Density-dependent stimulation and inhibition of cell growth by agents that disrupt microtubules

(cell cycle/growth control/colchicine/microtubules/contact inhibition)

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Contributed by Gerald M. Edelman, February 7, 1980

ABSTRACT We have previously reported that agents that disrupt microtubules, such as colchicine, inhibit the growth stimulation of lymphocytes and arrested fibroblasts; other workers have recently reported enhanced stimulation of fibroblasts in the presence of the same drugs. In the present studies, we resolve this conflict by demonstrating that the stimulatory and inhibitory effects of microtubule disruption depend upon the density and the cell type of the treated cultures. Our analysis included an examination of three variables: (i) cell density (sparse or confluent), (ii) cell type (resting fibroblasts from mouse or chicken embryos or from the permanent 3T3 mouse fibroblast line), and (iii) treatment with colchicine and related drugs in the presence or absence of various mitogens such as serum, insulin, and epidermal growth factor. We found that colchicine augmented mitogenesis in confluent cultures of all cell types. Colchicine by itself appeared to be mitogenic only for confluent chicken embryo fibroblasts. In sparse cultures with minimal cell-cell contacts, however, there were differences between embryonic cells and the 3T3 cell line. In confirmation of our previous reports, disruption of microtubules by colchicine inhibited the mitogenic stimulation of sparse cultures of embryonic chicken or mouse fibroblasts. In contrast, fibroblasts of the permanent 3T3 line in sparse cultures were stimulated by some mitogens despite the presence of colchicine. The augmentative effects of colchicine on the stimulation of confluent cultures were synergistic with the mitogens, and colchicine allowed response to otherwise submitogenic doses of growth factors. Kinetic studies indicated that the stimulatory and inhibitory effects of colchicine are separable and that both can operate simultaneously. The experiments suggest that the regulation of growth by nutrient deprivation and the regulation by density dependence proceed at least in part by different mechanisms. All of the results suggest that microtubular integrity can be associated with the expression of either negative or positive controls on cell growth, depending upon the confluence or lineage of the cells in culture.

The hypothesis (1, 2) that microtubules and other cytoskeletal structures are involved in the regulation of cell growth was originally based on experiments in which the growth state of lymphocytes was correlated with the mobility of their cell surface receptors. Cell surface receptor mobility, in turn, had been shown to be under the control of a surface-modulating assembly consisting of transmembrane receptors and submembranous arrays of microfilaments, microtubules, and their associated proteins (2).

An impressive body of evidence has accumulated to support the suggestion that the surface-modulating assembly is involved in regulating cell growth. Drugs that cause the dissolution of cellular microtubules, such as colchicine, inhibit the stimulation of lymphocytes by the lectin concanavalin A (3). Kinetic studies showed that this inhibition occurred at or immediately after the time when the cells became committed or stimulus-independent—that is, when removal of the concanavalin A did not

prevent them from reentering the cell cycle and proceeding on to eventual DNA replication and mitosis. Furthermore, the binding of appropriate lectins at higher doses to the cell surface led both to a microtubule-dependent restriction of the mobility of cell surface receptors and, in parallel, the delivery of a dominant negative signal that inhibited the cell stimulation induced by the lectin (2, 4). Thus, the inhibitory signal operated even in the face of ongoing stimulatory signals that had led to the commitment of the treated cells (4).

Colchicine and other microtubule-disrupting agents have also been shown to inhibit the stimulation of nonlymphoid cells (5–7). For example, we found that colchicine inhibited the stimulation by serum of serum-starved chicken embryo fibroblasts (CEF) (6). As was the case for lymphocytes, this inhibition occurred early in the G₁ phase of the cell cycle at a point operationally indistinguishable from the point of commitment to DNA synthesis.

In contrast, some workers have found that colchicine alone stimulated cell growth or enhanced the stimulation of cell growth by various mitogens (8–11) and therefore have questioned (10) the validity of our earlier conclusions.

In this paper, the apparent conflict in these data is resolved. We report studies on the effect of microtubule-disrupting drugs as a function of cell density in primary fibroblast cultures and an established fibroblast line. We found that cells respond to microtubule disruption differently depending upon the cell density and the cell's history in culture. Colchicine alone stimulated or augmented mitogenic stimulation mainly in cultures that were confluent. On the other hand, the stimulation of sparse cultures of embryonic cells by growth factors was inhibited by colchicine. Cell lines that have become adapted to artificial culture (such as Swiss 3T3) appear to be much less sensitive to the inhibitory effects of these drugs. Together, our findings appear to explain the apparently disparate results and lend additional support to the idea that cytoskeletal states are involved in the regulation of cell growth.

MATERIALS AND METHODS

Primary cultures of CEF and mouse embryo fibroblasts were prepared from the body walls of 10-day-old chicken embryos (SPAFAS, Norwich, CT) or 19-day-old mouse embryos (NCS, The Rockefeller University) as described (12, 13). After 2 days, secondary cultures were plated into 35-mm dishes in Dulbecco's modified Eagle's medium containing 10 mM Hepes and 0.5% fetal calf serum. These arrested cells were stimulated after 60 hr by addition of reagents in serum-free medium. DNA synthesis was assayed by adding 2 μ Ci of [³H]thymidine (1.9 Ci/mmol; 1 Ci = 3.7 $\times 10^{10}$ becquerels) to the dishes. After 2 hr the cells were washed in isotonic saline and disrupted in 0.1 M NaOH. Incorporation of the thymidine into trichloroacetic acid-precipitable material was measured as described (3).

Abbreviations: CEF, chicken embryo fibroblasts; EGF, epidermal growth factor; FGF, fibroblast-derived growth factor.

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Swiss mouse 3T3 fibroblasts, clone 4, were the gift of Howard Green. They were maintained as described (13) and arrested by passing into low-serum medium in the same manner as the embryonic cells. For autoradiographic experiments, cells were grown on glass cover slips, labeled as described above, and then processed as described (3).

Colchicine and bovine pancreatic insulin (26.8 international units/mg) were purchased from Sigma. Epidermal growth factor (EGF) and fibroblast-derived growth factor (FGF) were purchased from Collaborative Research (Waltham, MA). Lumicolchicine was prepared by the method of Wilson and Friedkin (14).

RESULTS

Serum-starved CEF could be stimulated to undergo further rounds of mitosis by the addition of serum (Fig. 1). We found that, in sparsely plated CEF, 1 μ M colchicine inhibited the stimulation by serum (Fig. 1A), in agreement with our previous report (6). In contrast, the stimulation of confluent cells was enhanced by colchicine (Fig. 1B). Addition of colchicine in the absence of serum had no effect on sparse cultures (Fig. 1A) but it did stimulate confluent cultures (Fig. 1B). The dense cells, which were plated at an 8-fold higher concentration than the sparse cells, had a relatively lower level of background DNA synthesis than the sparse cells. Nearly identical results, quali-

tatively and quantitatively, were obtained when cells were starved for 84 instead of 60 hr.

The effect of colchicine on the stimulation of mouse 3T3 fibroblasts by EGF plus insulin is shown in Fig. 2. In contrast to CEF, sparse 3T3 cells showed only slight inhibition by colchicine (Fig. 2A). Although colchicine augmented the mitogenic stimulation of dense cells (Fig. 2B), it caused only minimal stimulation when added alone. It should be pointed out that the cell cultures studied by other authors (8–11) who reported that colchicine augmented stimulation were confluent, although this was not specifically noted in their publications.

The observed stimulation or inhibition of the bulk counts of radioactive thymidine uptake could have resulted from differences in the number of cells responding or from differences in uptake or utilization of thymidine. To distinguish between these possibilities, the percentage of cells responding in these and the following experiments was quantitated by using autoradiography as an assay. In all cases, the bulk counts correlated well with the number of stimulated cells (data not shown). We conclude therefore that the diminution or enhancement of response caused by colchicine reflects a change in the number of stimulated cells and not, for example, a change in the amount of thymidine transported or incorporated per stimulated cell. The clear relationship between the number of stimulated cells and the bulk counts also allows normalization of the data in Figs. 1 and 2 on a per-cell basis. When this is done it appears that the stimulatory effect of colchicine is to allow confluent cells to act like sparse, untreated cells—that is, the *frequency* of stimulated cells in the colchicine-treated confluent cultures approaches but does not exceed the frequency of stimulated cells in the untreated sparse culture.

To check the generality of our results, CEF and 3T3 cells were stimulated with various agents in the presence and absence of colchicine. The inhibitory effects of colchicine on sparse CEF and the stimulatory effects of colchicine on dense CEF were seen with either insulin or serum (Table 1). Note also that colchicine allowed otherwise submitogenic doses of insulin (3 or 10 ng/ml) to stimulate the confluent CEF. For 3T3 cells, the picture was not as simple. The effect of colchicine on serum stimulation of 3T3 cells was analogous to that found for CEF—namely, the drug inhibited the stimulation of sparse cells

FIG. 1. Effect of colchicine on serum-stimulation of starved CEF. Sparse cells (A and C) were plated at 1.25×10^5 cells per 35-mm dish in Dulbecco's modified Eagle's medium containing 0.5% fetal calf serum. Confluent cells (Fig. B and D) were plated at 10^6 cells per dish. After 60 hr, serum was added to various final concentrations (abscissa) to these arrested cells. Cultures received either no colchicine (●) or colchicine at 1 μ M (○) at the same time. DNA synthesis (ordinate) was assayed at 13–15 hr. Phase-contrast micrographs (C and D) were made at this time. ($\times 320$).

FIG. 2. Effect of colchicine on EGF stimulation of starved 3T3 fibroblasts. Sparse cells (A) were plated at 1.6×10^4 cells per 35-mm dish. Confluent cells (B) were plated at 4×10^5 cells per dish. After 60 hr, EGF was added to various final concentrations (abscissa) along with insulin at 100 ng/ml. Cultures received either no colchicine (●) or colchicine at 1 μ M (○) concurrently. DNA synthesis (ordinate) was assayed at 20–22 hr.

Table 1. Effect of 1 μ M colchicine on stimulation of sparse and confluent CEF and 3T3 cells by various agents

Cells*	Stimulus†	[³ H]Thymidine incorporation,‡ cpm $\times 10^{-3}$		
		No drug	Colchicine	
Sparse CEF	None	13.9	14.0	(+0.7%)
	Insulin	22.5	16.5	(-26.7%)
	FCS (2.25%)	32.2	9.8	(-69.6%)
Dense CEF	None	12.3	17.3	(+40.6%)
	Insulin (3 ng/ml)	11.4	23.3	(+104.4%)
	Insulin (10 ng/ml)	11.8	27.9	(+136.4%)
	Insulin	25.2	47.2	(+87.3%)
	FCS (2.25%)	57.5	66.3	(+15.3%)
Sparse 3T3	None	1.5	0.7	(-53.3%)
	FCS (0.25%)	4.2	1.9	(-54.8%)
	FCS (1.25%)	12.8	8.2	(-35.9%)
	FCS (6.0%)	10.4	10.3	(-1.0%)
	EGF + insulin	5.7	5.5	(-3.5%)
	FGF + insulin	13.6	19.0	(+39.7%)
	Insulin	5.6	6.1	(+8.9%)
	Insulin	0.2	0.3	(+50.0%)
Dense 3T3	FCS (0.25%)	0.5	0.7	(+40.0%)
	FCS (1.25%)	2.2	2.7	(+22.7%)
	FCS (6.0%)	12.3	13.8	(+12.2%)
	EGF + insulin	7.8	28.7	(+267.9%)
	FGF + insulin	16.8	41.5	(+147.0%)
	Insulin	4.6	10.0	(+117.4%)

* Sparse CEF were plated at 1.25×10^5 cells per 35-mm dish; dense (confluent) CEF were at 10^6 cells per dish. Sparse 3T3 cultures had 1.6×10^4 cells per dish; confluent 3T3 cultures had 4×10^5 cells per dish.

† The agents—insulin (1 μ g/ml unless otherwise indicated), EGF (10 ng/ml), FGF (1 ng/ml), or fetal calf serum (FCS; various concentrations)—were added 60 hr after plating.

‡ At 13 hr (CEF) or 20 hr (3T3) after addition of stimulus, 2 μ Ci of [³H]thymidine was added to the cultures, and DNA synthesis was assayed 2 hr later. Results are means of duplicate determinations. Numbers in parentheses are the percentage changes in incorporation caused by colchicine compared to the stimulation without the drug.

but enhanced that of confluent cells. This result agrees with our previous report (6). More defined stimulants (EGF, FGF, and insulin) gave a different result, however. Slight to moderate enhancing effects of colchicine were seen even in sparse cells although in all cases the enhancement was much more pronounced in the dense, confluent cultures.

There are two obvious differences between CEF and mouse 3T3 fibroblasts that could be responsible for the observed differences in response to colchicine seen in Figs. 1 and 2 and Table 1. First, the cells are of different origin, avian and mammalian. Second, the CEF are largely unselected embryonic cells which, only 5 days prior to the experiments, had still been part of a whole, growing bird. The 3T3 cells are cloned descendants of cells that underwent a long selection and adaptation to permanent culture on plastic dishes in artificial medium. To resolve which of these differences is more important in these experiments, we studied the effect of colchicine on the stimulation of cultures of mouse embryo fibroblasts. The preparation of these cells was essentially identical to that of CEF and to the earliest stages of the preparation of 3T3 cells.

Fig. 3 and Table 2 show that the primary mouse embryo fibroblasts responded like the primary CEF rather than like the established line of mouse fibroblasts. In sparse cultures, colchicine markedly inhibited stimulation by various agents including EGF, FGF, serum, and insulin. Enhancement of stimulation was seen in the confluent starved cultures. Colchicine by itself had a novel effect in the mouse embryo fi-

FIG. 3. Effect of colchicine on serum stimulation of mouse embryo fibroblasts as a function of cell density. Cells were plated at various densities (abscissa) in 35-mm dishes. After 60 hr, cultures received either no serum (●, ○) or fetal calf serum to a final concentration of 0.3% (■, □), and either no colchicine (●, ■) or 1 μ M colchicine (○, □). DNA synthesis (ordinate) was assayed at 13–15 hr.

broblast cultures: it markedly reduced the background division of arrested cells in sparse cultures but not in dense cultures.

Further experiments were done to elucidate the nature of the colchicine effects on cell growth. Nocodazole augmented stimulation of dense cells and inhibited stimulation of sparse cells (Table 3). This chemical is structurally distinct from colchicine but also causes dissociation of microtubules by binding to tubulin (15). It lacks at least some of the probably microtubule-independent "side effects" of colchicine (16). Lumicolchicine, on the other hand, did not affect the action of growth-promoting agents. This chemical is a photoinactivated derivative of colchicine which does not disrupt microtubules

Table 2. Effect of 1 μ M colchicine on the stimulation of mouse embryo fibroblasts

Cell density	Stimulus	[³ H]Thymidine incorporation, cpm $\times 10^{-3}$		
		No drug	Colchicine	
Sparse	None	8.9	0.2	(-97.7%)
	Insulin	18.8	9.6	(-47.8%)
	FCS (3.0%)	31.7	20.7	(-35.1%)
	EGF + insulin	19.5	6.8	(-65.1%)
	FGF + insulin	22.3	15.6	(-30.0%)
Dense	None	26.4	26.2	(-0.7%)
	Insulin	50.8	99.5	(+95.9%)
	FCS (3.0%)	155.1	172.0	(+10.9%)
	EGF + insulin	119.0	153.2	(+28.7%)
	FGF + insulin	137.0	152.6	(+11.4%)

Sparse cells were plated at 0.5×10^5 cells per dish; dense cells were at 10^6 cells per dish. Conditions for stimulation and assay were as for CEF in Table 1. Numbers in parentheses in the last column are the percentage changes in incorporation caused by colchicine compared to the stimulation without the drug. FCS, fetal calf serum.

Table 3. Effects of 1 μ M colchicine, 1 μ M nocodazole, and 1 μ M lumicolchicine on cell stimulation*

Cell type and stimulus	[³ H]Thymidine incorporation, cpm $\times 10^{-3}$	Change due to drug, %
Dense 3T3		
None	0.8	—
EGF + Insulin	17.5	—
+ Colchicine	79.1	+352
+ Nocodazole	84.6	+384
+ Lumicolchicine	14.4	-12
Sparse CEF		
None	13.9	—
Insulin	22.3	—
+ Colchicine	16.5	-26
+ Nocodazole	16.1	-28
+ Lumicolchicine	20.8	-7

* For experimental conditions, see Table 1 legend.

(14). However, it does insert into membranes and has many of colchicine's microtubule-independent effects such as inhibition of thymidine transport. Thus, it is likely that the effects of colchicine on growth described above are due to the binding to tubulin or to the disruption of microtubules.

To define further the physiological basis of colchicine's action, the kinetics of the cells' response to growth stimulation were examined. The inhibitory effect of colchicine in sparse CEF was constant with time—that is, the drug caused a continuing suppression of a cell's ability to be stimulated (Fig. 4A). Of particular significance is the finding that the inhibition by colchicine was not due to a simple delay in the entry of these cells into their S phase.

FIG. 4. Effect of colchicine on kinetics of growth stimulation in sparse and confluent cultures. (A) Sparse cultures; 1.25×10^5 CEF were plated onto 18-mm glass coverslips in 35-mm dishes. After 60 hr, cultures were made 5% in fetal calf serum, and 1 μ Ci of [³H]thymidine was added to each dish. Cultures received either no colchicine (●) or 1 μ M colchicine (○) at this time. At various times (abscissa), coverslips were removed and processed for autoradiography. The cumulative frequency of cells that had entered the S phase by each time is plotted on the ordinate. (B) Confluent cultures; 10^6 CEF were plated in 35-mm dishes. After 60 hr, cultures received either 1 μ g of insulin per ml (●), insulin plus 1 μ M colchicine (○), colchicine alone (□), or no additives (■). At various times, cultures received 2 μ Ci of [³H]thymidine, and incorporation of label into DNA was determined 1 hr later.

Both the stimulatory and augmentative effects of colchicine, however, were delayed compared to the normal stimulation of confluent CEF by insulin (Fig. 4B). Analogous results were obtained with 3T3 cells stimulated by EGF plus insulin (data not shown). Note that even in confluent cultures, before any of the augmentative effects of colchicine were seen, the drug was also inhibitory (the 4–8 hr points in Fig. 4B). This strongly suggests that both inhibitory and stimulatory effects of colchicine operate simultaneously although incompletely in confluent cells and also that the inhibitory effect is masked by the stronger or dominant stimulatory one.

DISCUSSION

Agents that alter the functional state of microtubules in lymphocytes and normal and transformed fibroblasts also alter the growth state of these cells. This observation led to the hypothesis that the cytoskeleton and the surface-modulating assembly may regulate mitogenic signals from the cell surface (2). The hypothesis does not imply that these structures themselves carry these signals. We previously demonstrated both a stimulatory or permissive and an inhibitory or restrictive role for the surface-modulating assembly (3, 4). In this paper, we have confirmed that agents that disrupt microtubules can either interfere with or augment the stimulation of growth of various resting fibroblasts. The key finding is that the direction of the effect after disruption of microtubules depends on the state of the cell—specifically, on whether the cells are sparse or confluent and whether they are relatively naive or are adapted tissue culture lines.

The inhibitory effects of colchicine on nonlymphoid cell stimulation are entirely analogous to the effects on lymphoid cells (3) and have been reported before by us (6) and others (5, 7). From these experiments, we concluded that there was a state of tubulin or microtubules, destroyed by colchicine, which is necessary for the early normal control or partial processing of a mitogenic signal at the cell surface.

We had also previously inferred that there was a distinct microtubular state that was associated with the inhibitory regulation of cell growth. This conclusion was based on the well-correlated effects of relatively high doses of plant lectins which led to both global, microtubule-dependent anchorage modulation of cell surface receptors and the reversible inhibition of cell growth (1, 2). Furthermore, the release of normal growth control of CEF after transformation by Rous sarcoma virus is associated with morphological alterations in the cytoskeleton (6, 12). Thus, the stimulatory effects of colchicine on fibroblast growth and growth stimulation reported by others (8–11) are consistent with the basic conclusion that, in certain states, the surface-modulating assembly can also be a negative regulator of cell growth.

Superficially, however, some results with use of colchicine to alter the response of arrested fibroblasts to growth stimulants were directly contradictory: we had reported inhibition of growth by colchicine; others had reported stimulation. We discovered and report here that both sets of results are correct and that colchicine usually augments the growth of confluent cells only.

This finding has broad implications, suggesting that there is a distinct state of contact inhibition of cell growth that is different from simple nutrient depletion. Cells that are merely deprived of nutrients (Figs. 1A and 2A) respond differently to colchicine than do cells that are both deprived and confluent (Figs. 1B and 2B). Thus, contact inhibition might be seen as analogous to high-dose lectin inhibition of lymphocytes (4). However, our experiments cannot rule out the possibility that density-dependent phenomena are mediated by soluble factors

whose inhibitory effects are cancelled by colchicine. It is nevertheless attractive to propose that cell-cell contacts lead to inhibitory alteration in growth states, an old theory (17) which, after a period of disfavor, is now regaining some experimental support (18, 19). According to this view, the inhibitory signals from the cell surface would be mediated or regulated at some state via the surface-modulating assembly and microtubules, and the disruption of microtubules or the binding of drugs to tubulin would release the inhibition and allow the cells to respond more as if they were sparse.

That the stimulatory and inhibitory effects of colchicine operate simultaneously although incompletely is supported by our previous publications (4) and by the current kinetic data (Fig. 4). The kinetic differences seen in Fig. 4B also suggest that the biochemical pathway for the stimulatory effects of colchicine is at some level separate from the stimulatory pathway for hormone action. Arguing against total independence of these pathways is the fact that colchicine and growth factors show true synergism (Figs. 1 and 2; Table 1): the response due to hormone plus colchicine is greater than the sum of the responses to each agent alone. Furthermore, colchicine allowed stimulation by insulin at concentrations that would not be effective without the drug. Thus, there is a complex and still undefined dynamic balance associated with the phenomena connected with the effects of colchicine. The growth state of a cell is apparently the result of a complex integration of multiple signals, including cell density (Figs. 1 and 3), the strength of the stimulus, and undefined fluctuations in cytoskeletal assemblies. That the strength of the stimulus is important and can tend to override other effects is seen in Table 1: the higher the concentration of serum used to stimulate 3T3 cultures, the smaller the relative colchicine effect.

The molecular details underlying the effects of colchicine remain to be elucidated. We have hypothesized that transmembrane surface receptors are linked indirectly to microtubules (1, 2); colchicine, then, might either allow or prevent associations of enzymatic subunits or aggregation of the receptors necessary for mitogenesis (9). If such receptors had different transmembrane connectivities, this might also explain the apparently different effects of colchicine on EGF, FGF, and serum factor stimulation. Alternatively, the effects of colchicine may be more direct, acting on a central regulator of growth that consists of microtubules or depends for its organization on these structures. Whether colchicine has intrinsic mitogenicity or only allows response to existing low levels of growth factors is unresolved by these data. Table 1 suggests the latter interpretation, insofar as the drug can allow a submitogenic dose of insulin to stimulate. However, we also found that increasing the period of cell starvation (presumably further depleting the medium of growth factors) did not decrease colchicine's stimulatory effect. Furthermore, unless colchicine has effects on intrinsic regulatory centers in the cell, it is difficult to explain how colchicine by itself could allow the more-depleted medium of confluent CEF cultures to support the growth of a greater absolute number of cells than is supported by the less-depleted medium of the sparse cultures (e.g., Fig. 1).

A final result of this work which has broad implications is that there are fundamental differences in the way embryonic cells and permanent tissue culture lines behave in mitogenesis. In particular, the stimulation of the permanent 3T3 cell line was only minimally inhibited by colchicine whereas the stimulation of its embryonic ancestors could be inhibited by the drug. The particular cell traits distinguishing embryonic and 3T3 cells that are selected for or that occur during the process of adaptation to continuous artificial culture are not known.

The main conclusion of the present work is that microtubular integrity can affect the maintenance of both permissive and inhibitory growth states. Confluent cells are distinct from sparse cells with regard to these states, and embryonic cells differ from established culture lines. These facts lend support to the hypothesis that density-dependent inhibition of growth is separable from nutrient deprivation and operates in addition to it. The present findings are also consistent with our earlier hypothesis that signals from the cell surface relevant to control of cell growth and motility are mediated by various transmembrane connections to cytoskeletal elements, particularly microtubules. The biochemical details of these connections, however, still remain obscure.

This work was supported by U.S. Public Health Service Grants AI-11378, AI-09273, and AM-04256.

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