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Inhibition of Collagen Secretion from Bone and Cultured Fibroblasts by Microtubular Disruptive Drugs

(synthesis/hydroxylation/procollagen/colchicine/vinblastine/cytochalasin B)

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ABSTRACT **Collagen synthesis in chick-embryo frontal bone and 3T3 fibroblasts from mice was measured by incorporation *in vitro* of [^{14}C]proline into collagenase-digestible material. About 15–25% of the collagen synthesized by the frontal bone in 60 min, and 60% of that synthesized by the fibroblasts in 2 hr, was found to be soluble in the culture medium. The microtubular disruptive drugs colchicine and vinblastine, at 10 μM, inhibited collagen secretion in both systems almost completely. Formation of collagen hydroxyproline from proline was not inhibited by these drugs. Cytochalasin B, which impairs microfilament function, had no effect on collagen secretion. Our results support the theory that collagen is transported in vesicles to the cell membrane, where it is secreted. This conclusion is based on the similarity of the collagen-secreting system to other systems in which the movement of secretory vesicles or storage granules is inhibited by microtubule disruption.**

It has been known for some time that colchicine and vinblastine can bind to mitotic spindle protein and arrest mitosis; more recently, it has been shown that these alkaloids can dissociate the microtubule structure into its subunits (1). By the use of these agents, it has also been demonstrated that microtubules are involved in the secretion of thyroid hormone by mouse thyroid glands (2), of insulin by islets of Langerhans from the rat (3), of histamine by rat mast cells (4), and are involved in intracellular transport of amine granules by nerve cells (5), and melanin granules in melanocytes in frog skin (6).

In order to better understand the mechanism by which collagen is transported from the intracellular site where it is synthesized to the extracellular matrix, we have undertaken a series of studies with colchicine, vinblastine, and cytochalsin B and have obtained evidence strongly suggesting a role for microtubules in collagen secretion.

Materials

Uniformly labeled L-[^{14}C]proline (15.46 Ci/mol) was purchased from New England Nuclear Corp. Colchicine was obtained from Calbiochem. Vinblastine sulfate (Eli Lilly) was kindly supplied by Dr. M. Shelanski and cytochalasin B (Imperial Chemistry Industry) by Dr. J. Piatagorsky. Plastic tissue culture plates were obtained from Falcon Plastics.

Methods

Frontal bones were removed from 15-day-old chick embryos and cleaned of adhering tissues; individual bones were incubated in 0.5 ml of Eagle's minimal essential medium plus 0.25 mM sodium ascorbate in an atmosphere of 5% CO_2–95% air. After 30 min of incubation in the presence or absence of 10 μM colchicine, 10 μM vinblastine, or 10 μg/ml of cytochalasin B, 1 μCi of L-[^{14}C]proline was added and the incubation was extended for an additional 60 min. The bones were removed, homogenized in 1 ml of 0.05 M Tris·HCl (pH 7.6) in a stainless-steel mortar, and then sonicated for 20 sec with a Branson sonifier (3 amp). Protein was prepared from the sonicate and digested (7) with purified, protease-free bacterial collagenase. In this procedure, radioactivity released by collagenase and remaining in the supernatant after addition of 5% trichloroacetic acid–0.25% tannic acid is a measure of the collagen synthesized by the bone, while noncollagen protein is precipitated. Carrier protein (1 mg of chick-embryo protein) was added to the medium and this also was analyzed for [^{14}C]collagen and [^{14}C]noncollagen protein.

Mouse fibroblasts (Balb 3T3) were obtained from Dr. E. Scolnick and cultivated at 5×10^5 cells per plate in Eagle's minimal essential medium containing 10% fetal-calf serum, 25 mM tricine buffer (pH 7.4), and 13 mM bicarbonate in an atmosphere at 5% CO_2–95% air. When a cell concentration of 4×10^6 cells per plate (100 × 15 mm) was obtained, sodium ascorbate was added to the growth medium to give a final concentration of 0.25 mM. After 2 hr of incubation at 37°, the cells were washed free of growth medium and 3 ml of fresh medium containing ascorbate, but no serum, was added. In addition, the medium added to half of the plates contained 10 μM colchicine. After 30 min of incubation at 37°, 1.5 μCi of L-[^{14}C]proline was added and the cells were incubated for

TABLE 1. *Effect of 10 μM colchicine on collagen synthesis and secretion by frontal bone*

Exp.	Conditions	Radioactivity in collagen (dpm) Medium	Bone	Total	Fraction in medium (%)	Inhibition of secretion (%)
1.	Control	27,686	83,254	110,940	25.0	—
	Colchicine	5,314	60,386	65,700	8.1	67.7
2.	Control	14,271	89,871	104,142	13.7	—
	Colchicine	3,857	72,900	76,757	5.0	63.5

Each sample consisted of 2 frontal bones, incubated separately and then combined for analysis. Duplicate samples were used; the data are average values.

an additional 2 hr. The medium was removed, centrifuged at 240 × *g*, dialyzed against 0.01 M Tris·HCl (pH 7.6), and then lyophilized. After the residue was dissolved in 1.0 ml of 0.15 M NaCl, 2 mg of carrier protein was added. The cell layer was removed by scraping, the cells were washed twice with cold, phosphate-buffered saline (pH 7.6), resuspended in 1 ml of 0.05 M Tris·HCl (pH 7.6), and sonicated for 20 sec as above. Protein in the cells and medium was analyzed (7) to determine the amount of [^{14}C]collagen and [^{14}C]noncollagen protein.

RESULTS

Effect of colchicine on protein secretion in frontal bone

Chick-embyro frontal bone, when incubated *in vitro* in culture medium under the conditions used in these experiments, will incorporate [^{14}C]proline into collagen and noncollagen proteins at a linear rate for 2.5 hr. The total incubation time in the experiments described below was limited to 90 min. About 15–25% of the collagen and 8–10% of the noncollagen protein synthesized during the incubation appears in the medium. The results presented in Table 1 show that there is about a 65% inhibition of collagen secretion by frontal bone incubated in the presence of colchicine. This inhibition was calculated by comparing the fraction of collagen in the medium of the control to the fraction of collagen in the medium of the colchicine-treated tissue. This type of comparison was necessary, since there was a decreased amount of incorporation of [^{14}C]-proline into total collagen in the colchicine-treated bone (41% in experiment *1* and 26% in experiment *2*). This decrease may be due to an inhibition of isotope uptake or to an inhibition of one of the reactions involved in protein synthesis.

The effect of colchicine on the secretion of noncollagen protein in the same experiments is presented in Table 2. In this case also, there was decreased incorporation of isotope in the colchicine-treated samples (39% in experiment *1* and 19% in experiment *2*); secretion was inhibited about 63%.

Since it has been reported that the unhydroxylated form of collagen, which we call deoxycollagen*, is not secreted at a normal rate (11), it was necessary to determine if the effect of colchicine on secretion is due to inhibition of proline hydroxylation. Analyses were therefore made to determine the ratio of proline to hydroxyproline in the collagen found in the medium and the cell matrix of the colchicine-treated tissue. The results presented in Table 3 show that collagen in both the bone and medium of the colchicine-treated sample are fully hydroxylated, as indicated by a proline to hydroxyproline ratio of about 1.2. This is the ratio observed in fully hydroxylated collagen (12). Since the enzyme responsible for the conversion of lysine to hydroxylysine has almost identical properties and cofactor requirements as prolyl hydroxylase (13), we assume that colchicine does not inhibit lysyl hydroxylation. Therefore, inhibition of collagen secretion by colchicine cannot be attributed to a lack of deoxycollagen hydroxylation.

TABLE 2. *Effect of 10 μM colchicine on noncollagen protein synthesis and secretion by frontal bone*

Exp.	Conditions	Radioactivity in noncollagen protein (dpm) Medium	Bone	Total	Fraction in medium (%)	Inhibition of secretion (%)
1.	Control	7059	69,513	76,572	9.2	—
	Colchicine	1581	45,039	46,620	3.4	63.1
2.	Control	4654	39,390	44,044	10.6	—
	Colchicine	1419	34,451	35,870	4.0	62.7

See footnotes for Table 1; these samples represent the noncollagen protein from the samples in Table 1.

Effect of colchicine on protein secretion by 3T3 cells

The effect of colchicine on collagen secretion was also examined with 3T3 cells. This is an established line of mouse-embryo fibroblasts that actively secrete collagen into the culture medium. As shown in Table 4, control cells secrete up to 60% of the total collagen synthesized during a 2-hr incubation period. If the cells are first incubated in the presence of 10 μM colchicine, only about 12% of the collagen is secreted, an 80% inhibition. Although the fraction of noncollagen protein secreted into the medium is much lower than collagen secretion, there is about the same extent of inhibition by colchicine. There is no concomitant inhibition of either collagen or noncollagen protein synthesis.

Examination of photographs taken after 1 hr of incubation in the absence or presence of colchicine (Fig. 1*a* and *b*) reveal that the long processes characteristic of fibroblasts have been retracted in the colchicine-treated cells. These observations are consistent with results in other cell lines, such as neuroblastoma (14) and KB fibroblasts (15), in which this type of morphological change has been correlated with disruption of microtubules by colchicine.

Effect of vinblastine and cytochalasin B on protein secretion by frontal bone

Vinblastine is another drug that disrupts microtubular structure (16), while cytochalasin B inhibits the function of microfilaments without disrupting microtubules (17). In order to obtain further evidence that colchicine was acting specifically on microtubules, the effect of these two drugs on protein secretion by frontal bone was tested.

TABLE 3. *Effect of colchicine on collagen proline hydroxylation**

Exp.	Conditions	Medium (dpm) Pro	Hyp	Pro Hyp	Bone (dpm) Pro	Hyp	Pro Hyp
1.	Control	13,985	11,970	1.2	—	—	—
	Colchicine	3,081	2,478	1.2	—	—	—
2.	Control	8,385	5,883	1.4	50,394	41,112	1.2
	Colchicine	2,214	1,494	1.5	45,384	36,723	1.2

* Analyzed as in ref. 7.

* Recently, a possible precursor of collagen has been observed and has been called "procollagen" (8). The α-chains of this molecule have been called "pro-α-chains", while such chains in which proline and lysine are unhydroxylated have been termed "protopro-α-chains" (9). Since these terms may well be confused with the term "protocollagen," which has been used to describe unhydroxylated collagen (10), we suggest that the more chemically descriptive term "deoxycollagen" be used to describe this molecule. Individual chains of such unhydroxylated collagen would be deoxy-α-chains and precursors would be prodeoxy-α-chains.

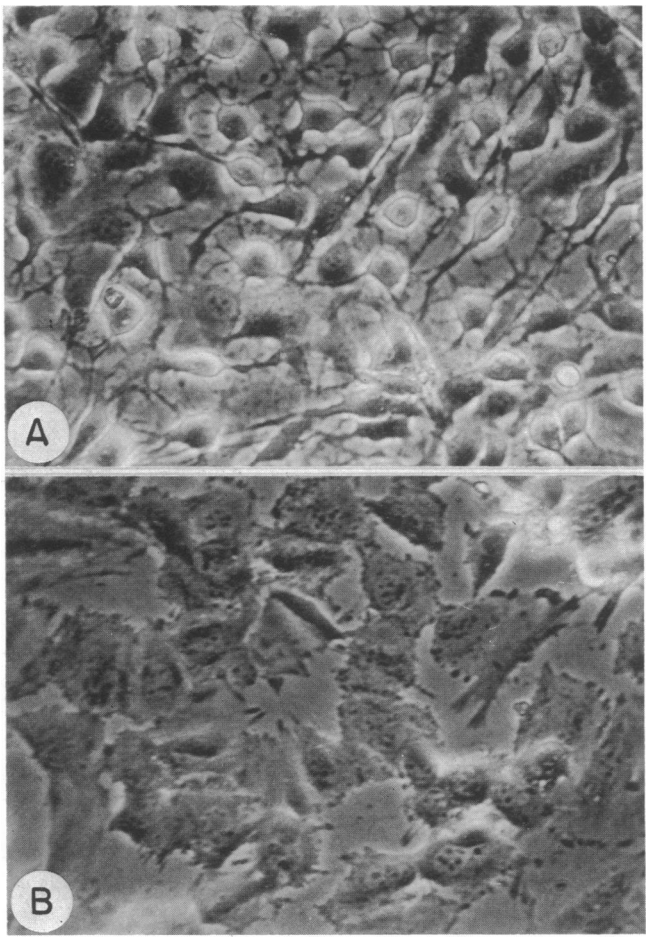

FIG. 1. The effect of colchicine on the morphology of 3T3 fibroblasts. Phase-contrast photograph taken after 1 hr of incubation in the absence (*A*) or presence (*B*) of 10 μM colchicine.

The results presented in Table 5 demonstrate that vinblastine almost completely inhibited collagen secretion (86%), while cytochalasin B had no effect. Noncollagen protein secretion was also inhibited by vinblastine, but not by cytochalasin B (Table 6). In this experiment, a separate control containing

TABLE 4. *Effect of 10 μM colchicine on collagen secretion by 3T3 fibroblasts*

Conditions	Radioactivity in collagen (dpm) Medium	Cell layer	Total	Fraction in medium (%)	Inhibition of secretion (%)
Control	5374	3,793	9,167	58.6	—
Colchicine	1029	7,611	8,640	11.9	79.7
*Radioactivity in noncollagen protein**					
Control	3266	85,281	88,547	3.7	—
Colchicine	831	86,499	87,330	1.0	74.3

Each sample consisted of three tissue culture plates (4×10^6 cells/plate), which were combined for analysis. Duplicate samples were used and the data are average values.

* These samples represent noncollagen protein remaining after collagenase digestion.

TABLE 5. *Effect of 10 μM vinblastine and cytochalasin B on collagen synthesis and secretion by frontal bone*

Conditions	Radioactivity in collagen (dpm) Medium	Bone	Total	Fraction in medium (%)	Inhibition of secretion (%)
Control	17,953	93,030	110,983	16.2	—
Vinblastine	1,661	72,073	73,734	2.3	85.8
Control*	14,715	50,886	65,601	22.4	—
Cytochalasin B†	16,378	44,876	61,254	26.7	0

* Medium contained 1% dimethylsulfoxide.

† 10 μg/ml, in medium containing 1% dimethylsulfoxide.

dimethylsulfoxide was prepared, since it was used to dissolve the cytochalasin B. This solvent reduced [^{14}C]proline incorporation into both collagen and noncollagen protein by about 40%.

Relative rates of collagen synthesis

It was found that the various drugs used in these studies inhibited the incorporation of [^{14}C]proline into both collagen and noncollagen protein by frontal bone. When the effect of these drugs on secretion was calculated, the fraction of protein secreted into the medium was compared to the total amount synthesized in order to eliminate this factor. This method of calculation appears to be valid, since the data presented in Table 7 show that the relative rate of collagen synthesis is not affected by these various drugs, even though incorporation was decreased in frontal bone. In addition, the relative rate of collagen synthesis in 3T3 cells is less than 10% than that of the bone.

DISCUSSION

The results of these studies demonstrate that two different microtubular disruptive agents, colchicine and vinblastine, inhibit collagen and noncollagen protein secretion by frontal bone (Tables 1, 2, 5, and 6); colchicine also inhibited secretion in 3T3 fibroblasts (Table 4). The concentration of colchicine and vinblastine used in these studies (10 μM) is similar to the low concentrations used to effectively inhibit microtubule function in other systems (2–6). It has been reported in a preliminary study that [^{14}C]colchicine, at concentrations 100 times that used in our experiments†, interacts in a reversible but nonspecific manner with salt-soluble collagen. Since the binding of colchicine to microtubular protein occurs at low concentrations, it would appear that this specific binding is responsible for the observed effects on secretion. The specificity of this effect on microtubles was further tested by incubation of bones with cytochalasin B, which impairs the function of microfilaments, another cellular structure thought to be involved in cellular movement (17). This compound had no effect on the secretion of collagen and noncollagen proteins.

Microscopic examination of normal 3T3 cells and those treated with colchicine (Fig. 1*a* and *b*) showed morphological alterations consistent with those observed by other investigators (14, 15) for cells in which microtubule disruption occurred. Furthermore, microtubules have been reported to be

† Nimni, M. E. (1969) *Arthritis Rheum.* **12**, 684.

TABLE 6. *Effect of vinblastine and cytochalasin B on noncollagen protein synthesis and secretion by frontal bone*

Conditions	Radioactivity (dpm) in noncollagen protein			Fraction in medium (%)	Inhibition of secretion (%)
	Medium	Bone	Total		
Control	4,937	54,448	59,385	8.3	—
Vinblastine	1,459	40,406	41,865	3.5	57.8
Control	4,766	30,510	35,276	13.5	—
Cytochalasin B	5,387	31,594	36,981	14.6	0

See footnotes for Table 5; these samples represent the noncollagen protein from the samples in Table 5.

present in osteoblasts and osteocytes of rat and rabbit bone‡; these cells actively synthesize collagen.

Although there was some inhibition of protein synthesis during incubation of frontal bone with these drugs (Tables 1, 2, 5, and 6), the calculation of the extent of inhibition of collagen secretion was based on a comparison of the fraction of collagen found in the medium to the total collagen content in the control and treated sample. Colchicine did not inhibit protein synthesis in 3T3 cells (Table 4).

Colchicine and vinblastine also inhibited the secretion of noncollagen proteins to about the same extent as collagen secretion in both the frontal bone (Table 2) and 3T3 cells (Table 4). Recent reports have presented evidence that collagen is initially synthesized as a procollagen molecule, which has a molecular weight about 20% greater than the native collagen molecule (8, 9). The additional polypeptide sequence occurs at the N-terminus (18), but it is not clear whether all of this sequence, or only a portion of it, is cleaved by collagenase. Any part that is not cleaved would assay as noncollagen protein in our experiments. However, since collagen represents about 40% of the total protein in the medium of frontal bone, and only 20% of the protein in the 3T3 cell medium, the additional polypeptide sequence in procollagen, even if not cleaved at all, would be a minor contribution to the noncollagen protein fraction. This observation indicates that other types of proteins, possibly glycoproteins, are being secreted by these cells.

The possibility that colchicine acts at other steps in collagen synthesis that are prerequisite for secretion has been considered. Since inhibition of hydroxylation of proline and lysine in deoxycollagen decreases secretion, the effect of colchicine on this step was investigated. The results presented in Table 3 indicate that colchicine does not inhibit hydroxylation. Some of the hydroxylysines in collagen have glucosyl-galactosyl residues covalently linked to the hydroxyl groups (19). It has been speculated that the decreased secretion resulting from the inhibition of hydroxylation may, in fact, be due to the inability to attach the glycosyl moiety to hydroxylysine. In our studies, it was assumed that colchicine and vinblastine did not affect glycosylation, since these drugs also inhibited the secretion of noncollagen proteins that do not contain hydroxylysine.

Three models have been proposed, on the basis of electron micrographs, to describe collagen secretion by fibroblasts

‡ Whitson, S. W. (1971) *Anat. Rec.* **169**, 454.

TABLE 7. *Relative rates of collagen synthesis under various conditions used in these studies*

Table	Conditions*	Relative rate† of collagen synthesis (%)
1	Control	21.2
	Colchicine	20.7
	Control	30.4
	Colchicine	28.4
5	Control	25.7
	Vinblastine	24.6
	Control + dimethylsulfoxide	25.6
	Cytochalasin B + dimethylsulfoxide	23.5
4	Control (3T3 cells)	1.9
	Colchicine (3T3 cells)	1.8

* See table listed.

† Calculated by the following formula to correct for the enriched imino acid content of collagen (22%) as compared to other proteins (4.1%):

$$\% \text{ collagen} = \frac{\text{dpm in collagenase digest}}{(\text{dpm in residue} \times 5.4) + (\text{dpm in collagenase digest})} \times 100.$$

from various sources. One is the merocrine type of secretion, in which soluble collagen precursor (procollagen) is transported to the cell surface in vesicles; the membrane of the secretory vesicle would fuse with the cell membrane to allow the collagen molecules to be released from the cell (20–22). A second model would be one in which the collagen molecule passes directly from the cytoplasm through the cell membrane to the extracellular matrix, while a third type would be direct communication between the cisternae of the rough endoplasmic reticulum and the extracellular space (22). Since many, if not all, of the secretory (2–4) or intracytoplasmic migratory (5, 6) processes inhibited by colchicine or vinblastine involve transport of granules or vesicles, it may be that collagen secretion is accomplished by the merocrine model, which involves vesicle formation. The role of microtubules would be to transport these vesicles containing procollagen to the cell membrane where extrusion into the extracellular space and conversion to collagen would occur. Since procollagen has been found in the medium of cultured cells (23–25), and in the skin of dermatosparaxic cattle (26), it is clear that this molecule may be secreted from cells without prior cleavage by procollagen peptidase. Vinblastine and colchicine should prove to be useful tools for the study of procollagen, since inhibition of secretion results in accumulation of the intracellular precursor (Table 4).

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1. Adelman, M. R., Borisy, G. G., Shelanski, M. L., Weisenberg, R. C. & Taylor, E. W. (1968) *Fed. Proc.* **27**, 1186–1193.
2. Williams, J. A. & Wolff, J. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1901–1908.
3. Lacy, P. E., Howell, S. L., Young, O. A. & Fink, C. J. (1968) *Nature* **219**, 1177–1179.
4. Gillespie, E., Levine, R. J. & Malawista, S. E. (1968) *J. Pharmacol. Exp. Ther.* **164**, 158–165.
5. Dahlstrom, A. (1968) *Eur. J. Pharmacol.* **5**, 111–113.

6. Malawista, S. E. (1965) *J. Exp. Med.* **122,** 361–384.
7. Peterkofsky, B. & Diegelmann, R. F. (1971) *Biochemistry* **10,** 988–994.
8. Bellamy, G. & Bornstein, P. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1138–1142.
9. Müller, P. K., McGoodwin, E. & Martin, G. R. (1971) *Biochem. Biophys. Res. Commun.* **44,** 110–117.
10. Kivirikko, K. I. & Prockop, D. J. (1967) *Proc. Nat. Acad. Sci. USA* **57,** 782–789.
11. Bhatnagar, R. S., Kivirikko, K. I. & Prockop, D. J. (1968) *Biochim. Biophys. Acta* **154,** 196–207.
12. Miller, E. J. (1969) *Fed. Proc.* **28,** 1839–1845.
13. Miller, R. (1971) *Arch. Biochem. Biophys.* **147,** 339–342.
14. Seeds, N. W., Gilman, A. G., Amano, T. & Nirenberg, M. W. (1970) *Proc. Nat. Acad. Sci. USA* **66,** 160–167.
15. Goldman, R. D. (1971) *J. Cell Biol.* **51,** 752–762.
16. Bensch, K. G. & Malawista, S. E. (1969) *J. Cell Biol.* **40,** 95–107.
17. Wessells, N. K., Spooner, B. S., Ash, J. F., Bradley, M. O., Luduena, M. A., Taylor, E. L., Wrenn, J. T. & Yamada, K. M. (1971) *Science* **171,** 135–143.
18. Dehm, P., Jimenez, S., Olsen, B. & Prockop, D. J. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 60–64.
19. Butler, W. T. (1969) *Biochemistry* **9,** 44–50.
20. Goldberg, B. & Green, H. (1964) *J. Cell Biol.* **22,** 227–258.
21. Revel, J. P. & Hay, E. D. (1963) *Z. Zellforsch.* **61,** 110–144.
22. Ross, R. (1968) *Biol. Rev.* **43,** 51–96.
23. Layman, D. L., McGoodwin, E. R. & Martin, G. R. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 454–458.
24. Dehm, P. & Prockop, D. J. (1971) *Biochim. Biophys. Acta* **240,** 358–369.
25. Church, R. L., Pfeiffer, S. E. & Tanzer, M. L. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 2638–2642.
26. Lapière, C. M., Lenaers, A. & Kohn, L. D. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 3054–3058.