Proc. Natl. Acad. Sci. USA
Vol. 75, No. 4, pp. 1864–1866, April 1978
Cell Biology

Density-dependent regulation of growth of BSC-1 cells in cell culture: Growth inhibitors formed by the cells

(ammonia/epithelial cells/lactic acid/unstable protein inhibitor)

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Contributed by Robert W. Holley, January 20, 1978

ABSTRACT **Inhibitors formed by a monkey epithelial cell line, BSC-1, play an important role in limiting growth at high cell densities. At least three inhibitors are formed: lactic acid, ammonia, and an unidentified inhibitor that may be an unstable protein. The unidentified inhibitor is destroyed by shaking the conditioned medium, by bubbling gas through the medium, or by heating or storing the medium in the absence of cells. The concentrations of lactic acid and ammonia that accumulate in conditioned medium inhibit growth when added to fresh medium. These results, together with earlier studies, indicate that density-dependent regulation of growth of BSC-1 cells results from the combined effects of (*a*) inhibitors formed by the cells, (*b*) decreased availability of receptor sites for serum growth factors as the cells become crowded, and (*c*) limiting concentrations of low molecular weight nutrients in the medium. In contrast, density-dependent regulation of growth in 3T3 mouse embryo fibroblasts results almost entirely from inactivation of serum factors.**

Evidence in the literature suggests that there are differences between the growth controls of epithelial cells and fibroblasts in cell culture (1–4). Due to these differences and to the importance of epithelial cells in the origin of tumors, we have made a thorough study of the factors that control the growth of BSC-1 cells, an epithelial cell line of African green monkey kidney origin (5). In previous papers (4, 6) we demonstrated that (*a*) serum factors and (*b*) the concentrations of low molecular weight nutrients in the medium contribute to growth regulation of this cell line. The present paper describes the regulation of growth of BSC-1 cells by the formation of inhibitory materials in the culture medium.

The initial evidence for the presence of inhibitors was a slight but consistent stimulation of the labeling index in crowded cells whenever conditioned medium with 0.1% serum was replaced by fresh medium without serum. Although the labeling index along the edge of a "wound" in the cell layer often decreased after this treatment, there was a consistent increase of the labeling index in the crowded cell layer. The increase could not be attributed to fresh serum factors, for serum was not added. Nor could it be due to an increase in the concentrations of nutrients in the fresh medium: the concentrations of nutrients in conditioned medium were found to be at least half those in fresh medium; changing the cultures to fresh serum-free medium with only half the normal concentrations of the nutrients still stimulated DNA synthesis in the cell layer.

Tests of known metabolites identified lactate and ammonium ions as significant growth inhibitors in BSC-1 cell cultures. However, the inhibition observed when these ions were added to fresh medium, at the concentrations found in conditioned medium, was not adequate to explain the above results.

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Evidence that the cells produce an additional, unidentified inhibitor was obtained initially from experiments in which conditioned medium was removed from the cells, placed briefly in a glass bottle, and then replaced on the cultures. This led to an increase in the labeling index compared with undisturbed cultures. Conditioned medium was then deliberately placed in a bottle, shaken, and replaced on cells. This resulted in pronounced stimulation of DNA synthesis in the cultures. DNA synthesis was not stimulated by careful removal and immediate replacement of the conditioned medium without shaking.

This paper describes some properties of the unidentified inhibitor and discusses the contribution of inhibitors to density-dependent regulation of growth of BSC-1 cells.

MATERIALS AND METHODS

Cell Cultures. BSC-1 cell cultures were maintained as previously described (4) in Dulbecco-modified Eagle's medium (DME) (ref. 7, with 0.45% glucose). Cells were detached from the culture dishes with 0.025% trypsin in calcium- and magnesium-free Tris/saline buffer (8) with 0.5 mM Na_2EDTA. Autoradiography was carried out as described (9).

Conditioned medium is that which has been on confluent cultures for 3–4 days.

Analytical Methods. Lactate was analyzed with Sigma Chemical Co. kit no 826, which uses lactate dehydrogenase, reduction of NAD, and measurement of the increase in absorbance at 340 nm. Ammonium ion was analyzed with Sigma Chemical Co. kit no. 170-A, which uses reductive amination of α-ketoglutaric acid and measurement of the decrease in absorbance at 340 nm. Conditioned medium was analyzed for amino acids using an amino acid analyzer (Beckman model 121), and for glucose using glucose reagent (9% wt/vol *o*-toluidine in glacial acetic acid) obtained from Dow Diagnostics, Dow Chemical Co.

RESULTS

Inhibition by Lactate and Ammonium Ions. Analyses of conditioned medium revealed approximately 11 mM lactate ion and 2 mM ammonium ion. Growth of BSC-1 cells is reduced about 30% when both lactate and ammonium ions are added at these concentrations to fresh medium with 0.1% serum. This inhibitory effect is an additive result of approximately equal individual inhibitory effects of the two ions. The growth inhibition by lactate and ammonium ions is less in medium with 10% serum.

The concentrations of lactate and ammonium ions present inside or adjacent to the cells are higher than the concentrations in the medium. If the conditioned medium is withdrawn from the culture dish, the lactate concentration found in the small

Abbreviations: DME, Dulbecco-modified Eagle's medium; EGF, epidermal growth factor.

Table 1. Evidence for the presence of inhibitors in medium conditioned by BSC-1 cells

Treatment	Thymidine incorporation, cpm
Control	4,400
Control + 0.1% fresh serum	6,800
Medium change	
DME + 0.1% serum	31,000
½ DME + 0.1% serum	28,000
Remove 2.5 ml of conditioned medium	
+ 2.5 ml of DME + 0.1% serum	11,000
+ 2.5 ml of NaCl/$NaHCO_3$ + 0.1% serum	9,000
Remove 4 ml of conditioned medium	
+ 4 ml of DME + 0.1% serum	14,000
+ 4 ml of NaCl/$NaHCO_3$ + 0.1% serum	15,000
Conditioned medium removed completely, medium shaken 60 sec and replaced	24,000

Cells were plated at 10^6 cells per 5-cm dish in 5 ml of DME with 0.1% calf serum. The medium was changed after 3, 7, and 10 days. Three days after the last medium change the cells were treated as indicated in the table. One-half DME was prepared by diluting DME with an equal volume of a solution of NaCl and $NaHCO_3$ made with the salt concentrations present in DME. In the last treatment, the conditioned medium was shaken in a 125-ml glass medium bottle. Incorporation of [*methyl*-^{3}H]thymidine (25 μCi/dish) was from 20–25 hr after the treatments. The cells were then washed and the acid-insoluble material was collected by filtration and the radioactivity was determined as previously described (9). The results were confirmed using autoradiography. The labeling index was approximately 2% in the controls and 10% after the conditioned medium had been shaken and replaced.

volume of residual medium plus cells is 10–20% higher than that found in the conditioned medium. Washing the cells once with cold Tris-buffered saline, after withdrawal of the conditioned medium, removes all of the lactate. However, the ammonium ion concentration found in the washed cells remains high, sometimes twice that found in the conditioned medium.

Evidence for an Unidentified Inhibitor. As shown in Table 1, dilution of the conditioned medium to one-fifth its original concentration with fresh medium only partially restores activity. Dilution should eliminate the inhibition due to lactate and ammonium ions, because 2 mM lactate and 0.4 mM ammonium ions are not inhibitory when added to fresh medium. Shaking conditioned medium by hand for 60 sec (Table 1) largely restores its original acitivity, apparently by destroying an inhibitor. Shaking conditioned medium does not change the concentrations of lactate and ammonium ions. The activation of conditioned medium by shaking is the same whether the shaking is in glass or plastic vessels, in the light or dark.

Activity is also restored to conditioned medium by bubbling N_2, CO_2, or O_2 through the medium for one minute, or by freezing and thawing the medium, or by storing the medium at 37° for 6 hr or longer, or by heating the medium for 5 min at 100°. It seems likely that the inhibitor is destroyed or removed by these treatments. The inhibitor survives adjustment of the medium to pH 9 and preliminary experiments suggest it is adsorbed less by surfaces at high pH. All attempts to trap a volatile inhibitor have failed, and lowering the pressure over a culture dish to 100 mm Hg (13 kPa) does not restore activity of the medium. The inhibitor is nondialyzable.

Response of BSC-1 Cells to Two Concentrations of Inhibitor. The relative ability of BSC-1 cells to initiate DNA synthesis in the presence of different amounts of the inhibitor is shown in Table 2.

Table 2. Inhibition of the initiation of DNA synthesis by the addition of conditioned medium

Addition	Thymidine incorporation, cpm
1.5 ml of DME	58,000
1.5 ml of conditioned medium	31,000
0.5 ml of conditioned medium + 1.0 ml of DME	50,000
1.5 ml of heated conditioned medium	62,000
0.5 ml of heated conditioned medium + 1.0 ml of DME	65,000

Quiescent BSC-1 cell cultures were grown as described in Table 1. The medium was changed to 2.5 ml of fresh DME + 0.05% calf serum plus the additions shown. Methods used for measuring thymidine incorporation and determining radioactivity were the same as in Table 1. Heating was for 5 min at 100°.

Production of the Inhibitor in Cell Cultures. Inhibitor production seems to be dependent on cell number rather than on the serum concentration. Dilution of conditioned medium containing 10% serum suggests that there is only 3 to 4 times more inhibitor produced in medium with 10% serum than in medium with 0.1% serum. The inhibitor is formed in medium with as little as 0.05% serum.

The following experiment suggests that the inhibitor is formed continuously. The medium on cultures was replaced with fresh medium, and 6 hr later the fresh medium was removed from some cultures, shaken and replaced on these cultures. There was then a 30% increase in the labeling index compared with the cultures left in unshaken fresh medium.

Conditioned medium from 3T3 cells does not have inhibitory activity for growth of BSC-1 cells.

DISCUSSION

The experimental evidence suggests that BSC-1 cells form growth-inhibitory materials when cultured. These inhibitory materials include lactate and ammonium ions, and an as yet unidentified inhibitor. The inhibitory effect on cell growth is due largely to the unidentified inhibitor; lactate and ammonium ions are probably less important (Table 1), because shaking conditioned medium restores most of the growth activity.

The unidentified inhibitor is inactivated when conditioned medium is shaken, bubbled with gases, frozen and thawed, heated, or stored at 37°. These results suggest that the inhibitor is easily surface denatured. Its characteristics are consistent with the hypothesis that the inhibitor is an unstable protein or polypeptide present at a low concentration.

Although inhibitors are important in density-dependent regulation of BSC-1 cells, they are only one of three classes of factors that determine the saturation density of these cells. Under normal culture conditions all three classes of factors—(*a*) serum factors such as epidermal growth factor (EGF), (*b*) the concentrations of low molecular weight nutrients in the medium, and (*c*) inhibitors formed by the cells—influence the final cell density.

The growth regulation of BSC-1 cells in culture is much more complicated than growth regulation of 3T3 mouse embryo fibroblasts. A comparison is given in Table 3. The saturation density of 3T3 cells is determined almost entirely by inactivation of serum factors, as is summarized in the footnote to the table. The basis for the semi-quantitative assignments, given in Table 3, of contributions of the various factors to growth regulation of BSC-1 cells can be summarized as follows.

Assays of spent culture medium from BSC-1 cells were made

Table 3. Density-dependent regulation of growth of 3T3 and BSC-1 cells

Growth-controlling factor	Contribution to density-dependent regulation of growth, %	
	3T3 cells (fibroblast)	BSC-1 cells (epithelial)
Serum factors		
Inactivation	>90*	<10
Decrease in receptor sites	Not observed	~50
Concentration of low molecular weight nutrients	Mechanisms present, but not important at normal concentrations	10 or less
Inhibitors produced by the cells	<10	~50

* A simple experiment, performed several years ago (10), established the significance of the inactivation of serum factors by 3T3 cells. Under normal culture conditions, in medium with 1% calf serum, the growth of 3T3 cells is arrested when the cells are sparse. If, instead of being allowed to distribute over the entire dish, the 3T3 cells are restricted to a coverslip in a large volume of 1% calf serum, the cells grow to a density 10 times higher, that is, to the cell density normally attained in 10% serum. Thus, 1% calf serum has a concentration of serum factors sufficient to support 3T3 cell growth to 10 times the density normally attained, as long as depletion of serum factors is avoided. From this, one can conclude that approximately 90% of normal growth restriction is due to inactivation or depletion of serum factors. In addition, a diffusion boundary layer, caused by inactivation of serum factor in the medium adjacent to quiescent 3T3 cells, has been demonstrated, and this accounts for the "wound healing" experiment (11).

by addition of aliquots of spent medium to fresh medium with low serum. These assays indicate there is little inactivation of serum factors during growth.

The number of EGF receptor sites per cell has been shown to decrease rapidly as the cell density increases (4). Quiescent cells in medium with a low serum concentration are stimulated to grow by increasing the serum concentration or by adding EGF. (The labeling index rises from 2–3% in the quiescent cells to 10–20% after these additions.) Cell growth is also strongly stimulated by brief exposure to medium without calcium, which uncovers many cryptic EGF receptor sites (4). These results suggest that the number of available receptor sites for serum factors such as EGF has decreased sufficiently at high cell density to restrict growth of BSC-1 cells. Therefore, availability of receptor sites for serum factors probably plays a major role in growth regulation of BSC-1 cells.

A summary of the effect of nutrient concentrations on the saturation density of BSC-1 cells has been given in previous work (6). Although the effect on saturation density is considerable, the difference in growth rate is small, and there is only a small difference in labeling index. Because cells in conditioned medium with a low serum concentration can be stimulated strongly either by increasing the concentration of serum or by shaking the medium, we feel that the decreases in nutrient concentrations that take place between medium changes play a relatively minor role in density-dependent regulation of growth under normal culture conditions.

The effect of inhibitors is demonstrated most dramatically by the results presented in Table 1, where shaking conditioned medium and replacing it on the cultures provides nearly as much stimulation as a change to fresh medium. The labeling index increases approximately 5-fold. The residual inhibition that exists after the medium is shaken is due, at least partially, to the lactate and ammonium ions in the conditioned medium. From these results, we conclude that inhibitors make a major contribution in the density-dependent regulation of growth of BSC-1 cells (Table 3).

The combined effect on cell growth of (*a*) serum factors, (*b*) concentrations of low molecular weight nutrients, and (*c*) inhibitors formed by the cells is sufficient to account for the observed growth behavior of the BSC-1 cell line.

The unidentified inhibitor described above is probably different from those inhibitors reported in the literature to date; a recent review of endogenous inhibitors of mammalian cells has been written by Lozzio *et al.* (12). We have excluded the possibility that the substance is a spermine complex, the major inhibitor in a "lymphocyte chalone" preparation (13). Our inhibitor is formed and is active when BSC-1 cells are grown in human serum, as well as in calf serum. Human serum is low in plasma amine oxidase activity, and as a result the spermine complex has little inhibitory activity in human serum (13). The BSC-1 inhibitor has very different properties than the inhibitor of *in vitro* protein synthesis obtained from frozen extracts of Vero M3 cells by Engelhardt (14).

As far as we are aware, no other growth inhibitor has been reported to be inactivated by shaking.

The importance of inhibitors in the growth regulation of BSC-1 cells suggests the need for purification and characterization of the unidentified inhibitor, and for study of the possibility that other epithelial cells also produce inhibitors that are important in the control of their growth.

This work was supported in part by American Cancer Society Grant BC-30, and National Institutes of Health Grant CA11176. R.W.H. is an American Cancer Society Professor of Molecular Biology.

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