

Establishment and Transformation Diminish the Ability of Fibroblasts to Contract a Native Collagen Gel

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ABSTRACT Cultures of established and transformed fibroblasts were less able to contract a hydrated collagen gel than normal precrisis cells. Postcrisis fibroblasts from different rodent strains and species underwent a further reduction in contraction ability after either spontaneous or simian virus 40 (SV40) transformation. Human precrisis fibroblasts contracted much more efficiently than two SV40-transformed human lines. Fibroblasts from a patient with Glanzmann's thrombasthenia were intermediate between all other human fibroblasts assayed and the SV40-transformed human lines. The absolute efficiency of contraction was dependent on temperature and serum concentration, but no conditions were found that resulted in equal efficiencies for the three types of cells. Precrisis cells were extremely sensitive to the passage procedures when assayed for collagen contraction.

The *in vitro* culture conditions routinely used to distinguish normal from transformed cells do not precisely reproduce an *in vivo* environment. Fibroblasts are in stromal collagen. Plastic or agar (13, 15), even with the addition of fibronectin (29), is a poor imitation of their *in vivo* situation. Although it is in no way identical to a tissue, a gel of native collagen more nearly duplicates the fibroblast's normal environment than does monolayer culture on a plastic dish. Indeed, Reid and Rojkind (20), Yang et al. (30), and others have reported that the culturing of cells on or in a collagen matrix helps to maintain their differentiated function.

Fibroblasts in the dermis, dispersed in a collagenous matrix, are capable of contraction (7, 8). When wounding occurs, there is a localized proliferation of the fibroblasts followed by a contraction of the tissue to close the wound. Therefore, the ability of fibroblasts to contract collagen fibers may be characteristic of their normal interaction with the environment. Disturbance of this interaction could result in the abnormal tissue repair found in Glanzmann's thrombasthenia (21). Several years ago, Niewiarowski et al. (14) found that fibroblasts could cause the contraction of a fibrin clot *in vitro*. Dolfine et al. (4) reported that the spontaneous transformation *in vitro* of human fibroblasts resulted in a partial loss of this retractability. More recently, it has been reported by Bell et al. (1) that human fibroblasts are capable of contracting a hydrated collagen gel.

We have assayed the ability of a large number of different cells to contract collagen, as an indication of their capacity to interact with their normal environment. We find that establishment leads to diminished capacity and that tumorigenic lines are even more deficient in this capacity.

MATERIALS AND METHODS

Cells and Culture

A large number of cells from various sources were used. Established cells have been previously described (17, 25, 26). Human precrisis cells were received from Dr. M. Lipkin at Sloan-Kettering Cancer Institute, New York and Dr. E. Pearlstein, New York University Medical Center, New York. SV80 and NG8 are SV40-transformed human cell lines obtained from Dr. S. Shin, Albert Einstein College of Medicine, New York. The SV40 dl 884 transformants 8842A, 3A, and 7A were received from Dr. W. Topp, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. Lines AD and AA were received from Dr. S. Chen (Columbia University). All human and rat cells had been grown on plastic dishes in Dulbecco's modified Eagle's medium (DME) (Grand Island Biological Co., Grand Island, N. Y. [GIBCO] H21) and 10% fetal calf serum (Reheis Co., Inc., Phoenix, Ariz.) before assay. Mouse cells had been grown in DME and 10% calf serum (GIBCO). These same sera were used for collagen gels. Culture conditions and procedures for passaging cells have been described (26). "3T3" and "3T6" passage protocols were essentially as described (27) for precrisis mouse embryo fibroblasts.

Collagen

Partially purified collagen solution was prepared from rat tail tendon bundles according to the procedure of Bell et al. (1). Determination of protein concentration was by the method of Lowry et al. (12), using purified collagen (Collagen Corp.) as a standard. Final collagen concentration in all gels was 150 $\mu\text{g}/\text{ml}$.

Preparation of Gels

Medium and collagen were mixed with a counted number of cells in a small tube and 0.5 ml of the mixture was immediately plated in duplicate in 16-mm tissue culture wells (Linbro Scientific, Hamden, Conn.) that had been precoated with a film of 0.66% agarose to prevent cell attachment to the plastic. Each tube contained 0.4 ml of cells in DME plus 10% serum, 0.26 ml of 2 \times DME plus 20%

serum, 0.09 ml of 0.1 N NaOH, and 0.3 ml (500 $\mu\text{g}/\text{ml}$) of collagen solution. After plating, the gels were incubated at 37°C in a 90% air, 10% CO₂ atmosphere. Solutions containing collagen formed a gel within 10 min of plating.

Measurement of Gel Size

Gel contraction did not occur in the absence of cells. Most contraction occurred within 24 h when high concentrations of cells were used. Diameters of contracted gels were routinely measured at 24 h after plating to minimize any difference in cell number that might result from differences in growth rates in collagen of the various cell types. Measurements were done on a Wild M3 dissecting microscope, using darkfield illumination. This procedure makes it very easy to visualize the edge of a partially contracted gel. Contraction occurred more rapidly as the cell number was increased. Contraction ability was thus defined as the number of cells required to reduce the gel to one-half its original surface area 24 h after plating ($NA_{1/2}$).

RESULTS

Collagen Contraction by Different Cell Types

Fig. 1 shows the appearance of a completely contracted collagen gel in a 16-mm well. Uncontracted gels fill the wells in the form of a thin disk. The gel floats in liquid medium released from the gel during contraction. Faint vertical lines at the top of the well are 1-mm delineations from a ruler placed under the well (see Materials and Methods).

Fig. 2 shows the amount of contraction of a collagen gel as a function of the number of cells present per gel. Three different types of cells were used: normal precrisis mouse embryo fibroblasts (MEF), normal established mouse cells (3T3), and SV40-transformed mouse cells (SV101). The solid lines reflect the mean values from at least five separate experiments. Noncontracted gels had a surface area of 200 mm² compared with 5 mm² for fully contracted gels. Bars indicate the SD in number of cells for each cell type at the value $NA_{1/2}$, where the gel is 100 mm².

All three cell types were capable of causing contraction, but

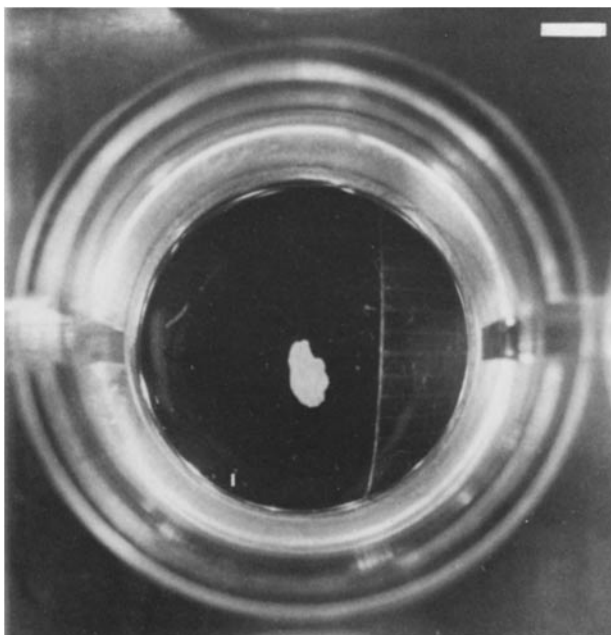


FIGURE 1 Appearance of fully contracted collagen gel. 3T3 was plated at 10^6 cells/well. The photograph was taken 24 h after plating with low power on a Wild M3 dissecting microscope and dark-field illumination. Final magnification, $\times 3$. Bar, 3 mm.

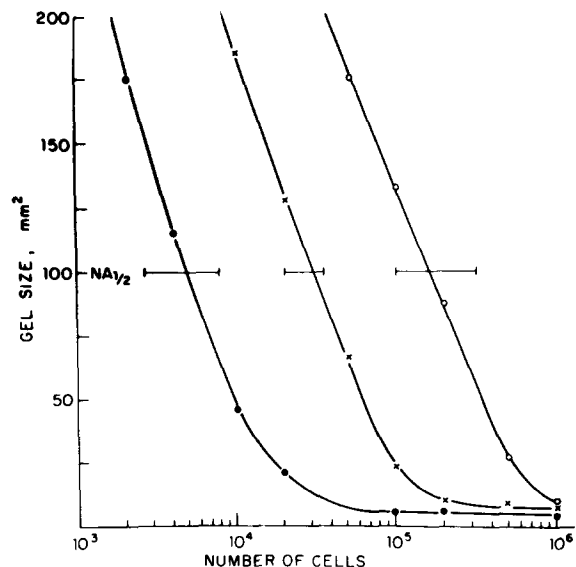


FIGURE 2 Relationship between contraction of collagen gels and the number of precrisis, established, and transformed fibroblasts plated in the gels. Gel diameters were measured 24 h after plating (see Materials and Methods) and values were converted to areas. Uncontracted gels have a surface area of 200 mm². The $NA_{1/2}$ is the number of cells required to contract the gel to 100 mm², one-half the original area. Bars indicate the SD for each cell type at $NA_{1/2}$. ●, precrisis mouse embryo fibroblasts (MEF Swiss); ×, established mouse fibroblasts (3T3 Swiss); ○, SV40-transformed fibroblasts (SV101 Swiss).

the number of cells required was very different. The transformed cells, SV101, were extremely inefficient when compared with the precrisis MEF. The number of cells necessary for contraction to half the area in 1 d ($NA_{1/2}$) for MEF was 5×10^3 , whereas for SV101 it was 1.8×10^5 . 3T3 was intermediate, with an $NA_{1/2}$ of 3×10^4 . There was no overlap in the range of values for the three types of cells. The transitions from precrisis to established to transformed cell apparently result in sequential reductions in the ability to contract collagen gels.

The $NA_{1/2}$ for a large number of other rodent cells was determined to ascertain the generality of these differences. These results are shown in Table I. All of the precrisis cells had $NA_{1/2}$ values of 3–10 $\times 10^3$, with a mean of 6.0×10^3 . Thus, the $NA_{1/2}$ of the Swiss MEF (Fig. 2) was characteristic of precrisis rodent cells.

The mean $NA_{1/2}$ for established cells was 58×10^3 , 10-fold higher than that of the precrisis cells. Therefore, as a group, established cells are clearly deficient, as compared with precrisis cells, in their ability to contract a collagen gel.

Spontaneously transformed cells had a mean $NA_{1/2}$ of 150×10^3 , whereas the SV40-transformed cells had a mean $NA_{1/2}$ of 160×10^3 . These two groups of cells, with the same ability to contract a collagen gel, are significantly less efficient than established normal cells, extending our initial observations with MEF, 3T3, and SV101 (Fig. 2).

Two other groups of cells are shown in Table I. The small t of SV40 is eliminated by deleting the region 0.54–0.59 map units. Such deletions do not effect the size of the SV40 large T antigen (23). Four different lines established by the transformation of REF with the SV40 0.54–0.59 deletion mutant dl 884 (23) had an $NA_{1/2}$ similar to that of normal established cells rather than wild type SV40 transformants. This is in keeping with other reports that dl 884 transformants ten-¹ to

TABLE I
Ability of Rodent Fibroblasts to Contract Collagen Gels

	Cells	Species	Strain	Tissue or parent cell	NA _{1/2} × 10 ^{-3*}	Mean ± SD‡
Precrisis	REF	Rat	Fisher	Embryo	5	6.0 ± 3.0
	MEF	Mouse	Balb/c	Embryo	3	
	MEF	Mouse	Swiss	Embryo	5	
	DBA	Mouse	DBA	Dermis	3	
	NIH	Mouse	NIH	Dermis	10	
	C58	Mouse	C58	Dermis	5	
	AKR	Mouse	AKR	Dermis	10	
Established	Rat-1	Rat	Fisher	Embryo	70	58 ± 28
	3Y1	Rat	Fisher	Embryo	100	
	3T3	Mouse	Balb/c	Embryo	40	
	3T3	Mouse	NIH	Embryo	50	
	3T3	Mouse	Swiss	Embryo	30	
Spontaneously Transformed	AD	Mouse	DBA	DBA	200	150 ± 70.7
	AA	Mouse	AKR	AKR	50	
	RIM	Rat	Fisher	Rat-1	200	
	MCA	Rat		FL ¹ 1-4	200	
	3T12	Mouse	Swiss	MEF	100	
SV40 wt transformed	14B	Rat	Fisher	Rat-1	100	160 ± 53
	WTA	Rat	Fisher	REF	200	
	SV101	Mouse	Swiss	3T3	180	
SV40 dl884 transformed	884B	Rat	Fisher	REF	30	52 ± 22
	884.2A	Rat	Fisher	REF	80	
	884.3A	Rat	Fisher	REF	40	
	884.7A	Rat	Fisher	REF	60	
Revertants	FL ¹ 1-4	Rat	Fisher	14B	600	600 ± 326
	FL ¹ 3-8	Rat	Fisher	14B	<1,000	
	FL SV	Mouse	Swiss	SV101	200	

* Mean number of cells required to reduce gel surface area to one-half, as measured in Fig. 1.

‡ Mean NA_{1/2} and SD for each group of cells.

exhibit a phenotype of partial transformation (3, 24). Certain T-antigen negative revertants of SV40-transformed rat cells have nearly normal growth control as assayed by saturation density, serum requirement, and anchorage independence (22, 26). These cells did not, however, revert to a high efficiency of collagen contraction but rather had an NA_{1/2} of 600 × 10³, similar to that of their fully transformed parent.

Human Skin Fibroblasts

Two SV40-transformed human cell lines were compared with precrisis fibroblasts from either normal individuals or persons with various inherited diseases of cellular growth control (Table II). Cultures 265A and 285 were from individuals with a parent carrying the autosomal dominant gene for adenopolyposis of the colon and rectum. The individuals were asymptomatic at the time of the skin biopsy and their cells were grouped with normal cells, but they have a 50% probability of developing the cancer. As a group, the normal precrisis cells varied more than did the rodent cells, as illustrated in Table I. There was no significant difference among cells from normal, asymptomatic, and symptomatic patients, with one exception. Cells from the patient with Glanzmann's thrombasthenia had an anomalously high NA_{1/2}. This reduced efficiency may be of significance because Glanzmann's cells are unable to cause contraction of a fibrin clot (5), and persons with this disease exhibit abnormal tissue repair (21). SV40-transformed

human cells had an NA_{1/2} significantly higher than that of any of the precrisis cells and comparable to that of SV40-transformed rodent cells.

Relationship Between Establishment and Contraction of Gel

The clear difference in NA_{1/2} between precrisis and postcrisis normal rodent cells (Table I) revealed a change in the cell population's ability to interact with collagen sometime during the passage procedure leading to establishment. In mouse cells, crisis and establishment do not occur before passage 15. Table III shows the effect of passage protocol and passage number on collagen contraction. Cells passaged by the "3T3" protocol (17) are never allowed to become confluent. Their NA_{1/2} was that of precrisis cells at passage 3 and 6, as expected. At passage 15, when these cells had become established, their NA_{1/2} was typical of an established cell line.

When precrisis cells were passaged by a 3T12 (17) protocol, they were always confluent. Under these conditions, there was a significant increase in the NA_{1/2} by the third passage, although these cells did not become established until passage 19. This change in NA_{1/2} was progressive with passage number. Apparently, even a short period of time in contact with other cells reduced the ability of precrisis cells to contract collagen. These data may explain some of the variability seen earlier

TABLE II
Ability of Human Fibroblasts to Contract Collagen Gels

	Cells	Tissue	Disease state	NA _{1/2} × 10 ⁻³ *	Mean ± SD‡
Precrisis	1379	Lung	Normal	9	
	1380	Lung	Normal	20	
	1381	Skin	Normal	10	
	189	Skin	Normal	5	
	301	Skin	Normal	2	
	167	Skin	Normal	7	
	265A	Skin	Adenopolyposis, asymptomatic	1	
	285	Skin	Adenopolyposis, asymptomatic	2	7.0 ± 6.1
Precrisis	CF	Skin	Cystic fibrosis	3	
	LSN	Skin	Lesch nyhan syndrome	5	
	166	Skin	Adenopolyposis	20	
	291	Skin	Adenopolyposis,	2	7.5 ± 7.9
	GT	Skin	Glanzmann's thrombas- thenia	50	—
SV40 trans- formed	SV80	Skin		200	
	NG8	Skin		200	200

* Mean number of cells required for gel reduction, as determined in Fig. 1.
‡ Mean NA_{1/2} value and SD for each group of cells.

TABLE III
The Relationship Between Establishment of MEF and Collagen Gel Contraction

Type of passage*	Passage number‡	NA _{1/2} × 10 ⁻³
3T3-like	3	6
	6	4
	15	30§
3T12-like	3	10
	9	20§
	15	30§

* Number of passages before cells were used for assay. All cells were assayed at one time in the same experiment.

‡ Passaged every 3 d at 3 × 10⁶ cells/60-mm dish for 3T3, passaged every 3 d at 12 × 10⁶/60-mm dish for 3T12.

§ Significantly different from precrisis cells, *P* < 0.001.

|| Significantly different from early passage "3T3" *P* < 0.002.

with precrisis cells (Tables I and II) because in small areas of the dishes cells became confluent during routine culture.

Effects of Culture Conditions on Collagen Contraction

We chose MEF (Swiss), 3T3 (Swiss), and SV101 (Swiss) cells as a set of related mouse cells to investigate the effects of changing culture conditions. The effects of temperature are shown in Table IV. With all three types of cells, reducing the temperature to 22°C decreased the efficiency of contraction. Raising the temperature to 39.5°C enhanced the contraction efficiency of the MEF and 3T3, but did not affect SV101. 3T3 cells were much more susceptible to increased temperature than to decreased temperature.

Varying the concentration of serum in the collagen gel also affected contraction (Table V). All three lines showed an increase in efficiency (lower NA_{1/2}) when plated in 15% serum and a marked decrease in efficiency when the serum contraction was lowered. This response parallels the growth response of these cells. However, the extent of variation was different for the three types of cells. MEF showed a 10-fold range in NA_{1/2}, the smallest deviation seen, with a continuous increase in NA_{1/2} as the serum concentration was lowered. The 3T3 cells showed the greatest range of NA_{1/2} values, 100-fold. At a concentration of 15% serum they were able to contract the gel and MEF, but at 0.1% serum they were 12-fold less efficient than MEF. Even 15% serum was not sufficient for SV101 to contract the gel at a level comparable to MEF but did reduce the NA_{1/2} to that seen for normal established cells in 10% serum (see Table I). At 0.1% serum, the NA_{1/2} of SV101 was one-thirtieth that seen with 15% serum. At no concentration of serum was the difference between normal and transformed cells abolished.

DISCUSSION

We have found clear differences in the abilities of various types of fibroblasts to interact with and contract a hydrated collagen gel. These distinctions are not a function of species. Rather, they appear to reflect primary differences among precrisis, established, and transformed cells. We generally consider established cell lines such as 3T3 and Rat-1 to be "normal" because by many criteria they are comparable to primary cells (22). However, by the criterion of collagen contraction they are clearly not normal, because their NA_{1/2} is intermediate between that of precrisis cells and that of fully transformed cells.

Human fibroblasts also appeared to fall into three classes. In the first class, normal human fibroblasts and fibroblasts from several people with unrelated inherited diseases had a relatively low NA_{1/2}. In the second class, the Glanzmann's thrombasthenia cells had an NA_{1/2} intermediate between that of normal

TABLE IV
Effect of Temperature on Collagen Gel Contraction

Cells	Relative contraction efficiency*			
	22°C	33°C	37°C	39.5°C
MEF	0.12 ± 0.4	0.45 ± 0.2	1.0	3.0 ± 1.0
3T3	0.50 ± 0.05	1.10 ± 0.01	1.0	12.2 ± 3.1
SV101	0.12 ± 0.04	0.20 ± 0.08	1.0	1.0 ± 0.01

* Calculated as NA_{1/2} 37°C/NA_{1/2} experimental temperature. NA_{1/2} were determined from titration curves as seen in Fig. 1. The higher the number, the more efficient the contraction.

TABLE V
Effect of Serum Concentration on Gel Contraction

Cells	NA _{1/2} × 10 ⁻³ in calf serum*				
	0.1	1.0	5	10	15
	%				
MEF	30	15	10	6	3.6
3T3	380	140	16	25	3.4
SV101	1500	700	100	100	50

* Cells were titrated in collagen gels containing each concentration of serum to determine NA_{1/2}, as shown in Fig. 1. A low NA_{1/2} reflects a high efficiency of contraction.

† Standard conditions in other experiments.

and that of transformed cells. This disease is marked by a poor ability to effect wound healing (21). Our results suggest the possibility that fibroblasts from such persons are defective in their interaction with collagen. In the third class, the two SV40-transformed human lines were very inefficient at contracting collagen, just as the SV40 transformed rodent cells were.

Whereas the intrinsic differences among the normal, established, and transformed cells resulting in the altered abilities to contract collagen must be determined, we consider the following to be reasonable contributing factors: (a) Cells differ in their distribution of actin. Transformed cells have fewer actin cables (18). Changes in actin patterns could alter the tension on the substrate created by fibroblasts (11). There are also small but reproducible differences in the size and distribution of actin cables between precrisis and postcrisis cells (28). (b) Extracellular protease produced by transformed cells (16) could reduce the affinity of the cells for their collagen substrate. It could also alter the actin cables and the morphology of the fibroblasts, as do exogenously added proteases (19, 31). However, preliminary mixing experiments with transformed and precrisis cells suggest that the $NA_{1/2}$ are approximately additive. No trans-active inhibition of contraction was detected (data not shown). (c) Alteration or loss of collagen-binding proteins on the surfaces of the cells could also affect contraction. Interaction between the cell surface and collagen or collagen-glycopeptide complexes has been previously reported (6, 9, 10).

Because MEF, 3T3, and SV101 cells responded in a similar though not identical manner to changes in culture conditions, we suggest that these culture conditions do not affect the inherent ability of a given class of cell to interact with and contract its collagen substrate. Changes in temperature or serum concentration modify this interaction but do not abolish the intrinsic differences among the three types of cells. Not only did cells demonstrate an altered $NA_{1/2}$ after undergoing the transition to established and transformed states, but even a short period of time in culture when cell-cell contacts and crowding occurred affected their subsequent interaction with the collagen gel. This is especially significant when one considers the report by Boone et al. (2) that even a few passages on plastic enhance the potential tumorigenicity of cultured fibroblasts. Apparently, the way in which precrisis cells are passaged may affect their normality, even after a few passages.

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