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Pertussis Toxin Inhibits Colchicine-Induced DNA Synthesis in Human Fibroblast

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Several lines of evidence indicate that microtubule depolymerization initiates DNA synthesis or enhances the effects of serum or purified growth factors in many types of fibroblasts. Yet little is known about the intracellular events responsible for the mitogenic effect of microtubule disrupting agents. The effects of antitubulin agents on DNA synthesis in sparse and dense cultures in the presence or absence of serum and possible involvement of G-proteins in their mitotic action were examined. In these studies, colchicine by itself appeared to be mitogenic only for confluent quiescent human lung fibroblasts. In sparse culture, however, colchicine inhibited serum-stimulated DNA synthesis. Colcemid, another antitubulin agent, showed similar effects of growth inhibition and stimulation in sparse and confluent cultures while lumicolchicine, inactive colchicine, did not. The mitogenic effect of two antitubulin agents, colchicine and colcemid, was partially inhibited by pertussis toxin. These data suggest that microtubular integrity is associated with the expression of either negative or positive controls on DNA synthesis and mitogenic effect of antitubulin agents may be partially mediated by pertussis toxin-sensitive G protein.

Key words: Colchicine, Colcemid, Lumicolchicine, Microtubule depolymerization, DNA synthesis, Pertussis toxin, G protein

INTRODUCTION

Microtubules are an integral component of the cellular cytoskeleton as well as of the mitotic apparatus. In addition to structural functions, microtubules carry out a number of other cellular functions including a crucial regulatory function in the DNA synthesis. Although several lines of evidence have accumulated to support the suggestion that the state of microtubule assembly is involved in regulating DNA synthesis, conflicting reports on the action of antitubulin agents on DNA synthesis have appeared, involving different cell types, different culture conditions and different methodologies (Baker, 1976, 1977; Edelman, 1976; Friedkin *et al.*, 1979; McClain and Edelman, 1980; Shinohara *et al.*, 1988, 1989; Walker, 1979). In many types of fibroblasts, colchicine and other antitubulin agents are known to initiate DNA synthesis or enhance the effects of serum or purified growth factors (Crossin and Carney, 1981; Friedkin *et al.*, 1979; McClain and Edelman, 1980; Vasiliev *et al.*, 1971). However, little is known about the intracellular events leading to initiate DNA synthesis by antitubulin agents.

Tubulin, the major constituent protein of microtubule, is itself a kind of GTP-binding protein with intrinsic GTPase activity and has significant functional and structural homology with the signal-transducing G proteins (Sternlicht *et al.*, 1987). Recently, tubulin is known to form complexes with specific G proteins and these complexes might provide a locus for the interaction of cytoskeletal components and signal transduction cascades(Wang *et al.*, 1990).

In this study, we determined the effects of colchicine, colcemid, analog of colchicine and lumicolchicine, inactive form of colchicine on DNA synthesis in fibroblasts. We also investigated whether pertussis toxin-sensitive G protein are involved in colchicine-induced DNA synthesis.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Grand Island, N.Y., USA). Trypsin, bovine serum albumin (BSA), colchicine, colcemid, lumicolchicine and

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pertussis toxin were obtained from Sigma (St. Louis, MO USA). [methyl-^{3}H] Thymidine (6.7 Ci/mmole) was perchased from New England Nuclear (Boston, Massachusetts, USA). Other chemicals used were of analytical grade.

Cell Culture

Human lung fibroblast (HEL299) was obtained from American Type Culture Collection (ATCC). The cells were grown in DMEM supplemented with 3.7 μg/L sodium bicarbonate, 100 units/ml penicillin G, 100 μg/ml streptomycin and 10% FBS. They were kept at 37℃ in a humidified atmosphere of 5% CO_2, 95% air incubator. All studies by human fibroblast were performed on subcultivations 10 to 20.

Quiescent cultures of these cells were obtained in the following way. Cells were rinsed, removed from stock 100 mm culture dishes with Hanks' balanced salts solution (HBSS) containing 0.53 mM EDTA and 0.05% trypsin. They were resuspended in DMEM with 10% FBS and plated in 96-well or 24-well plates (using 0.2 ml or 1 ml culture medium, respectively) at densities as desired for each experiment. After the cells had attached (overnight for sparse cultures or 72hr for confluent cultures), the serum-containing growth medium was removed and replaced with quiescent medium (DMEM containing 1% BSA). The cells were then incubated at 37℃ for 48 hr prior to the specified additions. These cells were quiescent under these conditions.

Quiescent cultures were treated with microtubule disrupting drugs which were present throughout the incubation unless otherwise indicated. For the experiments with pertussis toxin, cells were pretreated with pertussis toxin for 3 hr prior to exposure to antitubulin agents.

Measurement of DNA Synthesis

The mitogenic effect of the various additions was determined by incorporation of ^{3}H-thymidine for 24 hr after additions.

Quiescent human fibroblasts in 96-well plates were incubated for 24 hr with agents in 0.2 ml DMEM containing ^{3}H-thymidine (1 μCi/0.2 ml). The cells were rinsed twice in HBSS , trypsinized and harvested on glass fiber filter paper using a multiple automated sample harvester (Dynatech, USA). The glass fiber filter was dried at 100℃ for one hour and put into the vial containing five ml of scintillation cocktail (5.5 g PPO, 0.5 g dimethyl POPOP, 333 ml triton X-100 and 667 ml toluene). The radioactivity was counted for five minutes in a liquid scintillation counter (Packard, USA).

DNA synthesis was expressed as the mean dpm per well ± SD for quadruplicate determinations.

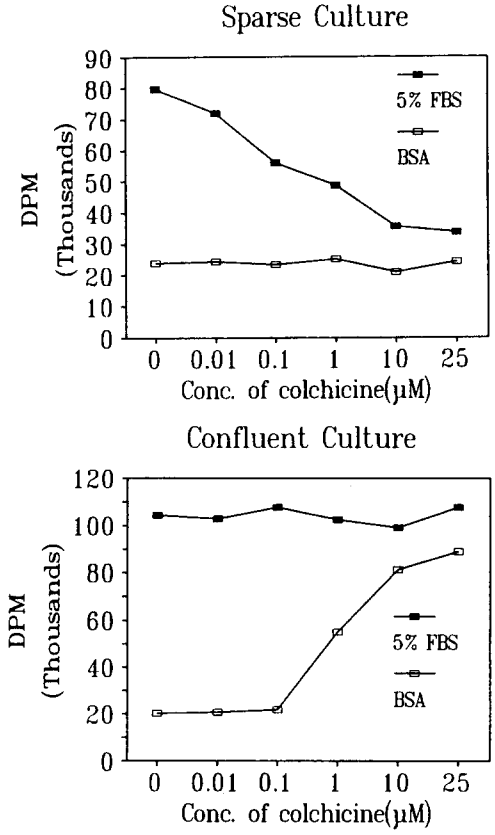

Fig. 1. Effect of colchicine on DNA synthesis by human lung fibroblast in different culture conditions.
Human fibroblasts were plated in 96-well plates in 0.2 ml DMEM with 10% FBS, at 5×10^3 cells per well for sparse culture and at 2×10^4 cells per well for confluent culture. Quiescent cultures were prepared as described in Materials and Methods. After a 24 hr exposure to various concentrations of colchicine in DMEM containing ^{3}H-thymidine (1 μCi) with or without 5% FBS, radioactivity was measured as described in Materials and Methods.

RESULTS

Effect of Antitubulin Agents on DNA Synthesis

In sparsely plated human lung fibroblasts, colchicine markedly inhibited the stimulation on DNA synthesis by 5% FBS but addition of colchicine in the absence of serum had no effect (Fig. 1). In contrast to sparse cultures, the addition of colchicine to the quiescent confluent human fibroblasts initiated thymidine incorporation into DNA in the absence of any growth factors or serum (Fig. 1). The thymidine incorporation in confluent human fibroblasts was stimulated half-maximally and maximally by 1 μM and 25 μM of colchicine, respectively. The maximal level was about 4-5 fold of control and attained about 80% of that induced by 5% FBS in several independent experiments.

Further experiments were done to elucidate the na-

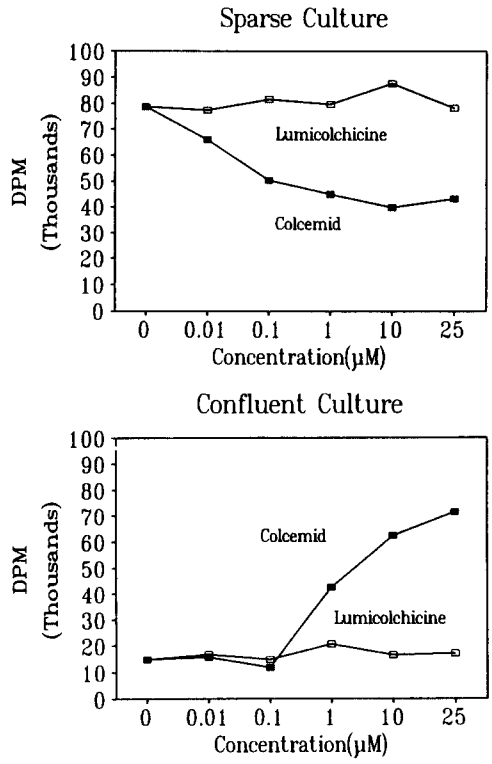

Fig. 2. Dose-response effect of colcemid and lumicolchicine on DNA synthesis by human lung fibroblast.
Quiescent cultures of human fibroblast were treated with various concentrations of colcemid and lumicolchicine. Sparse cultures (5000 cells per well in 96-well plates) were incubated in DMEM with 5% FBS and confluent cultures (2×10^4 cells per well in 96-well plates) in serum-free medium. DNA synthesis was measured as described in Materials and Methods.

ture of the colchicine effects on DNA synthesis. Colcemid augmented stimulation of dense cells and inhibited stimulation of sparse cell (Fig. 2). On the other hand, lumicolchicine at various concentrations did not stimulate or inhibit thymidine incorporation in both sparse and dense culture (Fig. 2).

Pertussis Toxin Inhibits Antitubulin Agents-Induced DNA Synthesis

The antitubulin agents-induced DNA synthesis in quiescent confluent culture was so marked that analysis of the mechanisms could be initiated. To understand the effects of pertussis toxin on confluent culture of mitogenically active cells, the amount of DNA synthesis induced by 10 μM colchicine in the presence of various pertussis toxin concentrations was measured. As shown in Figure 3, inhibition begins at 0.1 ng/ml pertussis toxin and is maximal (50% inhibition) at 100-1000 ng/ml pertussis toxin. In this case, the extent of inhibition by pertussis toxin was dose dependent while pertussis toxin alone did not show any effect on DNA synthesis. Figure 4 illustrates a parallel experiment with 10 μM colcemid and lumicolchicine in the presence of pertussis toxin. Cells pretreated with 100 ng/ml pertussis toxin, appoximately the same concentration required to block 50% of colchicine-induced DNA synthesis, were also inhibited about 50% of col-

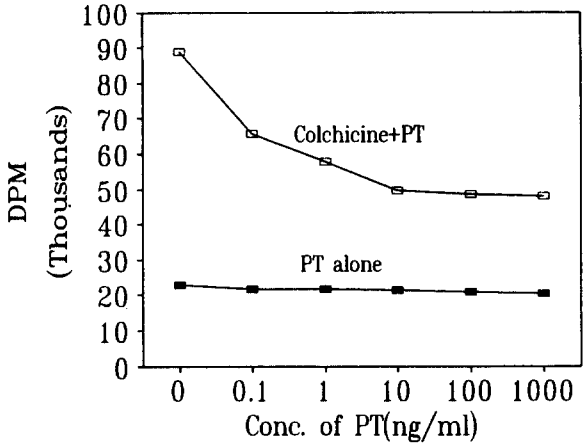

Fig. 3. Effect of pertussis toxin on colchicine-induced stimulation of DNA synthesis in human lung fibroblast.
Human fibroblasts were seeded at 2×10^4 cells per well in 96-well plates and cultured confluently. Quiescent cultures were pretreated with various concentrations of pertussis toxin in DMEM containing ^{3}H-thymidine (1 μCi) for 3 hr prior to addition of 10 μM colchicine. ^{3}H-Thymidine incorporation was terminated 24 hr after colchicine addition and pertussis toxin alone was incubated for 27 hr.

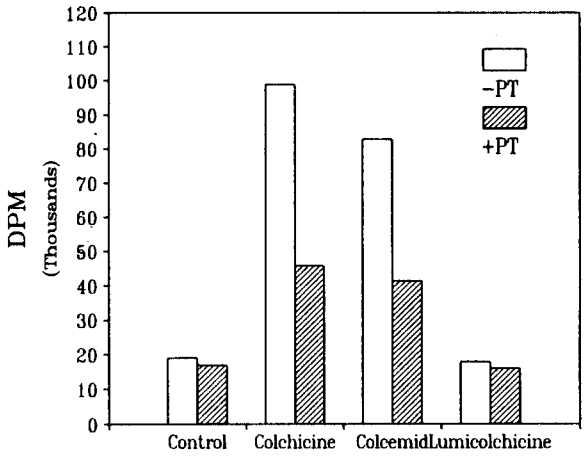

Fig. 4. Effect of pertussis toxin on colchicine and colcemid induced stimulation of DNA synthesis in human lung fibroblast.
Cells were seeded at 2×10^4 cells per well in 96-well plates and cultured confluently. Quiescent human fibroblasts were incubated for 27 hr with pertussis toxin 100 ng/ml in DMEM containing ^{3}H-thymidine (1 μCi). Colchicine (10 μM), colcemid (10 μM) and lumicolchicine (10 μM) were added for the last 24 hr DNA synthesis was measured as described in Materials and Methods.

cemid-stimulated DNA synthesis. On the other hand, lumicolchicine and pertussis toxin did not affect DNA synthesis (Fig. 4).

DISCUSSION

In this paper, it was found that the effect of microtubule disruption on DNA synthesis varied depending on cell confluency. Colchicine by itself appeared to be mitogenic only for confluent human lung fibroblast but it did not augment DNA synthesis stimulated by serum. In sparsely plated human lung fibroblasts, serum-induced DNA synthesis was inhibited by colchicine. Colcemid, another antitubulin agent, showed similar effects of growth inhibition and stimulation in sparse and confluent cultures of human lung fibroblast whereas lumicolchicine, a derivative that does not bind to tubulin and has no antimitotic action, did not. These results indicated that microtubule depolymerization by antitubulin agents could modulate the expression of either negative or positive controls on DNA synthesis in different culture conditions. Many conflicting data concerning the effect of colchicine on cell proliferation have been reported. For example, colchicine inhibits events leading to initiation of DNA synthesis in lymphocytes (Edelman, 1976), neuroblastoma cells (Baker, 1976), hepatocyte (Walker *et al.*, 1977, 1978), and some cultured fibroblasts (Walker *et al.*, 1977). By contrast, microtubule depolymerization initiates DNA synthesis (Crossin and Carney, 1981; Vasiliev *et al.*, 1971) or enhances the effects of serum or purified growth factors in many types of fibroblasts (Friedkin *et al.*, 1979; Friedkin and Rozengurt, 1981; McClain and Edelman, 1980; Otto *et al.*, 1979, 1981; Teng *et al.*, 1977). McClain and Edelman (1980) resolved these conflicts in part. They reported that microtubule assembly was involved in the regulation of DNA synthesis, depending on the density or lineage of the cells in culture. These results are well consistent with results presented here. The growth state of a cell is apparently the result of a complex integration of multiple signals, including cell density, the strength of the stimulus and undefined fluctuation in cytoskeletal assemblies. The molecular details of these connections remain to be elucidated. How then might the absence of the cytoplasmic microtubules by colchicine initiate DNA synthesis? This question is particularly difficult because depolymerization of microtubules leads to a multiplicity of effects in the cell.

Rapidly accumulating informations on hormone-receptor interactions suggest many alternative mechanisms by which disassembly of microtubules might enhance mitogenic stimulation of DNA synthesis (Kahn, 1976). The distribution of membrane components is markedly altered upon distribution of the cytoskeleton (Nicolson, 1976). It is conceivable that the interaction between receptor-bound mitogens and as-yet-unknown effectors could be facilitated by increased membrane fluidity characteristic of cells with deficient cytoskeleton integrity (Rozengurt, 1976; Teng *et al.*, 1977). Increased membrane fluidity might also increase the rate of return of newly synthesized receptors to the outer surface. Friedkin *et al.* (1981) support the premise that after internalization the receptor-mitogen complex may remain active within endocytic vesicles when cells are exposed to colchicine. Carney *et al.* (1986) suggest two possible mechanisms for the involvement of microtubules in initiating proliferative events. First, it might be possible that a subset of microtubules near the cell surface mediate normal mitogen signal generated by growth factor interaction with specific cell surface receptors. A second possibility is that perturbation of the equilibrium between free tubulin and polymerized microtubules is a part of the mitogenic signal. But, little is known about the intracellular events leading to initiate DNA synthesis by antitubulin agents.

In a variety of transmembrane signaling systems, cell surface receptors are linked to effectors by GTP-binding (G) proteins which function as signal transducers. Several of these proteins have been described, and their classification is based, primarily, on the effector molecules which each species of G protein appeared to engage. Pertussis toxin can covalently modify the G proteins by the addition of an ADP ribose group to the subunit. This toxin is enzyme which transfers ADP-ribose from NAD to specific receptor sites on the G protein (Katada *et al.*, 1980; West *et al.*, 1985). Tubulin, the main constitutive protein of microtubule, is itself a kind of GTP-binding protein with intrinsic GTPase activity and siginificant functional and structural homology with the signal-trasducing G proteins (Mandelkow *et al.*, 1985; Sternlicht *et al.*, 1987). Tubulin is also a substrate for pertussis toxin-catalized ADP-ribosylation (Lim *et al.*, 1985). The results presented here indicate mitogenic effect of antitubulin agents may be partially mediated by pertussis toxin-sensitive G protein. These observations leave open the possibility that the interaction and nucleotide exchange between G proteins and tubulin may exist. Lately, Wang *et al.* (1990) suggest that tubulin forms complexes with specific G proteins and these complexes might provide a locus for the interaction of cytoskeletal components and signal transducing cascades.

The current studies were undertaken in an effort to understand how colchicine alone initiates DNA synthesis by confluent human fibroblast. In the present study, it appeared that the mitogenic effect of antitubulin agents, colchicine and colcemid, was inhibited by pertussis toxin. These results suggest that antitubulin agents induce DNA synthesis by a mechanism involving pertussis toxin-sensitive G protein, in part.

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