DENSITY DEPENDENT REGULATION OF COLCHICINE INHIBITION OF MITOGENESIS IN 3T3 CELLS ARRESTED IN G_o

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SUMMARY

The effect of colchicine on mitogenesis in 3T3 cells arrested in G_o at different cell densities has been studied. Colchicine inhibits DNA synthesis in 3T3 cells arrested at low cell density. The inhibition of DNA synthesis by colchicine is diminished in high cell density cultures. Lumicolchicine does not inhibit DNA synthesis in low cell density cultures. These results suggest that: (1) structures that contain microtubules are involved in regulating the growth of 3T3 cells; (2) cell density can regulate the state of 3T3 cells arrested in G_o; and (3) in 3T3 cells arrested in G_o some biological processes that are dependent on microtubule containing structures may depend on cell density.

When mouse 3T3 cells are deprived of serum (1, 2) or low molecular weight nutrients (3), they arrest at a restriction point (4) in G_o (the "A" state of Smith and Martin (5)) that appears to be independent of the arrest procedure (3, 4). If the arrested cells are returned to complete growth medium, they begin synthesizing DNA with first-order kinetics after a lag phase of about 15 hours (6). In this report, I present evidence that colchicine can block the resumption of DNA synthesis in 3T3 cells arrested in G_o at low cell density and that the inhibition of DNA synthesis by colchicine is diminished in high cell density cultures. This indicates that 3T3 cells arrested in G_o at different cell densities by serum limitation are biochemically different. This information combined with current concepts about the regulation of the mobility and configuration of receptors on the cell membrane suggests that cell contact has a role in growth regulation.

MATERIALS AND METHODS

Swiss 3T3 cells (1) were obtained from Dr. R. Brooks. Cells were plated in 5 ml of

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0.5% calf serum in Vogt and Dulbecco modified Eagle's medium (7) (DME) into 60 mm Falcon tissue culture dishes and incubated at 37°C for 3 days. Under these conditions the cells are arrested in G_0 (6) (also Fig 1b). Then, 10% calf serum was added to the cultures. Colchicine was added 1/4, 10, 14 hours after the serum addition. The cultures were pulsed with 2 μCi/ml ^{3}H-thymidine at 3 μM from 15 to 24 hours after the serum addition. At this time, the cells in the cultures with colchicine were firmly attached to the dish. The cells were removed from the dish by replacing the medium with 5 ml of a solution containing 0.05% trypsin, 0.54 mM EDTA, 137 mM NaCl, 5.4 mM KCl, 6.9 mM Na HCO_3 and 5.5 mM glucose. The cells were collected on glass fiber papers and washed with 10% TCA and 95% ethanol. The filter papers were counted in 5 ml of Aquasol 2 (New England Nuclear Corp.) in a liquid scintillation counter. Each result for colchicine inhibition is the average of two measurements. The control results were done in quadruplicate. Cell number was determined in duplicate from parallel cultures using a Coulter counter.

Flow microfluorometric analysis. 3T3 cells were treated as described by Crissman and Tobey (8) to obtain a monodisperse suspension. The cells were then stained with mithramycin and passed through a Los Alamos design microfluorometer (9) equipped with an argon laser excited at 457 mm. The fluorescent light flash from each cell, which is proportional to the cell's DNA content, was converted to an electrical pulse by a photomultiplier and stored in a multichannel pulse-height analyzer. The data were displayed on an oscilloscope as shown in Figure 1.

RESULTS

I have extensively studied the effect of colchicine (0.5 to 5.0 μg/ml) on ^{3}H-thymidine incorporation into DNA in 3T3 cells that have been arrested in G_0 at different cell densities, 15 to 24 hours after they were returned to complete growth medium. Table 1 shows some of the results of my experiments. It is clear that colchicine inhibits DNA synthesis in 3T3 cells arrested at low cell density and that this inhibition decreases as the cell density increases. In other experiments, colchicine at a concentration as low as 0.1 μg/ml inhibited DNA synthesis in 3T3 cells arrested at low cell density. A second technique for measuring traverse through the cell cycle flow microfluorometric (FMF) analysis (8, 9) also shows that the effect of colchicine on mitogenesis in 3T3 cells is density dependent (Figure 1b, d, e).

Colchicine does not prevent cells in G_1 in an expontentially growing culture from entering S phase (Figure 1a, f), in agreement with other studies (4, 10-13).

Lumicolchicine (12) (2 μg/ml) (Fig. 2) does not inhibit mitogenesis in 3T3 cells arrested at low cell density, showing that colchicine is inhibiting DNA synthesis by disrupting microtubules. The data presented in Table 1 show that colchicine additions 10

TABLE 1: Effect of Colchicine on DNA Synthesis in 3T3 Cells

Cell Number/60mm Dish	Colchicine Concentration μg/ml	Time When Colchicine Was Added (Hours)	% Inhibition of ^{3}H-Thymidine Incorporation into DNA
4.3×10^5	5.	0.25	10
	2.		8
	.5		15
3.4×10^5	5.	0.25	28
	2.		31
	.5		32
$2. \times 10^5$	5.	0.25	63
	2.		65
	.5		65
	5.	10.	40
	2.		42
	.5		35
	5.	14.	22
	2.		17
	.5		7
$5. \times 10^4$	5.	0.25	76
	2.		74
	.5		80
	5.	10.	57
	2.		55
	.5		53
	5.	14.	27
	2.		20
	.5		19

Counts in cultures without colchicine: 4.3×10^5 cells/60 mm dish - 255,000 CPM; 3.4×10^5 cells/60 mm dish - 190,000 CPM; 2.0×10^5/60 mm dish - 107,000 CPM; 5×10^4/60 mm - 28,000 CPM.

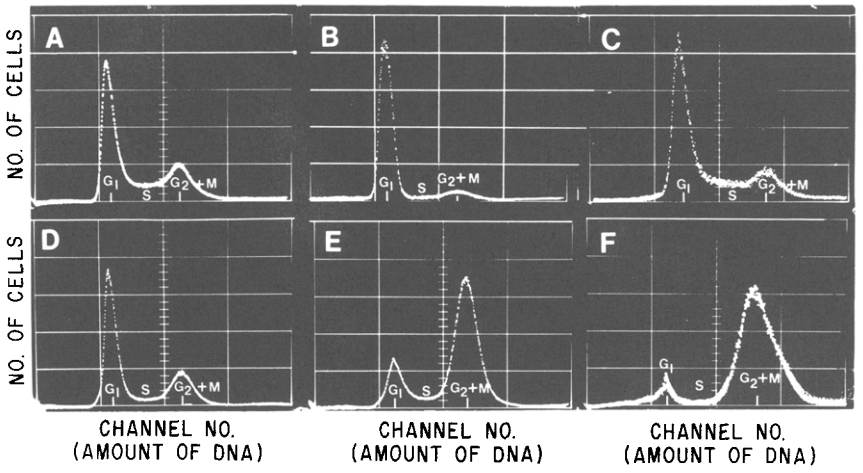

Figure 1: Effect of colchicine on mitogenesis in 3T3 cells. Flow microfluorometric analysis of 3T3 cells: (a) exponentially growing population. About 50% of the cells are in G_1. (b) G_0 arrested population. (c) 24 hours after adding serum, the cells leave G_0 and progress through the cell cycle. (d) Low density culture, 1.5×10^5/60mm dish, 24 hours after adding serum. Colchicine (2 μg/ml) added 1/4 hour after serum. Most of the cell population is still in G_0. (e) High density culture, 4×10^5/60mm dish, 24 hours after adding serum. Colchicine (2 μg/ml) added 1/4 hour after serum. The cells leave G_0 and progress through the cell cycle; some of the cells appear to be accumulating in metaphase. (f) Exponentially growing population incubated with colchicine (1 μg/ml) for 28 hours. The cells leave G_1 (Figure 1a) in the presence of colchicine.

and 14 hours after quiescent 3T3 cells are returned to DME + 10% calf serum, inhibit DNA synthesis. This indicates that disruption of microtubules late in G_0 inhibits DNA synthesis.

DISCUSSION

A role for cell density in regulating the arrest of 3T3 cells. Density dependent inhibition of 3T3 cells is thought to be due to depletion of serum factors in growth medium (2, 14, 15) or to a diffusion boundary layer that limits the accessibility of serum factors to the cells (16). The experiments reported here were with subconfluent cultures so that the variable was cell-cell contact. This varied from negligible contact at the lowest cell density to all cells having some intercellular contact at the highest density. Serum or medium components were not limiting (6) (also Figure 1) indicating that the

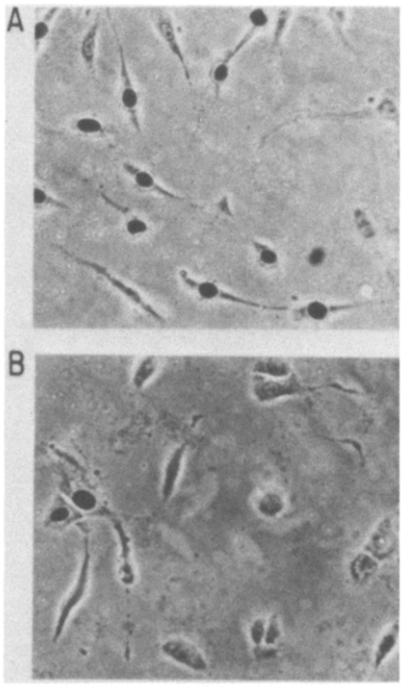

Figure 2: Autoradiographic analysis of the effect of lumicolchicine and colchicine on mitogenesis in sparse cultures of 3T3 cells arrested in G_o. Lumicolchicine (2 μg/ml) or colchicine (2 μg/ml) was added, one hour after serum, to cultures of 3T3 cells (1.2×10^5 cells/60mm dish) that had been arrested in G_o. The dishes were pulsed with ^{3}H-thymidine (2 μCi/ml, 3 μM) for 15 to 24 hours after serum was added. Then the medium was removed and autoradiographic analysis of ^{3}H-thymidine incorporation into DNA into cells in dishes containing serum, alone, or with colchicine, or lumicolchicine was carried out. Incorporation of ^{3}H-thymidine into acid precipitable DNA in parallel cultures was also determined as described in Materials and Methods. Figure 2a is an autoradiograph (100x magnification) of the dish which had 2 μg/ml of lumicolchicine added to the medium. The result is similar to that of the serum control (not shown). Figure 2b is an autoradiograph (100x magnification) of the dish which had 2 μg/ml of colchicine added to the medium. A comparison of figure 2a and b shows that colchicine inhibits mitogenesis in 3T3 cells arrested in G_o at 1.2×10^5 cells /60mm dish and that lumicolchicine had no effect on mitogenesis. The parallel cultures that were analyzed for incorporation of ^{3}H-thymidine into acid precipitable DNA showed that colchicine inhibited DNA synthesis by 74% while lumicolchicine had no effect on DNA synthesis.

density dependent effects (Table 1) are due to changes in the cells themselves.

At least two different restriction points or "A" states exist in 3T3 cells. The arrest of fibroblasts by limiting serum or low molecular weight nutrients in the growth medium can be described as occurring at a restriction point (4) in G_o or at an "A" state (5) in

G_1. The arrest state appears to be independent of the arrest procedure (3, 4). The results presented in Table 1 and Figure 1 show that 3T3 cells can arrest at either a restriction point or "A" state where they can re-enter the cell cycle in the presence of colchicine or a restriction point or "A" state were mitogenesis is inhibited by colchicine. Cell density appears to regulate at which restriction point or "A" state arrest occurs.

Growth regulation and microtubule containing structures. The results presented in Table 1 and the observation that lumicolchicine does not inhibit mitogenesis in 3T3 cells arrested at low cell density shows that these cells require an intact microtubule structure to enter S phase. Submembranous cytoskeletal structures containing microtubules appear to regulate the mobility and configuration of receptors on the cell surface (17-20). As discussed previously (13), disruption of receptor configurations would be expected to interfere with the action of serum factor(s) that are required for cell growth.

Colchicine inhibits mitogenesis in other types of cells arrested in G_o such as lymphocytes (21), mouse neuroblastoma C1300 cells (13), rat neuroblastoma B65 cells, dog kidney MDCK cells, and rat glioma C6 cells (M. Baker, unpublished results). This suggests that structures that contain microtubules have a general role in the regulation of mitogenesis from G_o.

Changes in the cell membrane accompany changes in cell growth (18, 22-26). For example, McNutt et al. (24) found that dense arrested 3T3 cells had a much more extensive submembranous cytoskeletal structure than exponentially growing 3T3 cells or dense SV40 transformed 3T3 cells. However, it has not been clear whether the changes in submembranous cytoskeletal structures are the result of, or the cause of, changes in cell growth. The results reported here (Table 1, Figure 1) and in previous study (13) show that colchicine has a different effect on mitogenesis in cells in G_1 and in G_o. This indicates that changes in submembranous cytoskeletal structures containing microtubules are coupled to differences in growth regulation.

The data presented in Table 1 show that colchicine additions 10 and 14 hours after quiescent 3T3 cells are returned to DEM + 10% calf serum inhibit DNA synthesis. This indicates that disruption of microtubule containing structures late in G_0 inhibits DNA synthesis. This observation and that of Brooks (6) showing a requirement for a serum-dependent event late in G_0 for the entrance of 3T3 cells into S phase indicate that the traverse through G_0 is effected by a dynamic interplay betweeen events on the cell surface and events inside the cell such as the pleiotype events (27), alterations in concentrations of cyclic nucleotides (28-30), or nutrients (27, 31).

The results reported here indicate that cell density must be considered in studies with 3T3 cells arrested in G_0. In particular, some biological processes dependent on microtubule containing structures would be expected to depend on cell density.

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