chondrocytes grown at clonal density whereas nonpermissive medium with embryo extract does not. As will be seen in the text to follow, such a distinction does not hold true for all culture conditions including clonal cultures. Therefore, our use of permissive and nonpermissive only refers to the presence or absence of embryo extract in the media and not to their relative capabilities of promoting chondrogenesis.

Permissive medium contains Ham's (26) F-10 with twice the amino acid concentration supplemented with 10% fetal calf serum and 1% bovine serum albumin (Grand Island Biological Co. Grand Island, N.Y.). A 0.1% concentration of antibiotic-antimycotic solution was added to the final mixture. Originally nonpermissive medium was prepared as described by Coon (10) and Coon and Cahn (12). Further experiments, however, revealed that cells in medium with 7% H-factor behaved as they did in medium prepared as follows: Two parts Simm's balanced salt, two parts horse serum (Grand Island Biological Co.), one part embryo extract (11-day chick embryos). Most of the following experiments used this nonpermissive medium.

Histological procedures for determining the presence or absence of metachromatic matrix involved fixing with glutaraldehyde (2.5% with 3.3 gm/liter of sodium cacodylate in Tyrode's salt solution) and overnight staining in 1% aqueous toluidine blue. Plates were rinsed repeatedly with 100% alcohol and air dried. Observations were made under immersion oil.

To facilitate description of the cytological observations, chondrocytes and their progeny are categorized as either "polygonal" or "fibroblastic." Virtually all investigators who have cultured cartilage cells (1, 6, 7, 10, 11) agree that the polygonal configuration of the chondrocyte is associated with the deposition of chondroitin sulfate, whereas the fibroblastic chondrocyte does not actively deposit metachromatic matrix.

A single primary floater cell may yield anywhere from 1–200 colonies in a 4 wk culture (Fig. 16). This is accounted for as follows: During the 1st wk many cell divisions yield daughter cells which adhere to the plastic. This leads to an increase in the area of the colony in the plane of the plastic substrate. During this period the colony is essentially one cell layer thick. However, not all daughter cells stick to the substrate. Many single cells detach from the dish, (Figs. 2, 3, and 4) establishing new colonies. Other cell divisions in the colony lead to vertically displaced daughter cells. This displacement of nascent cells changes the topography of the colony into an irregular, three-dimensional mound. The vertically displaced cells are round or polygonal and embedded in a transparent jelly (Figs. 17, 18, and 19). This jelly, when fixed, invariably stains metachromatically. The mounds (or strings) of cells, resembling frog egg masses, often extend upward into the medium, hundreds of microns from their epithelioid base (Figs. 18, 19). The photographs and descriptions by Coon (10), Coon and Cahn (11) and Cahn (14) indicate that this phenomenon occurs in their cultures. Masses of cells embedded in jelly may be released from the parent colony, particularly during the feeding procedures. These floating masses containing 1–60 (Fig. 17) cells can settle elsewhere in the dish and establish additional colonies. This detachment and reattachment of floating chondrocytes to form new colonies renders conventional determinations of plating efficiency; i.e.,

$$\frac{\text{No. of colonies}}{\text{No. of cells in original inoculum}} \times 100\%$$

subject to large errors. This mode of satellite colony formation probably accounts for the unprecedented high plating efficiencies reported for primary cells by Coon (10). The singularly weak binding of even single interphase chondrocytes to the substrate (Figs. 2, 3, and 4) may be due to their adhering shell of chondroitin sulfate (3). The detachment during mitosis reflects changes in the properties of the cell surface (Figs. 12 and 13) and is displayed by other cell types (e.g. 62). Results of experiments based exclusively on differences in plating efficiencies must be interpreted with great caution. Little can be concluded about the plating efficiency of *all* cells in the inoculum if colonies are counted after 16 days.

RESULTS

Transformation of the Progeny of a Single Chondrocyte into Fibroblastic Cells in Permissive Medium.—Fig. 1 is a phase photomicrograph of a living primary floater cell after 24 hr on plastic in permissive medium. Figs. 2–7 are photo-

micrographs of the same living culture 4, 7, 11, 16, 20, and 30 days later. The early progeny are round or polygonal and during the 1st wk are separated by one or two cell diameters. Subsequently they adhere to each other, forming characteristic epithelioid colonies of polygonal cells (Figs. 4, 8, 12). When centrally located cells enter mitosis (compare 61) they lose contact with surrounding cells and round up (Fig. 12). Within hours daughter cells again establish contacts with their neighbors. In older cultures (about 10 days) refringent material may be observed between polygonal cells. The epithelioid arrangement of cells in these colonies is reminiscent of the arrangement of replicating perichondrial cells in vivo (27).

Figs. 5, 12–15 demonstrate the variations in five different 16 day single cell cultures. Although the majority of cells adhere to one another and are polygonal, cells of a more fibroblastic cast are increasing in number, particularly along the fringes of the epithelioid colony. With time the central cells in the colony transform into fibroblastic cells and eventually the fibroblastic cells dominate the dish. There is great variation as to when fibroblastic cells first appear and when they become the dominant cell type (compare Figs. 8, 12 with Figs. 6, 14, and 15). The emergence of fibroblastic cells is not a simple function of the number of cell divisions (2, 28) or of the size of the colony. The fibroblastic phenotype may emerge when there are less than two dozen cells in the colony. In other instances there may be over 200 polygonal cells in a colony before fibroblastic cells appear.

Quantitative data on oscillations between polygonal and fibroblastic phenotypes are difficult to obtain, for the distinction between the two is not unequivocal under the phase microscope and there are many transitional stages. Both fibroblastic and polygonal properties have been observed in different parts of the same cell as well as in different parts of the same colony (Figs. 8–11). Nevertheless, colonies that consist largely of polygonal cells at the end of 2 wk become predominantly fibroblastic by the end of 3 wk of culture (Figs. 6, 7, 13–15). 3 wk cultures may contain between 5×10^3 and 30×10^3 cells/dish and the ratio of polygonal cells to fibroblastic cells varies between 1:3 to 1:8. By the end of the 4th wk over 95% of the cells are fibroblastic.

Primary epithelioid colonies of over 40 cells usually contain some cells surrounded by metachromatic matrix. The proportion of cells surrounded by matrix in a given colony varies enormously. In some young colonies (i.e., less than 12 days old) over 80% of the cells may be polygonal and surrounded by matrix; in others of the same age over 80% of the cells may be fibroblastic and only 20% surrounded by matrix.

Fibroblastic cells at the edge of or between epithelioid colonies do not exhibit metachromatic matrix. The transformation of epithelioid colonies into fibroblastic colonies is accompanied by the resorption of previously deposited matrix. Occasionally the center of a transformed colony is occupied by an irregular mound of metachromatic matrix devoid of cells. Presumably the

polygonal cells which had deposited the matrix died or emigrated. That functional, polygonal chondrocytes shed their capsules and transform into ameboid fibroblastic cells has been noted before (3).

Summary: Medium which is "permissive" for chondrogenesis in 1 and 2 wk-old cultures functions as "non-permissive" medium as the colony ages. It is unlikely, therefore, that a factor in the ambient medium transforms polygonal into fibroblastic cells. As there may be but 100 cells in the 60 mm Petri dish when transformation begins, it is unlikely that the transformation is due to an insufficiency of metabolites (10–12, 14). The deposition of metachromatic matrix is associated with polygonal and not with fibroblastic cells.

Behavior of Second-Passage Chondrocytes at Low Density in Permissive Medium.—For additional experiments large quantities of cells, all derived from a single cell, were required. These were secured as follows: 12–15 day single cell cultures of approximately 5×10^5 polygonal cells were individually suspended and replated at concentrations of 5×10^2 cells/dish. This procedure yields as many as 10 second-passage, low density cultures, the cells of which are all derived from a single chondrocyte.

Approximately 50% of the cells in the inoculum used to establish second-passage cultures do not attach to the substrate in the first 24 hr. Many of these floating cells remain viable and multiply. Some subsequently attach to the substrate and form epithelioid or fibroblastic colonies. In addition, nascent daughter cells detach from the plastic and contribute to the floating population. Estimates of cell multiplication in these cultures are subject to errors, since many floaters are lost when the medium is changed.

In 14 day, second-passage cultures there are about 10^6 cells/dish. Of these approximately 50% are polygonal, 40% fibroblastic, and 10% floaters. Fewer cell masses build up and detach from the epithelioid colonies of second-passage cultures than from primary cultures. The floater cells tend to be single, spherical cells and not embedded in metachromatic jelly. Furthermore, the area and number of cells in the second-passage epithelioid colonies are smaller at the time of transformation of polygonal into fibroblastic cells than in first-generation colonies. For the first 14 days the total number of fibroblastic cells is invariably smaller than the total number of polygonal cells; similarly the number of fibroblastic colonies is smaller than the number of adjacent epithelioid colonies. During this period, the frequency of mitotic figures is greater among polygonal cells than among fibroblastic cells. By the 3rd wk the majority of cells are fibroblastic. By the 4th wk over 95% of all cells are fibroblastic. As in primary cultures, metachromatic staining is associated with the polygonal cells in the epithelioid colonies. Occasionally, however, fibroblastic, aberrant polygonal, multinucleated, and giant cells stain metachromatically. The same data, not in terms of total numbers of cells in the dish, but in terms of cartilage-making colonies vs. fibroblastic colonies is shown in Table I.

Summary: Second-passage cultures differ from primary cultures in that: (*a*)

Epithelioid colonies are smaller in area and cell number; (*b*) Transformation from polygonal cells into fibroblastic cells occurs earlier; (*c*) Floater cells are single and do not secrete the copious amounts of metachromatic jelly characteristic of primary floaters.

Behavior of Dedifferentiated and Functional Chondrocytes at High Density in Permissive and Nonpermissive Medium.—Fibroblastic chondrocyte progeny which do not deposit metachromatic matrix, though reared in a medium

TABLE I

Change in Numbers of Metachromatic Colonies with Time

A primary culture derived from a single floater cell was trypsinized and used to establish eight second-passage cultures. The original inoculum for the second-passage cultures consisted of 10^3 cells/Petri dish. The peak in total number of cartilage making colonies occurs around day 12. Some of the cartilage-making colonies in 25 day cultures may be but a week old, having been seeded then from floaters given off by the older colonies. A colony consisted of a cluster of 25 or more cells. All colonies in each dish were counted.

Dishes	Colonies with less than 10% cells surrounded by matrix	Colonies with greater than 10% cells surrounded by matrix	Colonies with cells not surrounded by matrix (fibroblastic)
10 days			
1	301	463	479
2	204	562	576
3	242	493	226
4	280	402	458
25 days			
1	79	6	778
2	73	9	893
3	55	4	325
4	158	8	1324

known to support chondrogenesis, have been termed dedifferentiated chondrocytes. By this definition the majority of cells in 4-wk primary or secondary cultures are dedifferentiated. It was desirable to determine whether chondrocytes dedifferentiated in permissive medium had the same properties as those previously described for chondrocytes dedifferentiated in nonpermissive medium (1, 5, 28). Large numbers of fibroblastic chondrocytes, all derived from a single primary floater cell, were obtained by harvesting a cohort of 4-wk second-passage cultures. In each of the following experiments the response of the second generation fibroblastic cells is compared with chondrogenically competent primary floaters.

Six pellets, each of 10^6 fibroblastic cells were organ-cultured either in permissive medium or nonpermissive medium, on top of Millipore rafts. Six pellets, each of 10^6 primary floaters, were likewise grown in permissive or non-

permissive medium. Not one of the pellets of fibroblastic cells exhibited histologically recognizable matrix, regardless of the type of medium in which it was grown. Irregular masses of cartilage formed in all the pellets of primary floaters irrespective of whether permissive or nonpermissive medium was used.

5×10^5 second-passage fibroblastic chondrocytes or 5×10^5 primary floater cells were grown: (*a*) on a fibrin clot in nonpermissive medium; (*b*) on a fibrin clot in permissive medium; (*c*) on plastic in nonpermissive medium; (*d*) on plastic in permissive medium. Under all four culture conditions the fibroblastic cells multiply, become confluent and multilayered, but invariably remain fibroblastic. Occasional clusters of atypical polygonal cells appear, but even in these areas metachromatic matrix is wanting, or at most, barely detectable.

In high density cultures of primary floater cells matrix appears within a week under all four culture conditions. In permissive medium the cells remain polygonal and by the 3rd or 4th day there are many metachromatic areas. By day 10 most of the metachromatic material has disappeared. In contrast, in nonpermissive medium by 24 hr most of the cells are fibroblastic. Not until day 5 or 6 do polygonal cells appear. Their appearance is associated with the deposition of metachromatic matrix. Considerably more matrix is deposited in these cultures than in those with permissive medium. Furthermore, the matrix formed in nonpermissive medium tends to persist. This finding—more matrix and more stable matrix in nonpermissive medium than in permissive medium at high density—has also been observed by Lavietes and Weston (30). It may be coupled to the observation that collagen is synthesized by cells in nonpermissive medium (31) but is *not* synthesized by chondrocytes in permissive medium.[1]

Second-passage fibroblastic cells which clone very poorly as isolated cells (5) have been grown at high density as third, fourth, fifth, and sixth-passage cells for over 8 wk. During this time they essentially double every 2–3 days in either medium. After the 5th wk, the rate of increase of the population declines (see also 32). The number of dying and moribund cells in high density cultures of fibroblastic cells is higher than in comparable cultures of polygonal cells. Consequently, the data on time for cell doubling of fibroblastic cells as compared to polygonal cells could be misleading. Metachromatic matrix never appears in these fibroblastic cultures, irrespective of whether permissive or nonpermissive medium is used.

Summary: Whether a chondrogenic cell expresses its chondrogenic phenotype at high densities depends upon its past history rather than upon the type of medium—permissive or nonpermissive—in which it is grown. Primary floaters deposit matrix; fibroblastic chondrocytes do not deposit matrix in high density

[1] Schulte-Holthausen, H., S. Chacko, C. Davidson, and H. Holtzer. Unpublished observations.

cultures. More matrix and more stable matrix is deposited in cultures of floater cells in nonpermissive medium than in permissive medium. If there is an inhibitory factor in nonpermissive medium selectively suppressing the synthesis of chondroitin sulfate in *low density* cultures (10–12, 14), then its action in *high density* is completely reversed.

The Cytotoxicity of Nonpermissive Medium in Low Density Cultures.—Coon (10) and Coon and Cahn (11) state that nonpermissive medium is cytotoxic, that it selectively inhibits the synthesis of chondroitin sulfate and melanin, and that it stimulates cell multiplication.

To probe these partially contradictory claims, second-generation fibroblastic cells or primary floater cells were cultured at a density of 10^3 cells/dish. Cells were grown either (*a*) on a fibrin clot in nonpermissive medium; or (*b*) on plastic in nonpermissive medium. The fibrin series was introduced to keep cells anchored to the substrate. Irrespective of substrate both fibroblastic and primary floater cells attach, the latter more slowly, multiply sluggishly for several days, and vacuolate. By day 10 most of the cells have died or are moribund. Occasionally clusters of opaque cells are observed, suggesting that local higher densities permitted marginal survival. At this low density, nonpermissive medium is not adequate for either polygonal or fibroblastic cell multiplication or differentiation. Had these dishes been scored for colonies, we would have confirmed the finding of Coon (10) and Cahn (14) that the plating efficiency drops markedly in nonpermissive medium.

If, in contrast, nonpermissive medium is substituted for permissive medium over established clonal plates 5 days or older, within 24 hr, most of the polygonal cells transform into fibroblastic cells. These transformed cells continue to multiply as fibroblastic cells. Under these conditions it is operationally impossible to assess the mutually contradictory claims that nonpermissive medium (*a*) selectively inhibits the synthesis of chondroitin sulfate, (*b*) is cytotoxic, (*c*) promotes the rate of cell multiplication (10, 11, 14).

Failure of Most Fibroblastic Cells to Reexpress Their Chondrogenic Phenotype at Low Density in Permissive Medium.—Coon (10) and Coon and Marzullo (22) interpret their plating efficiency experiments to mean that *all* fibroblastic chondrocytes readily revert to polygonal chondrocytes when grown at low densities in permissive medium. On the other hand it has been reported that 5-Bromodeoxyuridine (BudR)-suppressed chondrocytes, though multiplying well in high density, clone poorly at low density (5, 23). To determine whether all or only a proportion of second-passage fibroblastic cells express their chondrogenic phenotype, two series of experiments were performed.

In the first series, single second-passage fibroblastic cells, single primary floater cells and single myogenic cells were challenged to establish colonies of 25 cells or more. Of the 47 cultures initiated from single fibroblastic cells in permissive medium, only 3 produced colonies of 25 or more cells after 10 days.

Two of these were epithelioid, one was fibroblastic (Table II). Of the 38 primary floaters that attached, 18 produced epithelioid colonies; the remaining either died, never divided, or produced a progeny of less than a dozen cells.

Mononucleated myogenic cells grown under these same clonal conditions do not multiply and do not form multinucleated myotubes (Table II). Likewise normal embryonic somite cells clone poorly in permissive medium on plastic (37). Myogenic cells and somite cells in nonpermissive medium at higher cell densities multiply and terminally differentiate (5, 24, 25, 62). Clearly, multipli-

TABLE II

Fate of Cultures Established with a Single Primary Floater, a Single Second-Passage Fibroblastic Chondrocyte or a Single Myogenic Cell

Cell Type	Series	No. of cultures established	No. of living cells attached after 24 hr	No. of colonies of more than 25 cells	No. of polygonal colonies	No. of fibroblastic colonies
Primary floater	a	15	11	8	8	0
	b	18	16	3	3	0
	c	11	6	4	4	0
	d	8	5	3	3	0
Second-passage fibroblastic	a	15	13	2	1	1
	b	10	9	1	1	0
	c	10	8	0	0	0
	d	12	10	0	0	0
Myogenic cells	a	15	12	0	0	0
	b	10	6	0	0	0
	c	12	7	0	0	0
	d	13	7	0	0	0

cation and differentiation of functional chondrocytes is selected for, whereas multiplication of fibroblastic chondrocytes and normal myogenic and somite cells are selected against, under these particular clonal culture conditions. Failure of fibroblastic chondrocytes to clone indicates an alteration in phenotypic properties from the parental cell. Failure to replicate under these clonal conditions is a property also shared by normal myogenic and normal somite cells and therefore in itself is not necessarily a degenerative change.

In the second series, cultures were set up with 10^4 second-passage fibroblastic cells/dish in permissive medium. For controls, the same numbers of primary floaters were cultured.

The viable fibroblastic cells attach in 24 hr. By the 3rd or 4th day both fibroblastic and polygonal cells are present. It is obvious from phase microscopic observations that many fibroblastic cells die after attaching. Whereas some

moribund cells are always present in fibroblastic colonies, they are never observed in adjacent clusters of polygonal cells. By day 6 the number of polygonal cells is considerably greater than the number of fibroblastic cells and floaters have appeared. As described earlier, these floater cells may form new epithelioid colonies. By day 10 approximately 80% of the cells are polygonal and no more than 20% can be termed fibroblastic. By the end of the 3rd wk, however, over 80% of the cells are fibroblastic.

TABLE III

Fate of Fibroblastic Cells Derived from a Single Primary Floater

Fibroblastic cells, derived from a 50 day clone established from a single primary floater, were cultured at a density of 2×10^3 cells/60 mm Petri dish. The theoretical number of attached cells after 24 hr assumes no cell division during this period. At days 1, 8, 12, and 14 all the cells in two separate rectangular areas (each 2 mm × 30 mm) were scored as either isolated fibroblastic cells or as cells in colonies.

Day 1		*Day 8*		*Day 12*		*Day 14*	
Theoretical no. of attached cells at 24 hr	Actual no. of attached cells at 24 hr	Isolated fibroblastic cells	Colonies of 5 or more cells	Isolated Fibroblastic Cells	Colonies of 5 or more cells	Isolated fibroblastic cells	Colonies of 5 or more cells
93	44	88	36	248	48	266	53 (23 colonies were metachromatic)

The variable fates of the fibroblastic cells from old primary cultures is shown in Table III. A 50 day clone established from a single primary floater was trypsinized and the cells used to establish second-passage cultures. A census of a marked area of the dish was taken on days 1, 8, 12, and 14. Three points emerge from this study: (*a*) Half of the cells of the original inoculum do not survive; (*b*) Only a minority in the inoculum actually attach and proliferate to the extent of forming colonies of five or more cells; and (*c*) Fibroblastic and polygonal cells replicate side by side in the same medium.

Although some of the fibroblastic cells which attach and proliferate form new colonies of polygonal cells, clearly many do not revert to the polygonal form. At low density fibroblastic cells experience a higher cell mortality, and probably a lower rate of multiplication, than do adjacent polygonal cells in the same dish. Nevertheless, many fibroblastic cells divide and yield only fibroblastic progeny. These considerations make it difficult to determine what percentage of the cells in the original inoculum revert to polygonal progenitors and what percentage proliferate as fibroblastic cells. The early appearance of floater cells under mass clonal conditions (i.e., 10^3 to 10^4 cells/dish) also make it impossible to determine what percentage of the cells in the inoculum revert to functional chondrocytes simply by counting cartilage-making colonies 18 days after the cultures are established.

Among the control cells, approximately half of the primary floaters attach in the first 24 hr, the remaining half behave as floaters or die. By day 4 or 5 less than 1% of the attached cells are fibroblastic. By day 10 over 95% are polygonal. By the end of the 3rd wk, over 90% of all cells in the dish are fibroblastic, though sizable cartilage-making colonies are still present.

Summary: Single fibroblastic cells clone much more poorly than do single polygonal cells. The mortality of fibroblastic cells at low density is higher than among polygonal cells. By focusing on cells in cartilage-making colonies, the fate of most of the cells in the dish is ignored. Furthermore, with respect to the cells in the original inoculum there is a spuriously high number of cartilage-making colonies, due to the seeding of new functional colonies by clusters or single floaters. Conditions in low density cultures select for polygonal chondrocytes and against the survival and multiplication of fibroblastic cells. Under these conditions, most of the original fibroblastic cells do not revert to the polygonal phenotype. The fact that both fibroblastic and polygonal cells replicate in the same dish makes it unlikely that there is a specific factor in the ambient medium which suppresses chondroitin sulfate synthesis.

TABLE IV

Adhesiveness of Third-Passage Fibroblastic Cells Compared with Primary Floater Cells

5×10^4 primary floater cells or 5×10^4 third-passage fibroblastic cells were introduced into non–tissue culture, plastic Petri dishes. The total number of cells in two separate rectangular areas (each 2 mm × 30 mm) that adhered to the substrate were counted.

Hr	Primary floater cells	Fibroblastic cells
4	9	270
10	10	411
24	160	1008

Surface Properties of Fibroblastic Cells.—Surface properties of chondrocytes dedifferentiated in nonpermissive medium or in BudR differ from those of polygonal chondrocytes (5, 23, 53). Dedifferentiated cells do not adhere to each other, are amoeboid, and have a total surface area averaging 4–10 times that of polygonal cells. The following experiments compare the rapidity with which polygonal and third-passage fibroblastic cells attach to the plastic substrate. 5×10^4 third-passage fibroblastic cells or 5×10^4 primary floaters were plated in nontissue culture dishes in permissive medium. As shown in Table IV fibroblastic cells attach more rapidly to the plastic substrate than do polygonal cells.

Two observations, one relating to cell division and one to amoeboid locomotion, also suggest differences between the surfaces of fibroblastic and polygonal cells. There is a high frequency of grossly asymmetrical cell divisions among fibroblastic cells. Fibroblastic cells, tightly adherent to the plastic, often do not retract all their processes and fail to round up during mitosis. The resulting

unequal-sized daughter cells reflect an unequal partitioning of cytoplasm, and particularly, of cell surface. The total cell surface area of one nascent daughter cell may be twice that of the other, depending upon the relationship of the cleavage plane to the unretracted processes. Cell divisions among polygonal cells have not revealed such obvious division asymmetries.

At times, migrating fibroblastic cells do not retract all their pseudopodia, resulting in self-amputation of sizeable portions of the cells. The cell processes are exceedingly thin and consist of relatively little endoplasm but disproportionately large amounts of cell membrane.

TABLE V

Effect of Frequent Subculturing on Expression of Polygonal Phenotype

In series *A*, 10^4 cells were grown for a total of 40 days without subculturing. In Series *B*, the same number of cells were subcultured every 10 days. T_D is the calculated time for cells to double in number; T_N is the calculated number of generations formed in the culture period. The latter value is obtained by using the formula: $N_0 \times 2^n = N(t)$ where N(t) is the number counted at the end of the culture period. The estimate of 25.2 cell divisions is the theoretical value based on the assumption that all of the progeny multiplied at the same rate and that they all survived. The estimation of 4×10^{11} cells is the number that would have been present if all the cells had been continuously subcultured at 10^4 cells/dish every 10 days. The estimate of 8.3 cell divisions is based on the number of cells counted. As this does not include cell death during the culture period, the figure is too low.

No. Cells in original inoculum	Days Cultured	No. Cells Recovered	Calculated T_D (days)	Calculated T_N (divisions)	Calculated No. Cells	Polygonal
						%
Series *A*: Cultured for 40 days without subculturing:						
10^4	1–40	3×10^6	4.8	8.3		2
Series *B*: Cultured for 40 days, but subcultured every 10 days.						
10^4	1–10	1.2×10^6	1.5	7.0		
10^4	10–20	1.0×10^6	1.5	6.6		
10^4	20–30	0.6×10^6	1.7	5.8		
10^4	30–40	0.6×10^6	1.7	5.8		
Total No.	40			25.2	4×10^{11}	60

Summary: The surface properties of fibroblastic cells differ from those of polygonal cells. The tight binding of the surfaces of fibroblastic cells to the substrate may directly or indirectly interfere with the mechanisms of extrusion or synthesis of chondroitin sulfate.

Frequent Subculturing and the Retention of Polygonal Phenotype.—It has been reported that the capacity of fibroblastic chondrocytes to function as progenitors of functional chondrocytes depends upon their prehistory (2, 5). This change in ability of fibroblastic chondrocytes could be due to: (*a*) the absolute time spent in vitro; (*b*) the absolute number of cell divisions among ancestral cells; (*c*) the time in the ancestral history when the transformation from polygonal to fibroblastic cells occurs; or (*d*) as yet undefined causes.

To approach these issues second-passage polygonal cells (i.e., 10-day cultures), all originally derived from the same parental cells, were cultured at 10^4 cells/dish. Some dishes were set aside to be inspected after 40 days (series *A* in Table V). Others, after 10 days, or when the majority of cells were still polygonal, were suspended and again subcultured at low density for 10 days (series *B* in Table IV). This procedure of subculturing every 10 days was repeated twice more. The 10-day culture in the series that had been subcultured three times in 40 days was compared to the 40-day culture that had not been subcultured. The total number of cell divisions in the frequently subcultured population was vastly greater than that in the population that was not subcultured—4×10^{11} cells vs. 3×10^6 cells (Table V). The percentage of polygonal cells in the dish of frequently subcultured cells is 60% versus only 2% in the nonsubcultured series. Prolonged culturing (i.e. greater than 10 days) without subculturing invariably results in fibroblastic cells. The polygonal phenotype, however, is favored by frequent subculturing in permissive medium at low density.

The *absolute* number of polygonal cells per dish declines as cells from later subcultures are used to initiate new cultures, whereas the numbers of fibroblastic cells increases. The number of polygonal cells/epithelioid colony and the amount of metachromatic material also diminishes in cultures made from successively later subcultures (27).

Summary: The retention of the capacity to form the polygonal phenotype is dramatically enhanced by frequent subculturing. Incident to frequent subculturing is the greater frequency of cell divisions in the system. These experiments make it unlikely that the absolute time cells spend in vitro or that the absolute number of cell divisions experienced by ancestral cells are sufficient in themselves to transform polygonal cells into fibroblastic cells (2, 5, 28, 63).

DISCUSSION

The altered behavior of specialized cells in vitro has been studied for many years and from many different view points (34–42). The point of departure for this series on chondrocytes has not been whether functional cells can or cannot be maintained in vitro, for this in all probability is a matter of technology (10, 43, 44). Nor is it whether dedifferentiation is reversible or not (1, 5), for this is more meaningfully analyzed on the level of nuclear, or cortical transplants (45, 46). Rather it is based on the assumption that by observing what activities may or may not be coupled when differentiated cells are reared under different conditions much can be learned about how extracellular cues determine nuclear responses. Unlike many investigations, then, we deliberately seek to exploit the fact that in vitro conditions often suppress the synthesis of sets of "luxury" molecules, yet do not grossly alter the synthesis of "essential" molecules (5, 37, 62, 63).

Chondrocytes, unlike terminally differentiated muscle, nerve, thyroid, or

pancreatic cells, are readily induced to reenter the mitotic cycle. Presumably this is a genetically determined property and reflects the in vivo capacity of chondrocytes for renewal. The progeny of genetically-programmed chondrocytes may assume a polygonal or fibroblastic phenotype. Coupled to these alternative gross morphological states are equally extensive alterations in the rough ER (endoplasmic reticulum) and Golgi complex (47), as well as alterations in the respective biosynthetic programs (4). Cell morphology and function are so coupled that it is not yet possible to determine whether the loss of the polygonal morphology results in failure to deposit matrix, or whether failure to deposit matrix results in the fibroblastic phenotype.

The majority of 10 day vertebral chondrocytes in vivo synthesize chondroitin sulfate, and collagen, but do not synthesize DNA (3, 4, 31). Whether this nuclear program is sustained in vitro depends on the in vitro microenvironment. The microenvironment is more than just permissive or nonpermissive medium; it includes the proximity of other cells. By confronting polygonal chondrocytes in pellets with surfaces of other polygonal chondrocytes, the in vivo nuclear program is sustained. The same cells, confronted with the fibrin-medium, or plastic-medium interface, alter their nuclear program and initiate the synthesis of DNA (4).

Chondrocytes mixed with skin fibroblasts, kidney or liver cells, and reared in monolayers transform into fibroblastic amoeboid cells and fail to deposit metachromatic matrix (3, 48). These findings were interpreted to mean that proximity of heterotypic cell surfaces or their exudates (20, 49–52) interfered with or suppressed the deposition of matrix by functional chondrocytes. The finding that fibroblastic, dedifferentiated chondrocytes do not deposit matrix at high density, but that some may yield a progeny of matrix-secreting cells at low density also suggests cell-cell interactions. One working hypothesis is that potential polygonal chondrocytes surrounded by fibroblastic chondrocytes are as suppressed as if they were surrounded by heterotypic cells. The surfaces or exudates of fibroblastic cells are as inimical to polygonal chondrocytes as the proximity of kidney or liver cells. A potential polygonal cell removed from the suppressing influence of contiguous fibroblastic cells may itself revert, or yield a daughter cell which may revert, to the polygonal phenotype. This postulated cell-cell suppression supports the observations that frequent subculturing at low density selects for expression of the polygonal phenotype. Minimizing the numbers of fibroblastic cells accumulating in a colony should minimize the rate at which polygonal cells are induced to transform by adjacent fibroblastic cells. Supporting this interpretation are recent experiments where primary floater cells are mixed with fibroblastic chondrocytes or BudR-suppressed chondrocytes. In these mixtures the fibroblastic chondrocytes suppress the synthesis of chondroitin sulfate by adjacent polygonal cells (5, 53).

Experiments with single cell cultures unequivocally demonstrate that in the

absence of high molecular weight, heat-labile factors in embryo extract, chondrocytes induce their own transformation into fibroblastic cells. The dedifferentiated progeny replicate, but do not deposit matrix. That the ambient medium is not the decisive factor in this reaction is the simple observation that polygonal and fibroblastic cells may replicate in the same dish, each phenotype retaining its characteristic properties for weeks.

Assuming that the initiation of DNA synthesis and the activation and inactivation of chondroitin sulfate synthesis reflects nuclear activities, it follows that extranuclear influences must trigger the appropriate nuclear controls. What kind of event in this closed system—constant medium and a single differentiated cell with a restricted genetic program—could induce replicating polygonal cells to transform into fibroblastic cells? One hypothesis, again implicating the cell surface, is that polygonal cells experience transitory changes affecting the adhesiveness of their cell surfaces. This leads to amoeboid movement and to novel interactions of the surface with substrate precluding the deposition of matrix. Enzymes required for chondroitin sulfate synthesis, or extrusion, anchored to membranes could be subject to conformational changes which alter their activity as the polygonal cells transform into the fibroblastic condition. Flattened cells sense very different microenvironments from polygonal cells. A large portion of the total surface faces the little understood plastic–medium interface (54, 55), the remaining surfaces confront the medium or other cells. This asymmetrical environment contrasts with the uniform microenvironment surrounding chondrocytes in vivo. The frequency of such postulated transitory changes in cell surface might correlate with age (i.e., length of time from last mitosis) of the cell. The unexpected finding that rapidly turning-over cells perpetuate the polygonal phenotype longer than more slowly dividing cells might mean that "young" cells are less likely to experience changes of surface properties than "old" cells. The diminution in secretory activity of tumor cells in older cultures which is reversed by remaining in vivo for a while might be a related phenomenon (56, 57). A more orthodox explanation of dedifferentiation is to invoke a mutation. Any mutation interfering with chondroitin sulfate synthesis would result in a fibroblastic cell. This cell would transform adjacent cells by cell-cell suppressor interactions. The mutated initiating cells and the transformed adjacent cells need not share the same intracellular lesions.

Earlier speculations relating dedifferentiation solely to the absolute numbers of mitosis has been questioned (1, 28). The results presented in this paper further support the view that factors other than mitosis per se lead to dedifferentiation. The observation that cells dedifferentiate more rapidly on a clot correlates with their tendency on fibrin to spread, become amoeboid, and their failure to adhere to one another. In contrast, on plastic the cells tend to resist spreading, forming colonies of sessile though replicating cells. Low density of

cells also appears to contribute to the retention of the polygonal morphology. In this connection it is interesting that several tumor lines which in vitro continue to synthesize their unique luxury molecules, exist as epithelioid colonies of adherent polygonal cells (57, 60).

Unequal cell divisions yield daughter cells which differ phenotypically from each other and from the mother cell (58, 59). The different fates of these daughter cells might reflect unequal inheritance of either cytoplasmic or cortical factors incident to the unequal cleavage. The frequency of such unequal divisions in fibroblastic cells or the loss of parts of the cell during locomotion could produce cells with unequal cytoplasmic or cortical endowments. If enzymes or cofactors required to synthesize or secrete chondroitin sulfate are not equally distributed throughout the cytoplasm or cortex, either of these events might lead to a cell unable to synthesize the polysaccharide.

When fibroblastic cells are subcultured at low density, they multiply and produce either fibroblastic or polygonal progeny. They do not all revert to functional chondrocytes. Are there then different kinds of fibroblastic cells, those that do and those that do not revert under these in vitro conditions? An unequivocal answer based on mass cloning requires that: (*a*) 100% of the fibroblastic cells used in the original inoculum yield identifiable progeny; and (*b*) the percentage of polygonal colonies derived from floaters be known. Currently this data is not available. Evidence based on cloning single second-generation fibroblastic cells suggests that no more than 5% of the original inoculum of dedifferentiated cells will produce polygonal progeny. Fewer functional chondrocytes are derived from fourth or fifth generation cultures (27).

Dedifferentiated chondrocytes which do not synthesize chondroitin sulfate are not "blank" cells; they are not "undifferentiated", uninstructed virginal embryonic cells (5, 63). The nuclear controls regulating dedifferentiated cells are as stringent and as ordered as those of normally functioning cells. The alkaline phosphatase activity per cell or the absorption spectra of five cytochromes of the dedifferentiated cells are indistinguishable from the corresponding proteins in polygonal cells (5). The orderly progression through the mitotic cycle indicates that the synthesis of the various classes of nucleic acids, membranes, microtubules, etc., is subject to regulation. From this it can be concluded that in the progeny of chondrocytes the synthesis of molecules such as chondroitin sulfate or collagen can be uncoupled from the synthesis of essential molecules.

Most current models of cell differentiation stress the controlling role of nuclear genes, the stability of the terminal phenotype being ascribed to the unique families of mRNA's transcribed in a given cell. The oscillation in the chondrogenic phenotype described in this paper, and in other cases of dedifferentiation, demonstrates that extracellular and cytoplasmic factors ultimately dictate the nuclear activity required to sustain a given phenotype.

SUMMARY

A single, functional, mitotically quiescent chondrocyte may be induced to reenter the mitotic cycle, and produce a progeny of over 10^{11} cells. Sessile, adherent, polygonal cells deposit matrix, whereas amoeboid, dispersed, flattened fibroblastic cells do not. The prior synthetic history of a cell is of greater importance in determining whether the characteristic chondrogenic phenotype will be expressed, rather than growth in "permissive" or "nonpermissive" medium. Clonal conditions select for stem-like cells, some of whose progeny may become polygonal chondrocytes. The retention of the characteristic chondrogenic phenotype in vitro is favored by pruning the dedifferentiated chondrocytes which arise in these cultures. Dedifferentiated chondrocytes interfere with the deposition and synthesis of chondroitin sulfate by neighboring functional chondrocytes. Possible mechanisms are proposed to explain this type of cell-cell or cell exudate interference.

If the progeny of a single, genetically programmed chondrocyte may or may not synthesize chondroitin sulfate, then extragenic sites in the cytoplasm or cell surface must influence the decision as to which cluster of "luxury" molecules the cell will synthesize.

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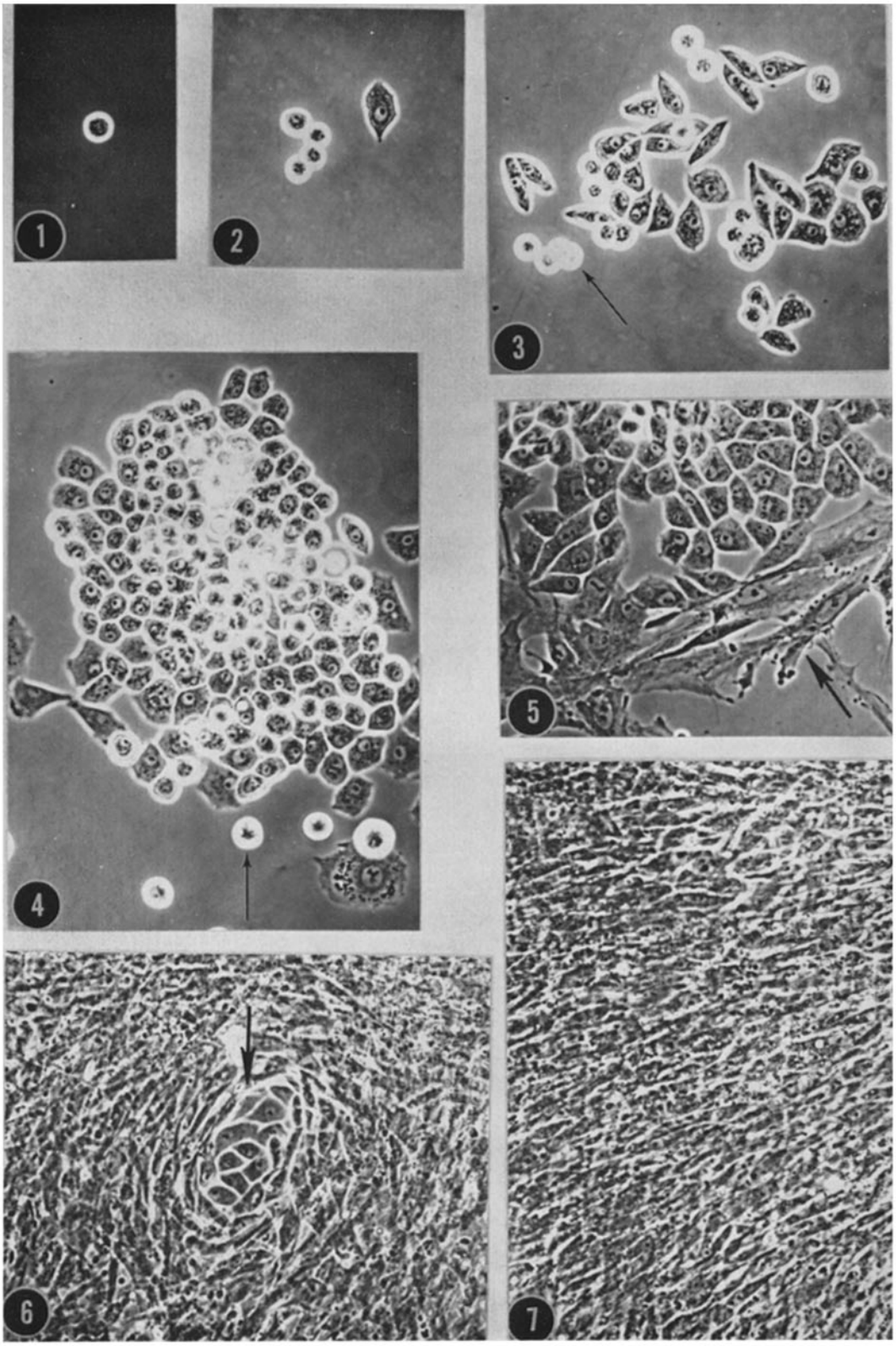
FIGS. 1–7. Low power, phase photomicrographs all at the same magnification of a single living culture established by plating a primary floater in a 60 mm Petri dish. Fig. 1 illustrates the culture after 1 day. Figs. 2–7 were taken when the culture was 4, 7, 11, 16, 20, and 30 days, respectively. Many of the round cells in Figs. 1–4 are but loosely attached to the plastic substrate and are readily dislodged when the cultures are fed. The arrows in Figs. 2 and 4 point to floating cells. The white areas in Fig. 4 are due to clusters of cells extending upward into the medium. These cells are not in the focal plane of the polygonal cells attached to the Petri dish. The arrow in Fig. 5 indicates the area where the cells at the periphery of the colony are transforming into fibroblastic cells. The arrow in Fig. 6 points to the remaining few polygonal cells left in the center of this particular 20 day colony. The center of the colony in Fig. 7 is multilayered and consists almost exclusively of spindle-shaped fibroblastic cells. At this stage the colony does not shed "floaters."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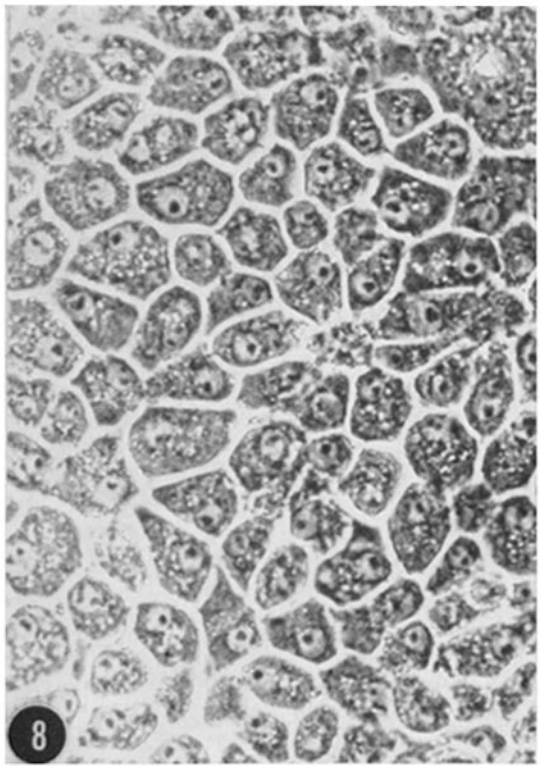
FIGS. 8–11. Intermediate power, phase photomicrographs illustrating the various cell morphologies observed in various regions of a single 15 day colony. Fig. 8 illustrates the epithelioid nature of the center of the colony, whereas Figs. 9–11 illustrate the peripherally distributed fibroblastic cells. Polygonal cells are not amoeboid and do not form long pseudopodial processes. They vary from 5 μ to 10 μ in thickness and form 10 μ to 15 μ in diameter. Observe the pseudopodia, stress fibers and microspikes of the fibroblastic cells. The exceedingly flattened condition of the fibroblastic cells is indicated by the apparent size of their flattened, pancake-shaped nuclei. All the polygonal cells in Fig. 8 will in time transform into fibroblastic cells. Fibroblastic cells may become exceedingly thin and stretched out, their surface areas ranging from 3–10 times that of polygonal cells and their long diameters measuring over 80 μ. Compare the apparent size of the cells in Figs. 8–11, taken at the same magnification, The thickest portion of the fibroblastic cell is about 2 μ measured through the nuclear region, whereas elsewhere the cells are less than 0.5 μ in thickness. Stress fibers (29), never observed in polygonal cells, are common in the cortex of fibroblastic cells.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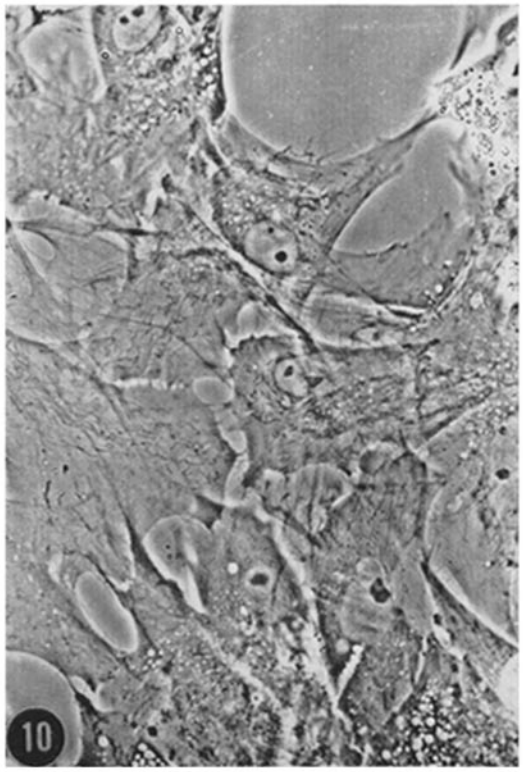
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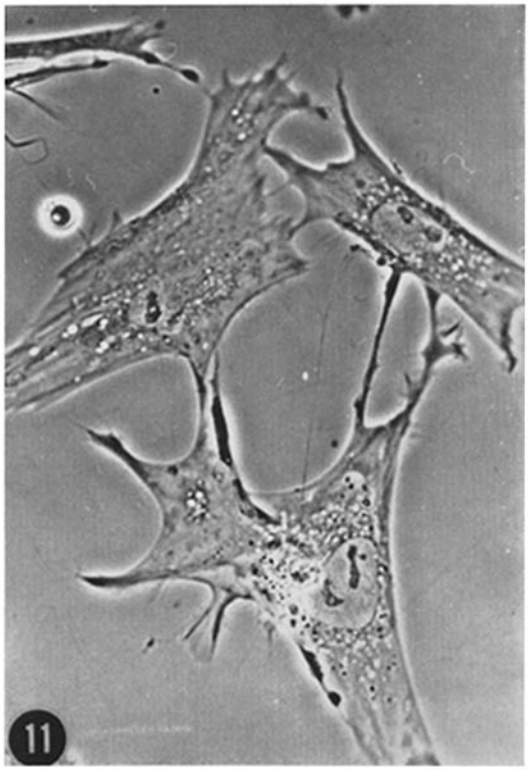
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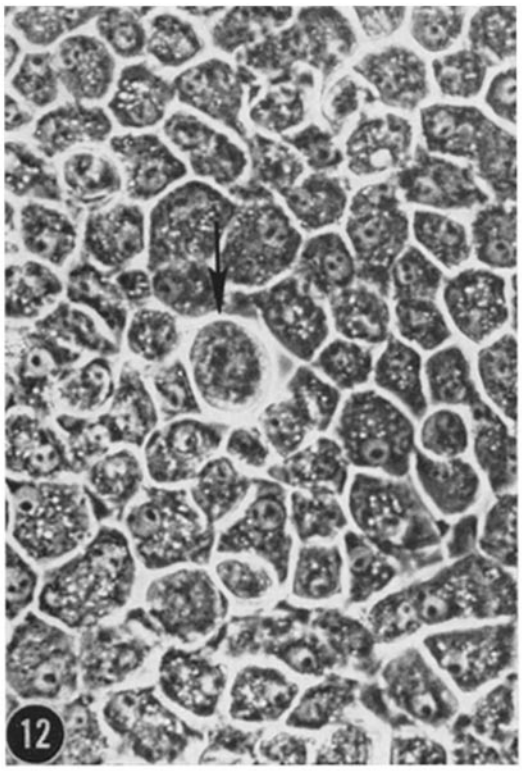
FIGS. 12–15. Phase photomicrographs all taken at the same magnification through the approximate center of four different 16 day cultures. During the 1st wk all these cultures consisted of predominantly polygonal cells. Mitotic figures are observed both in the center and at the periphery of the colony. Arrows in Figs. 12, 13, and 14 point to cells in mitosis. In some cultures the center of the colony becomes multilayered (Figs. 7 and 13); in others, as is illustrated in Figs. 14 and 15, the transformed cells disperse. Associated with this dispersal, there is a loss in the previously deposited metachromatic matrix. The sessile polygonal cells adhere to one another; the transformed fibroblastic cells repel rather than adhere to one another.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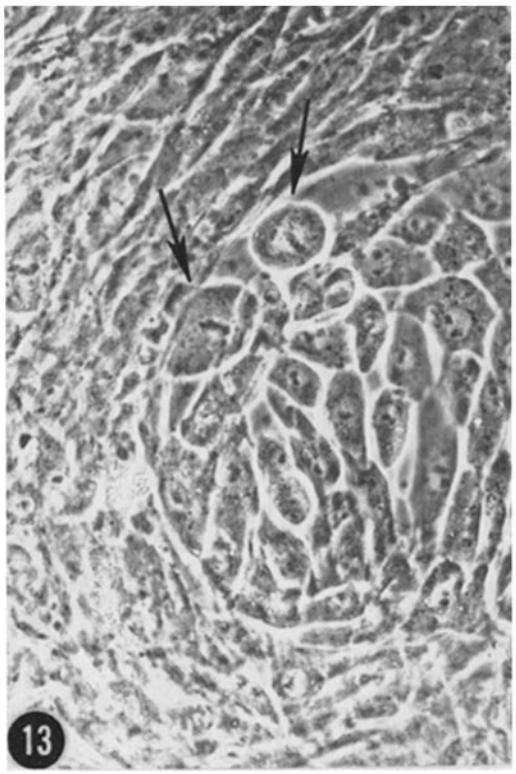
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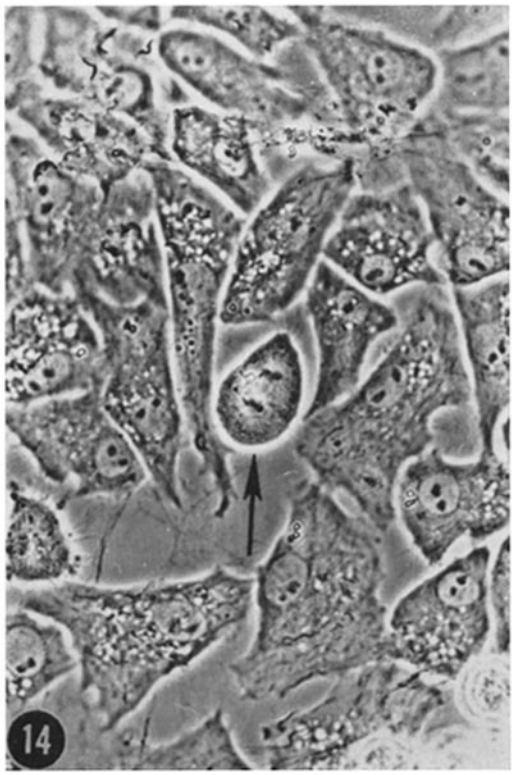
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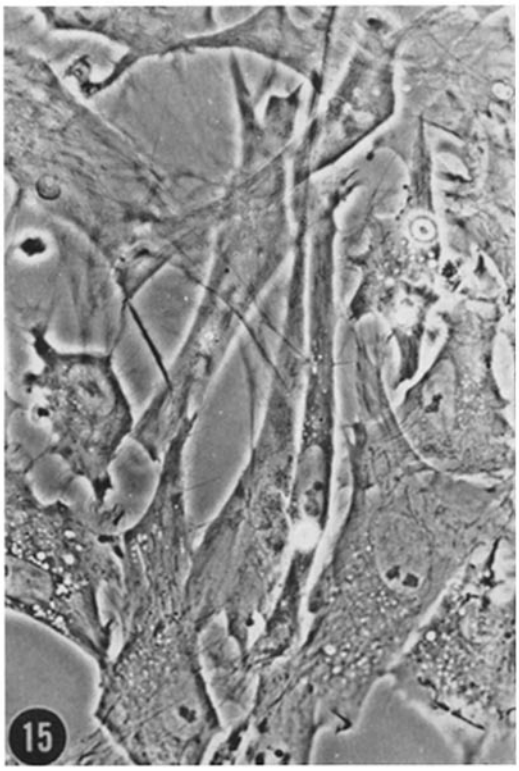
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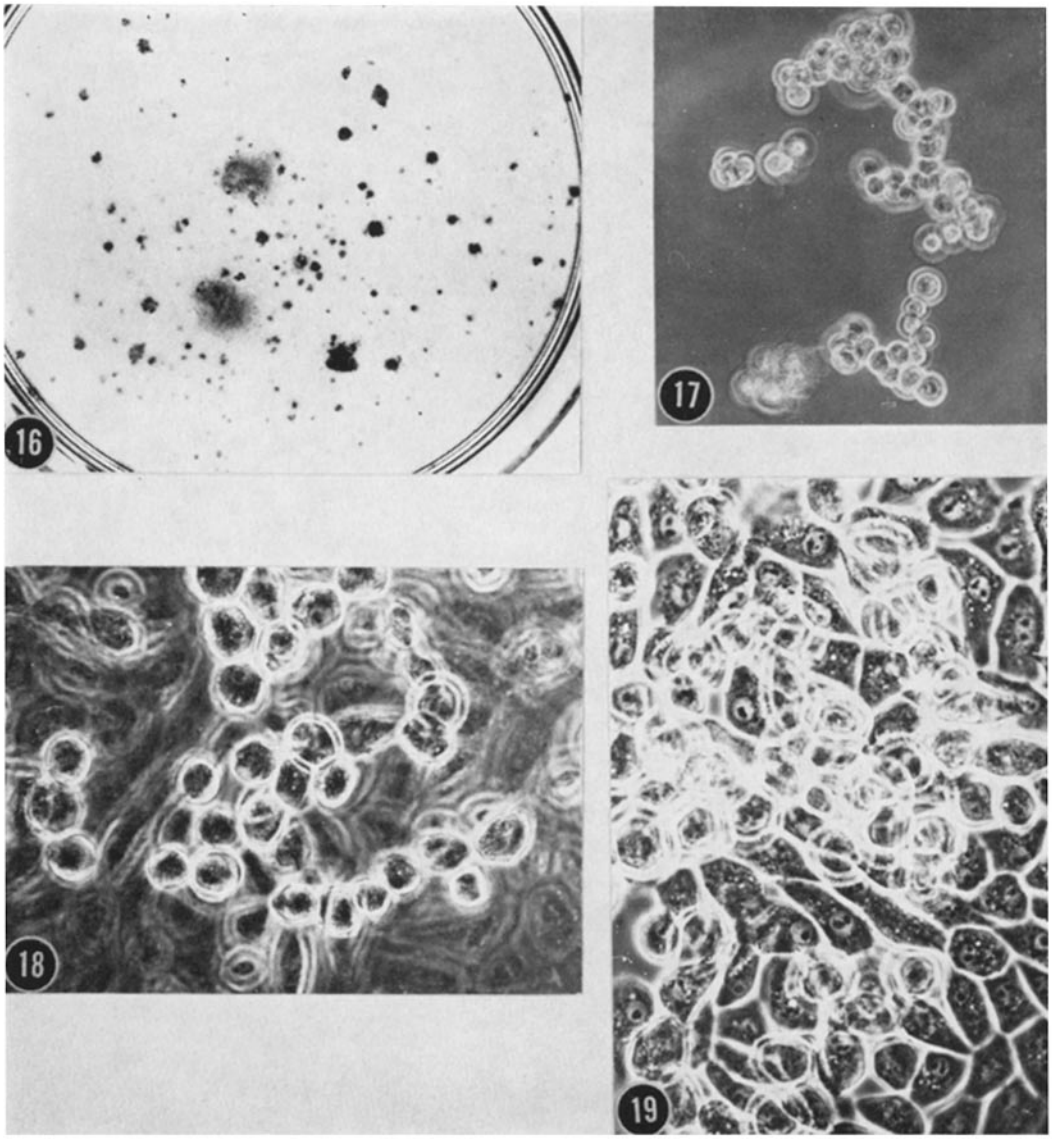

FIG. 16. A very low power photomicrograph of a 32 day culture established by plating a single primary floater cell. The plate has been fixed and stained with toluidine blue. The colonies are very unequal in size and the majority no longer display metachromatic matrix. Large numbers of dispersed fibroblastic cells cover the bottom of the dish between the colonies but cannot be detected at this low magnification.

FIG. 17. A low power, phase photomicrograph of a floating cluster from a 12 day old culture. This cluster of some 50 cells is embedded in a jelly-like matrix which would stain metachromatically. Cells divide in this mass. These floating masses may attach to the plastic substrate at another location and establish a new epithelioid colony which will eventually transform into a group of fibroblastic cells.

FIGS. 18 and 19. Intermediate power, phase photomicrographs of the same 11 day old culture to illustrate the upward growth of the vertically displaced polygonal cells. The cells in focus in Fig. 18 are 60 μ above the parent epithelioid layer. In Fig. 19 both vertically displaced cells and the epithelioid cells to which they are anchored may be observed.