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Initiation of DNA Synthesis in Cell Cultures by Colcemid

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ABSTRACT **Mitotic inhibitors (colcemid, colchicine, and vinblastine) initiate DNA synthesis in dense stationary cultures of mouse embryo fibroblast-like cells. This initiation is not due to any changes in local cell population density. Relationships between the activation of proliferation and other changes of interphase fibroblast-like cells produced by the mitotic inhibitors (disappearance of cytoplasmic microtubules and activation of movements of cell surface) are discussed.**

In dense cultures of embryo fibroblast-like cells proliferation is inhibited; this inhibition is local and seems to be associated with the changes of the cell surface (1). Experiments presented in this paper show that several substances interfering with the formation of microtubules in the cells (the so-called "metaphase inhibitors" colchicine, colcemid, vinblastine) are able to initiate DNA synthesis in these stationary cultures.

The starting point of these experiments was the finding that one of these inhibitors, colcemid, produces considerable changes in the locomotor activity of interphase fibroblast-like cells (2). In normal moving cells of this type it was found that only part of the edge (the so-called leading edge) actively changed its form by forming and withdrawing local protrusions; other parts of the edge, especially those that were in contact with neighbor cells, remained stable. In colcemid-treated cells, however, all of the edge eventually became active. The first stage of contact inhibition was unaffected by colcemid: the forward movement of the surface protrusion stopped when the protrusion touched the surface of another cell. However, subsequent formation of the stable areas of the surface in the vicinity of the contact did not take place; formation and withdrawal of protrusions continued to go on in these zones for a long time. This suppression of "stabilization" of cell surfaces was accompanied by the inhibition of the formation of microtubules under these surfaces. These data suggest that microtubule formation is essential for stabilization.

Activation of the whole cell edge made colcemid-treated cells unable to translocate directionally, e.g., to migrate into the wound in the culture. These colcemid-induced changes were completely reversible (2). Colcemid did not affect actions that were dependent on local movements of small parts of cell surface, e.g., phagocytosis (3) or migration from the grooves (4).

Activation of the movements of the cell surfaces in colcemid-treated cultures suggests that substances of this type can initiate proliferation in stationary monolayers. This suggestion is based on the assumption (see detailed discussion in ref. 5) that a set of reactions leading to growth and mitosis is switched on by surface changes which temporarily decrease the isolation of the cell interior from the external environment. Diverse agents that may cause surface changes of this type (e.g., by increasing the permeability of the cell membrane or by removing the outer cell coats) can be expected to initiate the proliferation. Activation of the surface movements is likely to be accompanied by these changes (5).

These considerations induced us to perform the experiments described below.

MATERIALS AND METHODS

Stationary cultures of the first passage of mouse embryo cells grown on coverslips in flasks containing penicillin were used. The following inhibitors were used: colcemid (Ciba, Switzerland) or chemically identical colchamine (Sojusreactiv, USSR); colchicine (Merck, DBR); vinblastine (Richter, Hungary). Culture medium consisted of 45% basal Eagle medium, 45% lactalbumin hydrolysate, and 10% bovine serum; the medium was changed every 48 hr.

Mitotic inhibitors, dissolved in 0.1 or 0.2 ml of saline, were added to the medium of the cultures 174 hr after seeding and 30 hr after the last medium change; the same volume of saline was added to control cultures. No change of the medium was made during the period of incubation. [^{3}H]thymidine (Radiochemical Centre, Amersham, England; 8.6 Ci/mmol) was added to the cultures to assay DNA synthesis; cultures were then fixed and examined autoradiographically. In the experiments with pulse labeling, [^{3}H]dT (1 μCi/ml) was added 30 min before fixation. In the experiments with continuous labeling, [^{3}H]dT (1 μCi/ml) was added together with the inhibitor and remained in the medium throughout the incubation (3–30 hr). The cultivation procedures and autoradiography were identical with those described earlier (6).

The percentage of labeled interphase cells (labeling index) was determined for each culture. The percentage of labeled mitoses and the mean number of grains per labeled nucleus were also determined in some cultures. Three or more cultures were used to test each concentration of an inhibitor in a single experiment; each experiment was repeated three or more times.

RESULTS

The labeling index (Table 1) of the cultures incubated for 24 hr with colcemid (effective concentrations (0.05–0.4 μg/ml), colchicine (0.05–0.1 μg/ml), or vinblastine (0.05 μg/ml) was in all the experiments considerably higher than in those of the control cultures. This increase was observed in the experiments with pulse-labeled and with continuously-labeled cultures. It was not accompanied by any significant changes in the mean number of grains per labeled nucleus. These results show that the three metaphase inhibitors are able to induce in-

TABLE 1. *Effects of mitotic inhibitors on DNA synthesis in stationary cultures of mouse embryo fibroblast-like cells*

Inhibitor	Concentration (μg/ml)	% Interphase cells labeled with [³H]dT after 24 hr of incubation			
		Pulse labeling (30 min)		Continuous labeling (24 hr)	
		Expt.	Control	Expt.	Control
Colcemid (Ciba)	0.1	13.3 ± 1.52	5.0 ± 0.78	42.1 ± 3.41	32.1 ± 2.16
	0.2	24.6 ± 1.86	4.6 ± 0.35	47.1 ± 2.48	
	0.4	26.4 ± 2.68		46.9 ± 2.38	
(Sojuzreaciv)	0.01*	5.0 ± 0.68	4.5 ± 0.40	NT	NT
	0.05	14.7 ± 0.96	4.3 ± 0.71	39.0 ± 2.55	29.2 ± 1.46
	0.1	19.9 ± 1.08	4.6 ± 0.51	43.3 ± 1.70	27.6 ± 1.28
Colchicine	0.05	24.8 ± 2.13	4.6 ± 0.35	40.0 ± 2.37	32.1 ± 2.16
	0.1	29.8 ± 3.07		43.6 ± 3.00	
Vinblastine	0.01	8.3 ± 0.86	5.7 ± 0.30	24.8 ± 1.03	27.4 ± 1.52
	0.02	7.5 ± 0.56	3.4 ± 0.12	28.6 ± 2.12	27.7 ± 1.22
	0.05	17.1 ± 2.66	4.9 ± 0.51	42.5 ± 3.21	28.1 ± 2.17

Each horizontal row summarizes the results of 3–4 Expts.; means ± SE shown.
NT, not tested.
* Metaphase block was not observed.

creased entry of the cell into the S phase of the mitotic cycle. All concentrations of the inhibitors that induced significant increases of the labeling index also caused complete metaphase block: anaphases and telophases were not seen in these cultures. These concentrations also produce changes in locomotion and in cell form in the same system (2).

To test the effect of cell population density on the cell entry into S phase, "density-adjusted labeling indices" were determined in several paired control and colcemid-treated cultures. The total number of cells and the number of labeled cells were counted separately in each of 100 randomly chosen fields of view of the microscope, the area of each being 8.7×10^{-3} mm². Then, labeling indices for areas with various cell densities were calculated. The indices in colcemid-treated cultures were always considerably higher than those in areas having the same cell densities in paired control cultures (Fig. 1). Thus, increased cell entry into S phase in colcemid-treated cultures was not a result of any changes in cell population densities.

Experiments with pulse-labeled cultures had shown that the fraction of DNA-synthesizing cells in the cultures increased significantly about 12 hr after the addition of the inhibitor and continued to increase thereafter. Fig. 2 shows the time course of the effects of colcemid in detail. In continuously labeled control cultures (Fig. 2*B*), the labeling index increased with time not only because new cells entered S phase but also because previously labeled cells were doubled in number after mitosis. This doubling did not take place in colcemid-treated cultures and, therefore, the labeling index was lower than in control cultures until 16 hr. Later this effect was overruled by increased entry of cells into S phase.

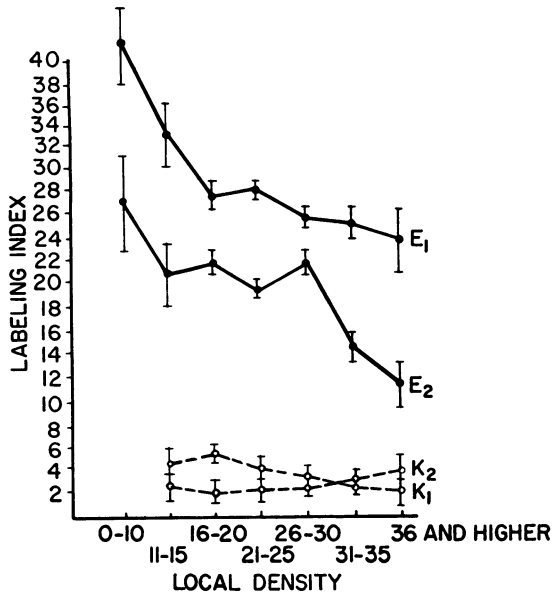

FIG. 1. Labeling indices in areas of cultures having various local cell densities after pulse labeling with [³H]dT. E_1, E_2, experimental cultures (incubated 24 hr with 0.1 μg/ml of Colcemid). K_1, K_2, controls. Abscissa, number of cells per field of view. Values are means ±SE.

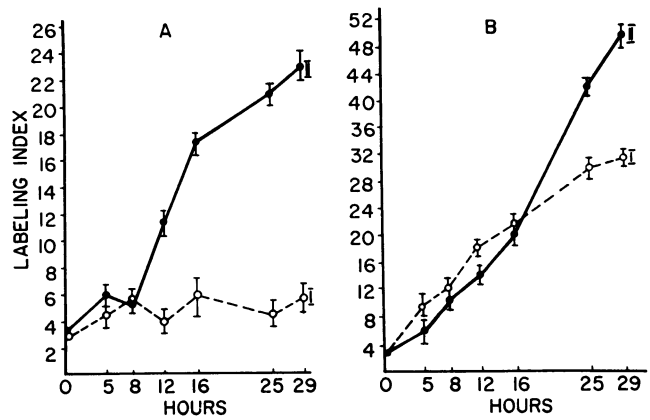

FIG. 2. Time course of the changes in [³H]dT incorporation induced by colcemid (0.1 μg/ml) in stationary cultures (means ±SE). Abscissa, time of incubation with colcemid. O, Control cultures. ●, Colcemid-treated cultures. *A*, Pulse labeling. *B*, Continuous labeling.

In the experiments with continuous labeling, all the mitotic figures in control cultures became labeled at about 8 hr; in colcemid-treated cultures, 75–100% of the mitoses became labeled after 12–16 hr. This result seems to indicate that cells in colcemid-treated cultures enter mitosis some time after passage through S phase. More exact determination of the kinetics of this entry into mitosis was impossible because the destruction of some mitotic cells and (or) reversal of blocked mitoses in colcemid-treated cultures (7) could affect the counts of mitoses.

CONCLUSION

Initiation of DNA synthesis in stationary cultures of fibroblast-like cells can be induced by agents of several types: (*a*) by wounding of the culture (1); (*b*) by the factor (or factors) contained in fresh serum (1, 6, 8); (*c*) by various enzymes such as proteases (9), hyaluronidases (6), and ribonuclease (6); (*d*) by detergents such as lysolecithin (10) and digitonin (6). Proliferative reactions induced by the agents of the last three groups are not preceded by any changes in cell population density. Experiments described in this paper show that a new group has to be added to this list of activating agents, namely substances that affect microtubular structures (mitotic inhibitors) (11). The time course of the initiating effect of these substances is similar to a few other agents (6).

Many activating agents were shown to become toxic when the conditions of these experiments are changed (6). In these and previous experiments (2), we observed no significant toxic changes in the interphase cells treated with mitotic inhibitors. However, in a few other experimental systems, certain toxic effects of these inhibitors, e.g., inhibition of DNA synthesis, have been described (12).

Thus, mitotic inhibitors, besides producing a characteristic metaphase block, cause at least three types of alterations in the interphase fibroblast-like cells: disappearance of cytoplasmatic microtubules (2), disappearance of the stable parts of the cell surface (2), and initiation of the DNA synthesis in dense cultures. Similar concentrations of inhibitors produce each of these alterations.

It would be interesting to discover the relationships between these alterations. We have mentioned above the suggestion that the initiation of proliferation by all types of activating agents is a consequence of surface changes (5). This generalization seems to have some heuristic value: it has helped to reveal the ability of mitotic inhibitors and of a few other activating agents (6) to initiate DNA synthesis. Little is known, however, about the real mechanisms of action of these agents.

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