Journal of Supramolecular Structure
and Cellular Biochemistry 15:29–40 (1981)
Control of Cellular Division and Development: Part A: 353–364

Interaction of Serum and Cell Spreading Affects the Growth of Neoplastic and Non-Neoplastic Fibroblasts

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Both growth factor availability and cell-to-cell contact have been mechanisms used to explain cell growth regulation at high cell density. Recently Folkman and colleagues have shown that changes in cell shape, rather than cell-to-cell contact, can regulate the growth of fibroblasts. However, in those studies the relation between serum and shape regulation of growth was not studied, nor were neoplastic and non-neoplastic cells compared. In this report we have studied these aspects by varying cell spreading and serum concentration independently for 2 non-neoplastic and 3 neoplastic cell lines. Cell spreading (projected cell area) was controlled by decreasing the adhesiveness of tissue culture plastic plates with poly (hydroxyethyl methacrylate) [poly (HEMA)]. Cell growth was measured as the increase in cell number/day. We have found that more spreading increased net growth of both neoplastic and non-neoplastic cells, while less spreading (toward rounded configuration) depressed growth. There were also quantitative differences between neoplastic and non-neoplastic cells. Neoplastic cells continued to grow under conditions of cell rounding, which completely prevented the growth of their non-neoplastic counterparts. Some neoplastic cells also tended to show little or no increase in net cell number for serum concentrations above 10% as cells became more spread; in contrast, all non-neoplastic cells grew more with increasing concentrations of serum as they became well spread. Thus, in normal cells, it appears that the sensitivity of cells to humoral factors is governed by cell spreading. This interaction between serum and cell shape is less prominent in some neoplastic cells.

Key words: **serum, neoplastic, non-neoplastic, growth, cell area, cell spreading**

It is well established that non-neoplastic fibroblasts stop growing at high cell density. Both humoral and cell contact mechanisms have been suggested to explain this phenomenon. For example, it has been proposed that cell crowding decreased the amount of serum available in the microenvironment of each cell [1–5]. Other studies suggested that contact between cells was itself sufficient to slow cell growth [6–9]. The fact that this effect of cell contact could be overcome by adding either serum [9] or pure mitogenic factors [10] suggested to some investigators that humoral mechanisms were the dominant factors.

Received June 2, 1980; accepted October 8, 1980.

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More recently, the implication that humoral and cell contact are competing factors has been questioned, as has the nature of the signals in cell contact. In fact, recent work [11] has shown that cell contact prevents growth simply by preventing cell spreading. Thus, sparsely plated cells held in the same rounded shape as crowded cells showed the same decreased growth rate. Similarly, Westermark [6] observed that restricted cell spreading on haptotatic palladium islands inhibited cell growth in the absence of cell-to-cell contact. Other workers have concentrated on the interaction between growth factors and cell shape. Using a combination of in vivo and in vitro studies on corneal cells, Gospodarowicz et al [12] showed that 2 different cell shapes had a profound influence on the cell's sensitivity to 2 different mitogens. O'Neill et al [1] recently demonstrated the importance of cell shape or cell spreading in the growth of hamster fibroblasts in vitro under conditions in which serum but not substratum conditions were varied. Thus, there are a number of studies demonstrating the importance of both cell shape and growth factors, but the description of the interaction of cell spreading and humoral factors is incomplete. No one study has examined the growth effect of serum and cell shape when they were varied independently.

In the present study, we have changed cell spreading and serum *independently* for both neoplastic and non-neoplastic cells. Cell spreading was varied by reducing the adhesiveness of tissue culture plastic with coatings of different concentrations of poly (hydroxyethyl methacrylate) [poly (HEMA)]. Cells were kept sparse, so that cell-to-cell contact was not a significant factor contributing to growth. We have found that over a wide range of different amounts of cell spreading (projected cell area) and serum concentrations, the growth of 3 non-neoplastic rodent fibroblasts was more sensitive to serum when the cells were extensively spread. Unspread (rounded) non-neoplastic cells were realtively resistant to growth stimulation by serum. In contrast, some neoplastic cells showed little change to serum above 10% as cells became more spread. Neoplastic cells also continued to grow under conditions of cell rounding and low serum concentration, which prevented the growth of non-neoplastic cells.

MATERIALS AND METHODS

Cell Cultures

3T3 and SV40-transformed 3T3 cells were both cultured in Dulbecco's modified Eagle's medium (DME, GIBCO Laboratories) supplemented with 10% calf serum (CS; GIBCO Laboratories). 3T3 cells, clone A-31, were obtained from C. Stiles, and SV40-transformed 3T3 cells were obtained from C. Scher. New ampules of 3T3 cells were thawed every 4 to 6 weeks to ensure that cells were growth-inhibited at high cell density.

The non-neoplastic cell line, CHEF 18-1, and the neoplastic line, CHEF 16-2, were established from the same Chinese hamster embryo [13]. Cells were grown in Alpha-MEM (Kansas City Biological) supplemented with 10% fetal calf serum (Microbiological Associates). New ampules of line 18-1 were thawed every 4 to 6 weeks to ensure that the cells remained density inhibited and nontumorigenic. Both cells lines were obtained from R. Sager and P. Novac.

HT1080, a human fibrosarcoma established from a tumor specimen [14], was grown in DME and 10% CS.

Poly (HEMA) Coating of Plastic

Plastic plates were coated with films of poly (2-hydroxyethyl methacrylate) [poly (HEMA)] to change the spreading of cells as previously described [11]. Poly (HEMA), obtained as a purified powder from Hydron Laboratories (New Brunswick, NJ), was dissolved in 95% ethanol to make a 12% stock solution (w/v). The stock solution was diluted from 10^{-1} to 10^{-4} with 95% ethanol. Various dilutions of the alcoholic solution of poly (HEMA) were distributed into Falcon 24 well multiwell culture plates with 200 μl in each well. The plates were allowed to dry in a 37°C warm room for 48 h with the covers in place. A hard, optically clear film remained bonded to the surface of the plate. Two to five $\times 10^4$ cells were then added to each well. Plates coated with the highest concentrations of poly (HEMA) (10^{-1} dilution) were the least adhesive, and cells remained spheroidal and weakly adherent. Plates coated with lower concentrations (10^{-2} to 10^{-4} dilutions) were more adhesive, and cell spreading increased as the poly (HEMA) dilutions increased.

Measurement of Cell Growth

Two to four $\times 10^4$ cells were plated initially into each well so that 3 days growth on plastic substratum was not quite confluent. Adherent cells were removed from the plates with trypsin (GIBCO Laboratories) 1, 2, and 3 days following plating of the cells. The total number of adherent and suspended cells was then counted in a Coulter Counter, and cell growth was expressed as the total number of cells accumulated over a 2- or 3-day period. Cell counts on the previous days were checked to be sure that an increase in the number of cells over 3 days represented progressive cell growth. When cells were clumped in suspension [eg, on 10^{-1} poly (HEMA)], 0.1% collagenase (grade II, Worthington Biochemical Inc.) was added to assist in breaking up the clumps. The incubation with collagenase was continued until gentle mechanical agitation produced a single cell suspension. Cell counts were done in triplicate and expressed as the mean ± SD.

Growth rates (ie, population doubling) from day 1 to day 3 were measured by dividing final cell number on day 3 by the initial cell number on day 1 after plating cells. Ratio of > 1 indicates net growth.

Measurement of Cell Spreading

The projected cell area for each individual cell was measured from the cell outline in 35 mm pictures. At 24 h after plating cells were photographed through an inverted Nikon phase microscope, and the developed film was projected onto a digital image analyzer (ZEISS MOP-3) on which the cell area was directly measured. The average cell area ± SEM was obtained for 40 cells for each combination of serum and substrata.

RESULTS

Well Spread Non-Neoplastic Cells on More Adhesive Substrata Were More Sensitive to Serum

As Figures 1A and 2A show, non-neoplastic cell lines 3T3 and 18-1 grew more (larger cell number on day 3) as serum was increased from 0.5% to 30%. Moreover, on the more adhesive surfaces [plastic, 10^{-3} poly (HEMA)], growth rate (fold increase) was increased compared to that on the less adhesive surfaces [10^{-2} and 10^{-1} poly (HEMA)]

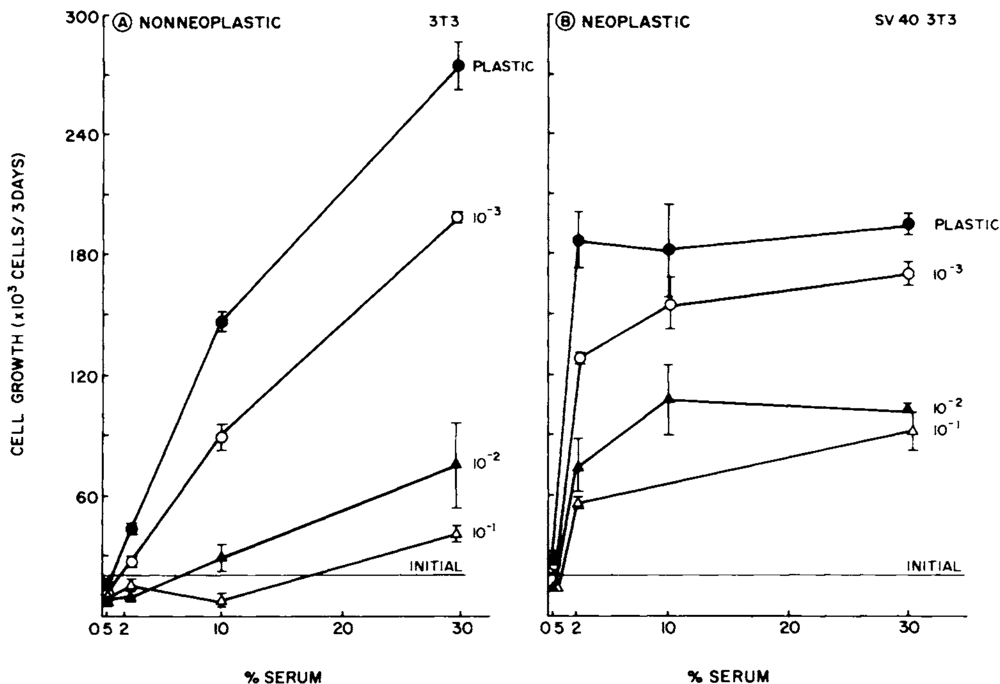

Fig. 1. Adhesiveness of the substratum changes of cells. 3T3 (A) and SV40-transformed 3T3 (B) cells were plated at same initial density (2×10^4/well) then counted daily. Total number of cells/well after 3 days of growth is plotted vs percent serum concentration in medium for different poly (HEMA) concentration. Note increasing slope of growth vs serum lines for 3T3 cells (A) as substrata were made more adhesive. In contrast, SV40-transformed 3T3 cells (B) showed little change in slope above 2% serum supplementation. Data plotted are mean ± SD. Symbols ● = for plastic; ○ = 10^{-3} poly (HEMA); ▲ = 10^{-2} poly (HEMA); △ = 10^{-1} poly (HEMA).

(see Table I). The increased cell number on day 3, therefore, primarily reflects changes in growth rate and not plating efficiency. We conclude that on the more adhesive surfaces, non-neoplastic cells were more sensitive to growth stimulation by serum, grew at a higher rate, and reached a higher final cell number.

Rounded Non-Neoplastic Cells on Poorly Adhesive Substrata Did Not Grow

Figures 1A and 2A also demonstrates that non-neoplastic cells on poorly adhesive substrata [10^{-1} and 10^{-2} poly (HEMA)] did not show net growth in 10% serum or less, and only marginal growth in 30% serum. In these conditions, the number of cells at day 3 was not above the initial number plated, and monitoring of cell counts on days 1 and 2 revealed no transient increase in growth (see Table I). Thus, these non-neoplastic fibroblasts did not grow when prevented from spreading on poorly adhesive substrata, although high serum concentration (30%) could partially overcome this growth inhibition.

Well-Spread Neoplastic Cells on Adhesive Substrata Were More Sensitive to Serum

As Table I shows, neoplastic lines (mouse SV40 3T3, hamster 16-2, and human HT1080) grew faster at any given serum concentration as substratum was made more adhesive. However, unlike non-neoplastic cells, on a given substratum net cell number did

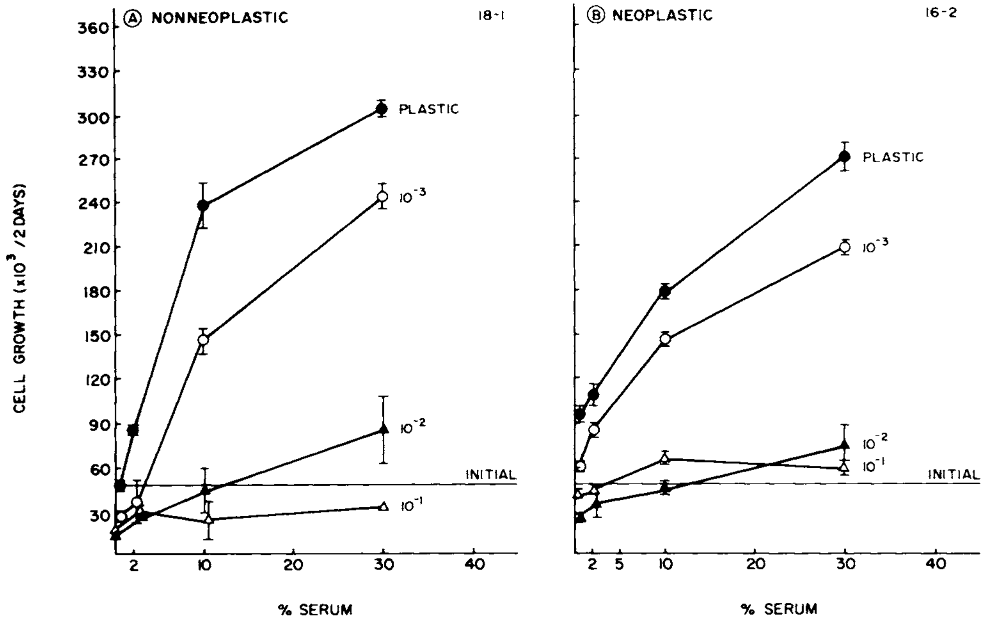

Fig. 2. Experimental details same as Figure 1. Cell lines 18-1, 16-2 were plated at same initial density (5×10^4/well). Note that slopes are similar, but that growth of neoplastic 16-2 is above initial cell number on poorly adhesive substrata (10^{-1}), whereas, that for 18-2 cells is not.

not increase above that stimulated by 2% serum for SV40 3T3 and by 10% for HT1080 (Figs. 1B, 2B, 3). Neoplastic line 16-2 showed an increase in net cell number similar to that of non-neoplastic line 18-1. Thus, neoplastic cell lines showed increased sensitivity to growth stimulation by serum as the adhesiveness of the substratum was increased, but growth for SV40 3T3 was maximal at 2%, and for HT1080 growth was maximal at 10% serum. This increase in sensitivity to growth stimulation by serum is also reflected in the net cell growth of all 3 neoplastic cells in 0.5% serum.

Rounded Neoplastic Cells on Poorly Adhesive Substrata Grew

As Figures 1B, 2B, and 3 show, neoplastic lines all grew above the initial number of cells plated on poorly adhesive substrata [10^{-1} and 10^{-2} poly (HEMA)]. This growth occurred even in the presence of low amounts of serum (2%) for cell lines SV40 3T3 and HT1080. This is in contrast to the absence of growth of non-neoplastic cells on 10^{-1} and 10^{-2} poly (HEMA).

Non-Neoplastic and Neoplastic Cell Pairs in Identical Culture Conditions Were Spread to the Same Extent

Despite the marked differences in the effect of substrata on growth, neoplastic and non-neoplastic cells from common origin changed their cell spreading to the same extent as substrata and serum concentrations were changed. As Figures 4 and 5 show, the amount of cell spreading on the different dilutions of poly (HEMA) was remarkably similar for both cell pairs – 3T3 vs SV40-transformed 3T3 cells, and 18-1 vs 16-2. All cell lines showed little change in the amount of spread cell area as serum concentrations changed

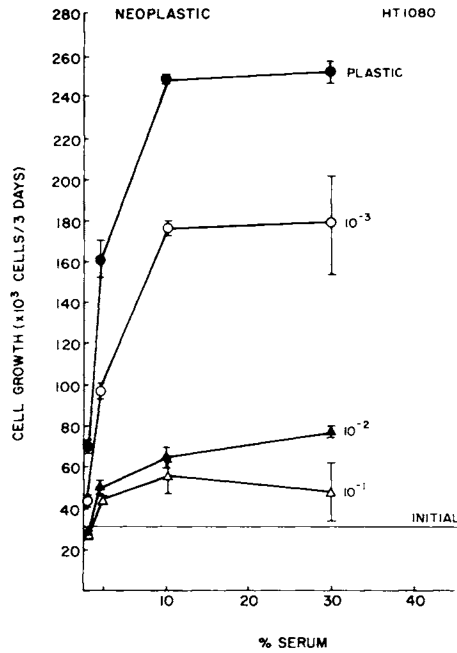

Fig. 3. Experimental details same as Figure 1. Cell line HT1080 was plated at initial density of 3×10^4 cells/well. Note flat curves (no change in cell number) above 10% serum supplementation.

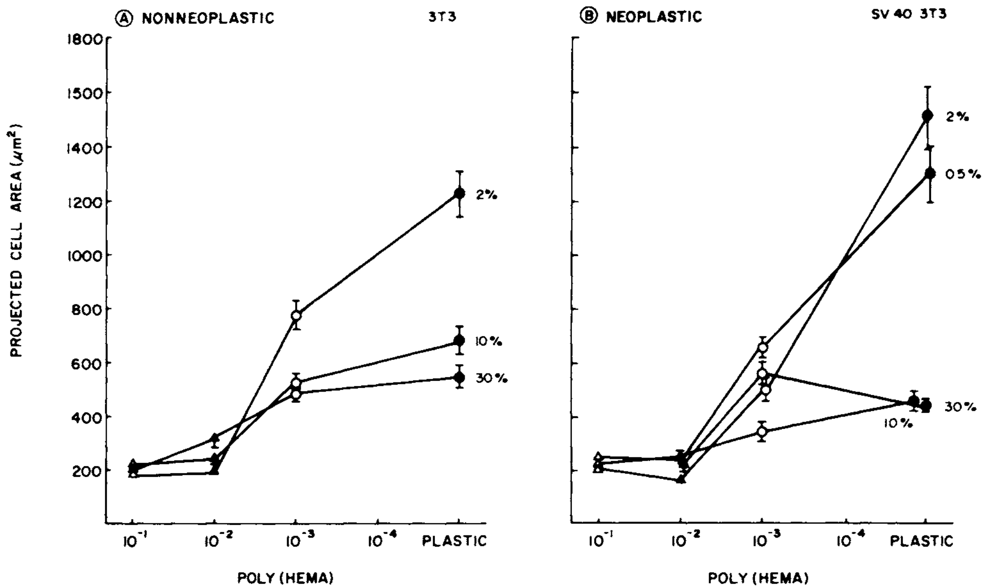

Fig. 4. The amount of cell spreading is different on substrata of different adhesiveness. See Figure 1 for experimental details. Cell area measured of at least 40 cells 24 h after plating. Note both 3T3 (A) and SV40-transformed 3T3 cells (B) showed increased cell spreading (projected cell area) on 10^{-3} poly (HEMA) and plastic with 2% serum supplementation. Data plated are mean ± SEM.

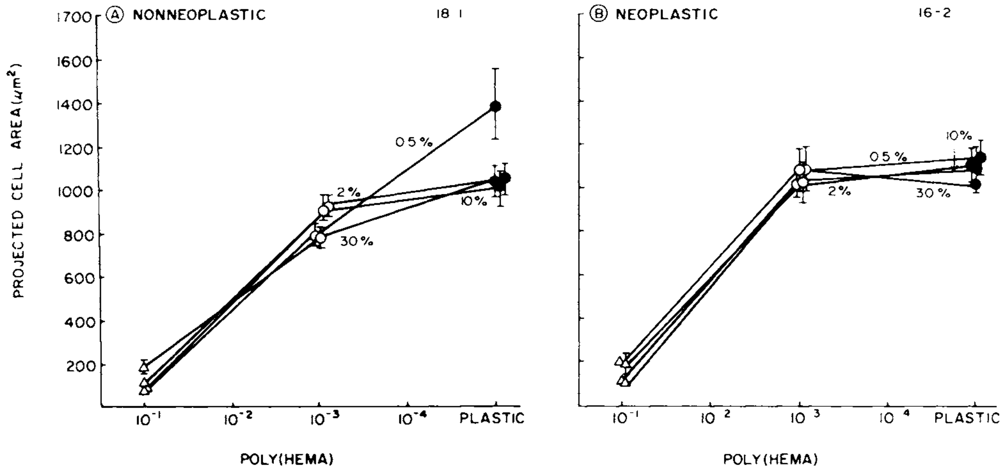

Fig. 5. Experimental details same as Figures 2 and 4. Note very little effect of serum on cell spreading except for 0.5% serum supplementation of 18-1 cells on plastic.

TABLE I. Effect of Serum and Substratum on Growth Rates

Non-neoplastic cell line	Serum (%)	Fold increase on[a] PL, 10^{-3}, 10^{-2}, 10^{-1}	Neoplastic cell line	Fold increase on[a] PL, 10^{-3}, 10^{-2}, 10^{-1}
3T3	30	3.6, 3.6, 1.4, 1.2	SV3T3	2.3, 1.7, 1.4, 1.2
	10	2.5, 1.5, 0.6, 1.0		1.6, 1.2, 1.0, –
	2	1.5, 0.6, 0.3, 0.7		6.0, 4.8, 3.8, 2.7[b]
	0.05	0.4, 0.2, 0.4, 0.5		2.0, 1.4, 1.4, 1.6
18-1	30	4.3, 4.7, 1.1, 0.3	16-2	1.8, 1.6, 0.9, 1.0
	10	5.1, 3.8, 0.8, 0.2		1.0, 1.1, 0.5, 0.7
	2	2.2, 2.9, 0.5, 0.2		1.1 1.1 0.8, 0.6
	0.05	1.8, 1.4, 0.6, 0.4		1.1, 0.8, 0.6, 0.8
	30		HT1080	2.8, 2.7, 2.9, 1.8
	10			5.6, 3.8, 1.5, 1.0
	2			2.2, 1.8, 1.1, 0.9
	0.5			1.5, 1.2, 1.2, 0.8

[a]Fold increase is net number of cells on day 3 divided by net number of cells on day 1 of growth.
[b]High values of fold increases in 2% serum reflect the high sensitivity of these cells to serum, so that growth was maximal in 2%, and in 10% and 30%, much of final growth had already occurred by day 1 after plating.

until poly (HEMA) was 10^{-3} diluted or 0 (plastic). Moreover, the amount of cell spreading was similar for cell line pairs at each combination of poly (HEMA) and serum; Figures 4A and 5A are virtually superimposable with 4B and 5B, respectively. Figures 4 and 5 also demonstrate that on an adhesive substratum like plastic, both cell lines were more spread in the medium with lower serum concentrations. Thus, both serum concentration and substratum adhesiveness interacted to affect the extent of cell spreading, and this amount of cell spreading was remarkably similar for paired non-neoplastic and neoplastic cell lines. Figure 6 demonstrates the very small changes in spreading by HT1080 in the different serum concentrations.

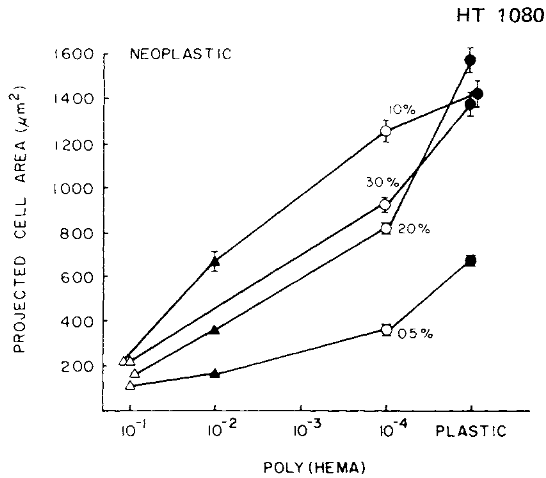

Fig. 6. Experimental details same as Figures 3 and 4. Note that lowered serum did not cause increased cell spreading for this neoplastic cell line, HT1080.

Serum and Cell Spreading Interacted in the Growth Stimulation of Non-Neoplastic Cells

Figures 7A and 8A show that both 3T3 and 18-1 cells were more sensitive to serum as they became more spread (larger projected area). For a given increment in cell area, there was a larger increment in net cell growth at the higher serum concentrations. As we have seen before, these changes in net cell growth for a given serum concentration reflect increased growth rates (see Table I). Thus, increased spreading of non-neoplastic cells increased their sensitivity to growth factors in serum.

Serum and Cell Spreading Were Not Coupled in the Growth Stimulation of Some Neoplastic Cells

Figures 7B and 9 show that neoplastic cells SV403T3 and HT1080 showed little change in the increment of net cell growth for a given change in projected cell area as serum concentration was changed from 10% to 30%. Cell line 16-2 (Fig. 8B) showed some change in the net cell growth as cell area was changed at different serum concentrations, but quantitatively less than its non-neoplastic counterpart (line 18-1 in Fig. 8A). Thus, the relationship between net cell growth and projected cell area for some neoplastic lines showed little or no change as serum concentration was varied. In other words, growth of these neoplastic cells was already maximal at low serum concentrations, so that changes in projected cell area on different substrata affected growth, but changes in serum concentration did not. In this sense, exogenous serum and cell spreading effects on cell growth were not coupled for neoplastic cells as they were for non-neoplastic cells (see Figs. 7A and 8A).

DISCUSSION

For the first time it has been possible to vary serum and cell shape independently and to study their combined effects on cell growth. This study shows that cell spreading and serum effects on growth are much more tightly coupled in 2 non-neoplastic cell lines

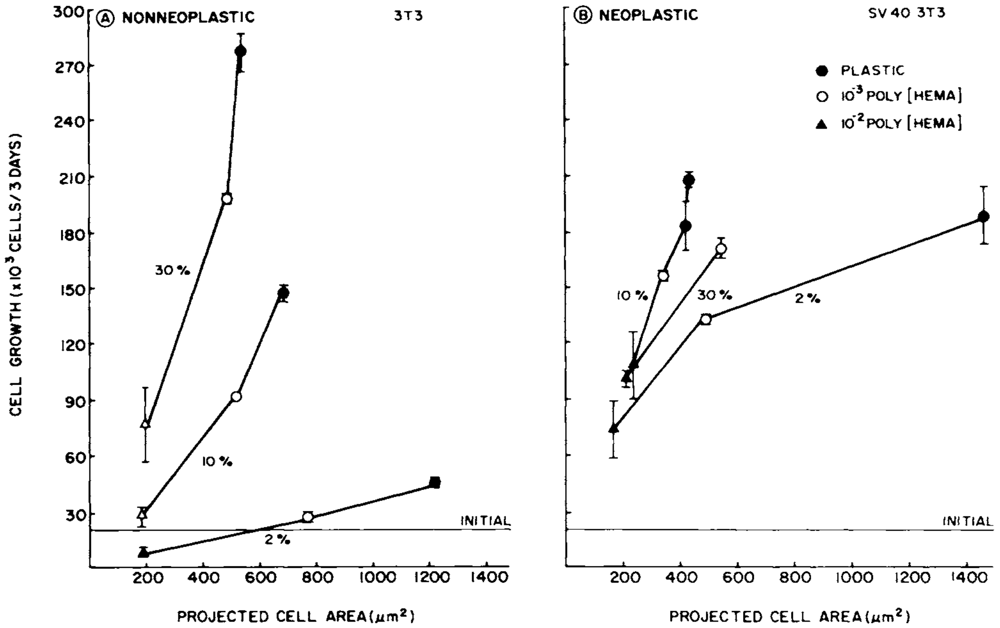

Fig. 7. Cell spreading (projected cell area) and serum concentration interact in affecting cell growth. See Figures 1 and 4 for experimental details. Note the marked differences in graphs of cell growth vs projected cell area of 3T3 at different serum concentrations (A). In contrast, the corresponding curves for SV40-transformed 3T3 cell (B) are more similar.

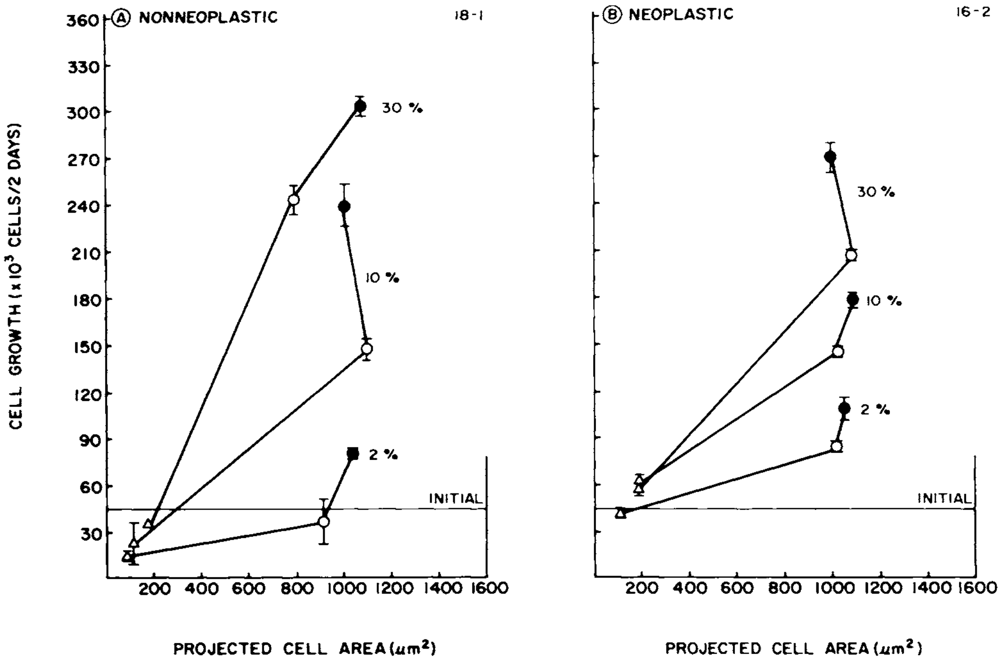

Fig. 8. Experimental details same as Figure 7. Note that the graphs of cell growth vs projected cell area at different serum concentrations for cell line 16-2 are closer together than the same graphs for line 18-1.

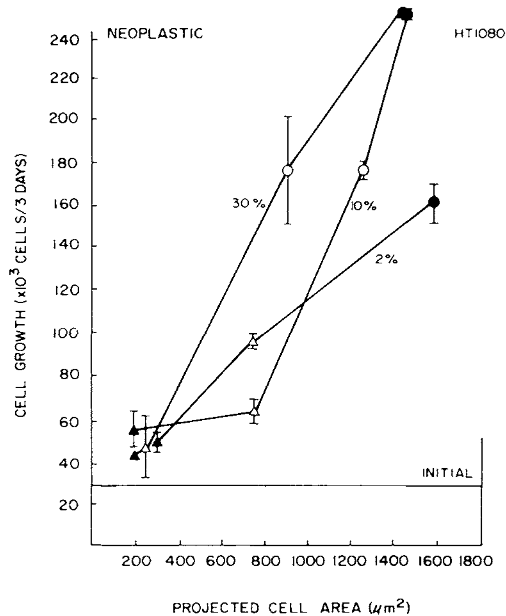

Fig. 9. Experimental detail same as Figure 7. Note the marked similarity in the slope of the graphs of cell growth vs projected cell area in 10% and 3% serum concentrations.

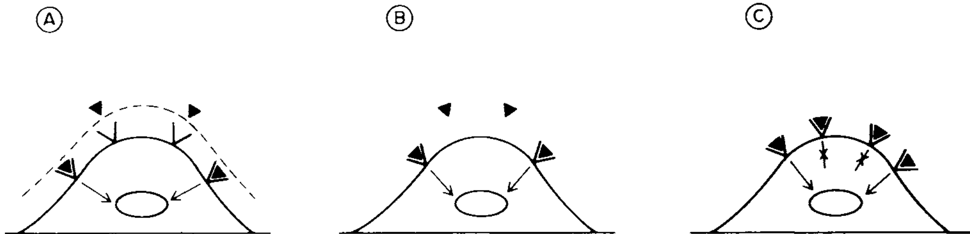

Fig. 10. Different theoretical reasons for decreased growth in rounded non-neoplastic cells secondary to decreased effective growth factor effect. A. Diffusion limited hypothesis. Growth factors (▲) are prevented from reaching receptors (V) on cell membrane. B. Receptor hypothesis. Receptors (V) for growth factors decrease in number so that fewer growth factors (▲) are bound. C. Cytoskeletal hypothesis. Growth factors bind to receptors, but the cytoskeletal link between receptor binding and nuclear DNA synthesis is ineffective.

than in 3 neoplastic cell lines we studied. In the 2 non-neoplastic cell lines increased cell spreading was associated with an increased sensitivity to serum concentration; neoplastic cells also increased net cell growth with similar changes in cell spreading, but growth was already maximal at low serum concentrations. At the same time, rounded cells of all 3 neoplastic lines grew on poorly adhesive substrata even in low (2%) serum. Thus, these neoplastic cells required little serum and no attachment to grow, but still increased their growth rate if allowed to spread. Neoplastic cells, which depended the least on increases

to serum (SV403T3), were either producing their own growth factors [15] whose function was also sensitive to cell shape or were so sensitive to exogenous serum that concentrations above 2% or 10% did not stimulate additional growth. The important point is that cell spreading and exogenous serum do not always cooperate in producing neoplastic cell growth to the same extent that they do in non-neoplastic cells. The most fundamental control appears to be the effect of cell spreading with which exogenous serum and other growth factors interact in various ways.

The interdependence of serum (growth factor) and cell spreading in producing growth also provides new interpretations of previously reported phenomena. The requirement for increased serum [9] or increased growth factor [10, 16] to overcome density inhibition of growth can now be viewed as resulting from the resistance of rounded non-neoplastic cells to growth stimulation by serum. Similarly, the increased growth of neoplastic cells in suspension when attachment is provided by floating beads [17] shows the sensitivity of even neoplastic cells to changes in cell spreading. The growth of some non-neoplastic cells in suspension when very high serum concentrations are used [1] emphasizes that the effect of cell shape on serum sensitivity is a quantitative, not an all-or-none, change.

The mechanism of the coupling of cell spreading and serum effects in non-neoplastic cells is unknown. Possible explanations of the inhibiton of growth of rounded non-neoplastic fibroblasts include either increased inhibitors or decreased stimulators. So far, increased inhibitors have been found only for epithelial cells [18], so we will concentrate here on stimulators in discussing fibroblasts. Decreased actions of growth-stimulatory factors could result from a diminished availability of diffusion-limited growth factors associated with the postulated decreased surface area in rounded cells [1]. The result would be fewer growth factor receptors occupied (Fig. 10A). Fewer bound receptors could also result from changes in number or distribution of receptors (Fig. 10B), although Westermark [19] has shown that changes in number of receptors are not important in EGF stimulation of glial cells. Another possibility is that receptors are occupied but unable to stimulate DNA synthesis in the rounded cell (Fig. 10C). How cell shape itself might affect response to growth factors is unclear, but Benecke et al [20] have recently shown that cytoskeletal events associated with cell spreading affect protein synthesis and mRNA metabolism. Whatever the mechanism, the coupling of humoral information and conformational (spatial) information is important in the regulation of cell growth. Aspects of this coupling appear quantitatively different in neoplastic cells. Our work here suggests that changes in a cell that occur with increased spreading (increased projected area) are associated with growth regulatory changes.

ACKNOWLEDGMENTS

Supported by NCI grant CA14019-04 (to Dr. Folkman) and a grant to Harvard University from the Monsanto Company. Dr. Tucker was supported by a grant from the American Cancer Society, Massachusetts Division, and a fellowship from the Medical Foundation, Incorporated, Boston, Mass. The generous support and advice of Dr. A. B. Pardee is also gratefully acknowledged.

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