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Effects of Mebendazole on Protein Biosynthesis and Secretion in Human-Derived Fibroblast Cultures

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ABSTRACT. Previous results of our group revealed that mebendazole, a broad spectrum anthelmintic drug with antimicrotubular properties, used for the treatment of liver cirrhosis, decreased total collagen content and biosynthesis in liver upon treatment. In the present study, we have evaluated the effects of mebendazole (5–50 μg/mL) on protein synthesis, secretion, and deposition in human-derived fibroblast cultures. The results showed a decrease in cell viability (18.5 ± 0.9%) at 50 μg/mL. [^{3}H]Thymidine incorporation diminished gradually with increasing mebendazole concentrations, reaching a plateau (53.67%) between 30 and 50 μg/mL. In late logarithmic phase cultures, the drug caused a decrease of [^{3}H]proline incorporation (43.10%) and collagen biosynthesis (58.61%) in the extracellular matrix. This correlated with an increase in radioactivity in total proteins (51.28%) of the intracellular fraction. Similar results were obtained when mebendazole was assayed in post-confluent fibroblast cultures. The electrophoretic patterns of the extracellular matrix showed a decrease of radioactive collagenous components (α chains and β dimers). By contrast, in the intracellular fraction an increase of radioactive collagen precursors (pro α chains) was observed. Immunofluorescence studies and immunotransfer analysis, using polyclonal anti-type I collagen antibodies, revealed an accumulation of intracellular collagen which included: collagen pro α chains, α chains, and low molecular weight peptides. The results obtained suggest that mebendazole interferes with the transcellular mobilization of proteins, resulting in a decrease of secretion and deposition of extracellular matrix proteins, and an accumulation of intracellular collagenous components. The intracellular accumulation of newly synthesized proteins could cause a feedback regulation in fibroblast cultures. BIOCHEM PHARMACOL 52;2:289–299, 1996.

KEY WORDS. fibroblasts; collagen biosynthesis; mebendazole

ECMs† play a critical role in several biological functions such as cell adhesion, migration, proliferation, and differentiation, in addition to providing the supporting matrix. They are composed of several proteins (collagen, elastin, fibronectin, laminin, proteoglycans, and others), which are synthesized by mesenchyme-derived cells, such as fibroblasts, chondroblasts, osteoblasts, and other mesenchymal cells. Collagen is the major component of the ECMs in most connective tissues.

Thus far, at least 19 genetically different types of collagen have been reported and grouped into several categories according to their structural properties. The overall features of collagen biosynthesis have been described [1, 2] and distinguish it from other proteins. One unusual characteristic of collagens is the large number of posttranslational modifications occurring during its biosynthesis. Thus, theoretically, a collagen disease can be produced by a defect in any of the steps involved in collagen biosynthesis. The onset of events leading to the establishment of fibrotic processes requires the interaction or presence of different cellular elements, such as platelets, neutrophils, and macrophages. There is an increase in the number of ECM-producing cells, production of cytokines and chemoattractants by recruited monocytes and macrophages, and a decrease in collagenolytic activity [3].

Among the most relevant diseases are those characterized by an increase in the rate of synthesis and deposition of collagen, such as pulmonary fibrosis, liver cirrhosis, systemic scleroderma, and others. Several different compounds are being used for the treatment of fibrotic diseases including colchicine [3], malotilate [4], and silymarin [5], among others. We have evaluated mebendazole, a broad spectrum anthelmintic drug with antimicrotubular properties [6], for the treatment of CCl_4-induced cirrhosis [7]. The results obtained in this study revealed a decrease in total collagen content and biosynthesis in rat liver upon treatment with mebendazole. Also, a marked improvement of liver histological features was observed.

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† *Abbreviations:* ECM, extracellular matrix; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; and TCA, trichloroacetic acid.

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Mebendazole is an antimicrotubular agent that possesses a higher affinity for tubulin than colchicine (1×10^3 times) [8]; therefore, it was assumed that this compound could exert an inhibitory effect on collagen biosynthesis and secretion in cell cultures. Based on the above-mentioned considerations, in the present study we have evaluated the effects of mebendazole on collagen synthesis, secretion, and deposition in human-derived fibroblast cultures.

MATERIALS AND METHODS

Cell Cultures

Human fibroblasts were derived from normal skin biopsies obtained from individuals undergoing general surgery. Cultures were routinely maintained in DMEM (Gibco Laboratories, Grand Island, NY), containing 10% FBS (Fisher Scientific Co., Pittsburgh, PA) [9]. Flasks were incubated at 37° in an NAPCO metabolic incubator under an atmosphere of air–CO_2 (95%–5%). Fibroblasts were subcultured each week at a 1:5 dilution by 0.25% trypsin (Sigma Chemical Co., St. Louis, MO) treatment in versene solution, pH 7.5. All experiments were performed between 4 and 10 passages of the cultures. Growth curves were monitored by cell counting, using Neubauer chambers [9]. Also, total protein was determined by the method of Lowry *et al.* [10], and total collagen content in cell monolayers was measured according to the method of Woessner [11]. For this purpose, cell cultures were rinsed thoroughly with PBS, pH 7.4, to eliminate all DMEM and FBS. Cell monolayers were resuspended in 2 mL of PBS and were homogenized with a polytron (Brinkmann, 4 × 15 sec bursts). The homogenate (2.0 mL total volume) was divided in 0.5-mL aliquots for total protein and in 1.5-mL aliquots for total collagen content determinations.

Effect of Mebendazole in Cell Viability

To determine the effect of mebendazole (Janssen Pharmaceutic, Mexico City, México) on cell viability, 24-well tissue culture plates, containing confluent fibroblast cultures, were exposed to different concentrations of mebendazole (5–50 μg/mL) for 20 hr at 37°. Cell viability was determined by trypan blue exclusion. The effect of mebendazole on cell proliferation was determined by [^{3}H]thymidine (Amersham Co., Arlington Heights, IL; sp. act. 120 Ci/mmol) incorporation, by incubating 96-well tissue culture plates, containing logarithmic cultures previously exposed to mebendazole (5–50 μg/mL) for 18 hr at 37°. After this time, the medium was removed, and fresh medium containing mebendazole and 0.5 μCi of the isotope/well was added. The incubation was continued for an additional 18 hr at 37°, under conditions similar to those described to maintain cell cultures. After incubation, cell monolayers were harvested, and incorporated radioactivity was determined by 10% TCA precipitation.

Protein Biosynthesis by Cell Cultures

Total protein and collagenous protein biosynthesis were determined together with the development of the growth curve of the cultures by incorporation of [^{3}H]leucine (Amersham Co.; sp. act. 25–50 Ci/mmol) and [^{3}H]proline (New England Nuclear Corp., Boston, MA; sp. act. 102.Ci/mmol), respectively. For this purpose, 1×10^5 cells/dish were seeded on P_{50} petri dishes, and 24 hr prior to labeling cells were incubated with fresh DMEM containing 10% FBS and 50 μg/mL ascorbic acid (Sigma Chemical Co.). The medium was withdrawn, DMEM without FBS was added, and cells were incubated for 30 min to equilibrate the amino acid intracellular pools. Cultures were incubated in 3.0 mL of DMEM containing ascorbic acid (50 μg/mL), 0.02% glutamine (Sigma Chemical Co.), and 20 μCi of [^{3}H]leucine or [^{3}H]proline. Cultures were incubated for 24 hr at 37° in an NAPCO metabolic incubator. After incubation, medium was collected, and the cell monolayer was rinsed with PBS and resuspended in 3 mL of DMEM for its homogenization. Appropriate aliquots of medium and homogenate were adjusted to a final volume of 1.0 mL with distilled H_2O and incubated with 250 μL of BSA (0.4 mg/mL), 250 μL of 5% tannic acid (J. T. Baker Chemical Co., Phillipsburg, NJ) and 1 mL of 25% TCA for 30 min at 4°. TCA-insoluble material was collected in glass fiber filters, and washed with 10% TCA containing L-proline (1 mg/mL) or 0.1% casamino acids. Filters were dried, and radioactivity was measured in 5 mL of toluene-based solution (0.4%) with a liquid scintillation counter (Beckman LS-1801).

For the determination of collagenous protein biosynthesis, prior to the precipitation with 10% TCA, aliquots of medium and/or homogenates were incubated with pepsin (Sigma Chemical Co.) (100 μg/mL) for 72 hr at 4°. TCA-precipitable radioactivity, but resistant to pepsin digestion, was considered as isotope incorporated into collagenous proteins.

Effect of Mebendazole on Collagen and Total Protein Biosynthesis

Different concentrations of mebendazole were assayed in confluent fibroblast cultures. For this purpose, saturated solutions of mebendazole in DMEM were prepared (0.01%, w/v) by overnight vigorous shaking [12]. Labeling conditions were similar to those described above, except that the medium with the isotope contained, in addition, different concentrations of mebendazole (5–30 μg/mL), and the cultures were incubated for 20 hr at 37°. After the incubation period, the medium was collected, and the monolayer was rinsed with 2 mL of DMEM without FBS. This volume was mixed with the withdrawn medium to obtain a final volume of 5 mL. The monolayer was washed with PBS (3×) and was incubated with 3 mL of 0.01 M NH_4OH for 15 min at room temperature. The supernatant fraction was collected, and petri dishes were washed with 2 mL of DMEM without FBS. These were pooled and considered as the intracellular material. The remaining extracellular matrix was resuspended in 3 mL of DMEM, and petri dishes were washed with an additional 2 mL of DMEM, to obtain the extracellular ma-

trix fraction in a final volume of 5 mL. From the three generated fractions, medium, extracellular matrix, and intracellular material, appropriate aliquots were used for the determination of incorporated radioactivity. In addition, pepsin-resistant incorporated radioactivity (collagenous protein) was measured in all three fractions. In some experiments, 0.1% Triton X-100 was used to obtain the intracellular material [13]. In these conditions, the monolayer was incubated for 15 min at room temperature, the medium was collected, and the remaining extracellular matrix was washed with an additional volume of DMEM.

Effect of Mebendazole on Intracellular Radioactive Protein Secretion Determined by Pulse-Chase Analysis

Late logarithmic fibroblast cultures were exposed to different concentrations of mebendazole (10–30 μg/mL) and labeled with [^{14}C]proline (3 μCi/assay) for 20 hr at 37° in a final volume of 1.5 mL as described above, using 6-well cell culture plates. After incubation, medium was collected, and the monolayer was rinsed with 2 mL of DMEM without FBS. Fresh DMEM, containing ascorbic acid (50 μg/mL) and L-proline (10 mM) and without mebendazole, was added, and the incubation was continued for different periods (30, 60, and 90 min). Also, pulse-chase analysis was carried out by exposing fibroblast cultures to mebendazole (10–30 μg/mL) for 2 hr and then labeling them with [^{3}H]proline (10 μCi/mL) for 40 min followed by a chase of 30 and 60 min, as described above. After each time, medium was collected, and the monolayer was rinsed with PBS. The intracellular fraction was obtained by 0.01 M NH_4OH treatment, as described above. TCA-precipitable radioactive material was determined in both fractions, medium and intracellular.

Electrophoretic Analysis of the Generated Fractions

All three generated fractions, medium, extracellular matrix, and intracellular material, were analyzed by SDS–PAGE. For this purpose, 100 μL of each fraction was added to 50 mM Tris–HCl, pH 6.8, 5% glycerol, 2% SDS, and 100 mM 1,4-dithiothreitol. Samples were boiled for 5 min and subjected to electrophoresis on 6% acrylamide, 0.16% bisacrylamide slab gels for 2.5 hr at 40 mA [14]. Gels were prepared for fluorography by treatment with a 20% solution of 2,5-diphenyloxazole in DMSO, as described by Laskey and Mills [15], and dried on a Bio-Rad slab gel drier. The gels were exposed to Kodak XRP-5 film for varying periods at −70°. The films were visualized by conventional photographic methods.

Characterization of Intracellular Collagen

IMMUNOFLUORESCENCE STUDIES. Fibroblasts were seeded on coverslips, and before reaching confluence were incubated with different concentrations of mebendazole as described above. Following incubation, cells were fixed with 3.5% formalin, treated with methyl alcohol and acetone at −20°, and air dried.

Purified antibodies were prepared from rabbit antisera to type I collagen by immunoadsorption procedures as previously described [16]. Their specificity was evaluated by immunoprecipitation of cell-free products [17] and in the characterization of murine sarcomas [18]. The reaction with antibodies against type I collagen was as follows: cells were incubated with the first antibody for 1 hr at 37° in an NAPCO metabolic incubator. Samples were rinsed with PBS (6×) and incubated with the second antibody (fluorescein isothiocyanate = anti-rabbit IgG). Immediately thereafter, coverslips were rinsed with PBS (6×) and mounted with 50% glycerol on slides and were sealed with nail polish. Preparations were observed and photographed in an epifluorescence microscope [19]. In addition, fibroblasts were also incubated with anti-fibronectin antibodies (Sigma Chemical Co.) to evaluate the effect of different concentrations of mebendazole on another extracellular matrix protein.

WESTERN BLOT ANALYSIS. Following SDS–PAGE, gels containing the intracellular material were transferred to nitrocellulose paper (0.45 μm) for 3 hr at constant voltage (230 V). Nitrocellulose paper was blocked with BSA for 12 hr at 4°. Then it was incubated with polyclonal anti-type I collagen antibodies for 60 min at room temperature. Nitrocellulose paper was washed thoroughly with PBS–Tween-20 (0.3%), and rinsed with PBS before incubating with the second antibody (peroxidase-anti-rabbit IgG) for 60 min at room temperature. Papers were washed and rinsed as described previously and were developed with orthochloronaphthol (0.5 mg/mL) and H_2O_2 [20].

Collagenase Assay

Collagenase activity from fibroblast cultures was assayed as described previously [21], using ^{3}H-labeled acid-soluble collagen from rat skin (600,000 cpm/mg/mL). For this purpose, conditioned media, of cultures exposed to different concentrations of mebendazole for 24 hr, were lyophilized and resuspended in 250 μL of 0.05 M Tris–HCl, pH 7.4, with 5 mM $CaCl_2$ and dialyzed extensively against the same buffer. Samples were activated with trypsin (10 μg/mL) by incubating at room temperature for 30 min. Immediately thereafter, soybean inhibitor was added (50 μg/mL), and incubation was continued for an additional 20 min.

Samples were divided into two aliquots, 10 μL of ^{3}H-labeled collagen was added to each aliquot, and samples were incubated for 24 hr at 37° without or with 20 mM EDTA. Following incubation, samples were subjected to SDS–PAGE and fluorography as described previously.

Statistical Analysis

All results are expressed as means ± SD unless otherwise mentioned. Student's *t*-test was used to evaluate the differ-

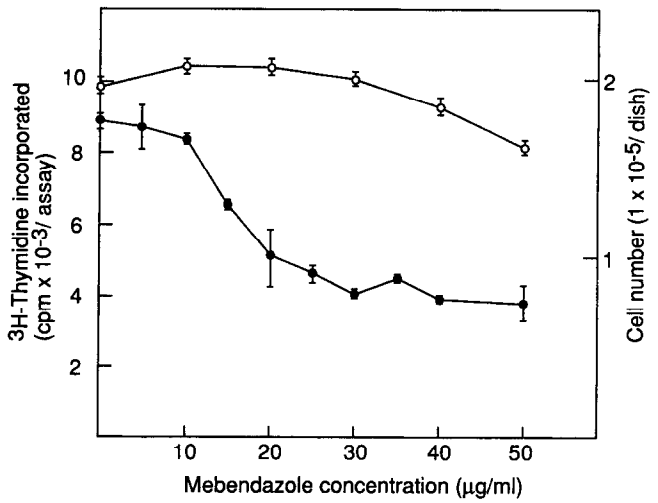

FIG. 1. Effect of mebendazole on cell viability and [^{3}H]thymidine incorporation by fibroblast cultures. Confluent cultures were treated with different concentrations of mebendazole to determine its effect on cell viability (○—○) measured by trypan blue exclusion. The effect of mebendazole on [^{3}H]thymidine incorporation (●—●) was determined in logarithmic cultures. Each point represents the mean ± SD (N = 6).

ence of means between groups, accepting $P < 0.05$ as the level of significance [22].

RESULTS

Effect of Mebendazole on Cell Viability

Prior to the evaluation of the effect of mebendazole on cell viability, the basal conditions for growth, isotope incorporation, as well as other biochemical parameters, such as total protein and collagen content of fibroblast cultures, were established. It was observed that total protein and collagen content followed growth curves. Labeling studies revealed that [^{3}H]leucine incorporation also followed growth curves, whereas [^{3}H]proline incorporation showed a lag period, and increased just before reaching confluence. Also, radioactivity incorporated into collagenous protein was more apparent at day 6 of the cultures (results not shown).

The effect of different concentrations of mebendazole (5–50 μg/mL) was assayed in confluent cultures to measure cell number and in logarithmic cultures for [^{3}H]thymidine incorporation. The results obtained are presented in Fig. 1. A decrease in cell number was observed between 40 and 50 μg/mL of mebendazole, corresponding to 18.5 ± 0.9% at the highest concentration. The incorporation of [^{3}H]thymidine diminished gradually with increasing concentrations of mebendazole and reached a plateau between 30 and 50 μg/mL. The maximal value for the inhibition of [^{3}H]thymidine incorporation was obtained with 50 μg/mL of mebendazole and corresponded to 53.67%.

Effect of Mebendazole on Total Protein and Collagen Biosynthesis

Based on the above-mentioned results, it was decided to study the effect of mebendazole on protein biosynthesis at concentrations below 30 μg/mL, in late logarithmic phase cultures. The results obtained are presented in Table 1, where a marked decrease of [^{3}H]proline incorporation was observed in the extracellular matrix fraction for total proteins (43.10%, $P < 0.05$) with increasing mebendazole concentrations. This decrease correlated with an increase in incorporated radioactivity in total proteins from the intracellular fraction (51.28%, $P < 0.05$). The decrease of incorporated radioactivity in the medium corresponded to 21.90% ($P < 0.05$) for total protein.

The electrophoretic patterns corresponding to the extracellular matrix and intracellular fractions are presented in Fig. 2. The extracellular matrix fractions showed a decrease in its radioactive collagenous components (α chains and β dimers), with increasing concentrations of mebendazole. A densitometric scan showed a reduction of 75.63% at 20 μg/mL for α_2 in these fractions (Table 2). By contrast, in the intracellular fractions, the radioactive collagen precursors (pro α chains) were similar, except for an increase (205.24%) at the highest concentration. Also, several lower molecular weight radioactive bands were present;

TABLE 1. Effect of mebendazole on total protein biosynthesis in human fibroblast cultures determined by [^{3}H]proline incorporation into different fractions*

Mebendazole concentration (μg/mL)	[^{3}H]Proline incorporated in the different fractions (cpm × 10^{-3}/cell)						
	Total incorporation† (cpm)	Culture medium (cpm)	(%)‡	Extracellular matrix (cpm)	(%)	Intracellular material (cpm)	(%)
0	40.0 ± 0.84	19.1 ± 0.45	47.27	17.4 ± 1.42	43.06	3.9 ± 0.65	9.65
5	37.6 ± 0.54	19.0 ± 0.67	50.53	14.9 ± 0.58	39.62	3.7 ± 0.38	9.84
10	40.6 ± 0.64	19.4 ± 1.28	47.78	17.9 ± 0.25	44.08	3.3 ± 0.41	8.12
15	35.5 ± 0.63§	17.0 ± 0.26§	47.88	13.7 ± 0.75§	38.59	4.8 ± 0.89	13.52
20	30.7 ± 0.53§	14.9 ± 0.48§	48.53	9.9 ± 0.58§	29.31	5.9 ± 0.53§	19.21

Results are the means ± SD of three different experiments.

* Cells were incubated with [^{3}H]proline (20 μCi/assay) for 20 hr at 37° as described under Materials and Methods.

† Total incorporation represents the addition of the three different fractions.

‡ Corresponding percentage from the total incorporation.

§ Significantly different from control (0 μg/mL), $P < 0.05$ (Student's t-test).

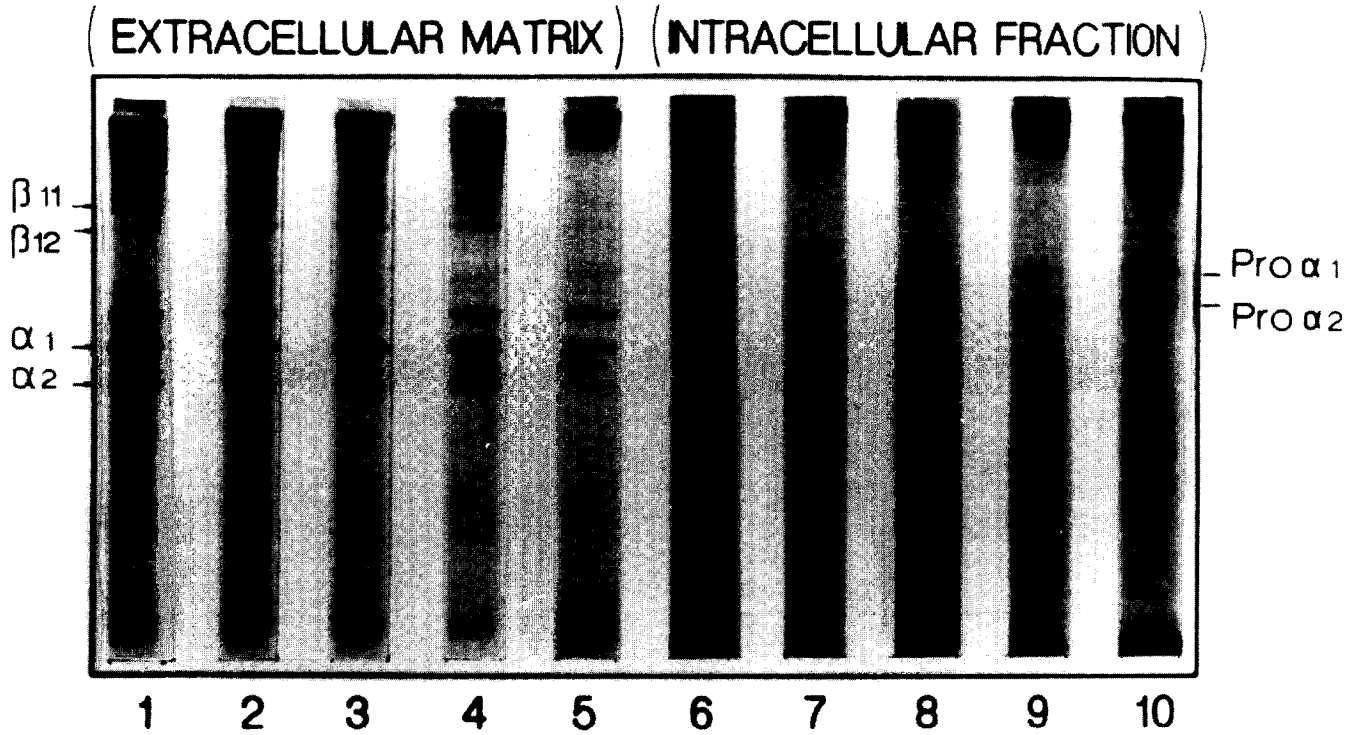

FIG. 2. Effect of different concentrations of mebendazole on protein biosynthesis and its distribution on extracellular matrix (lanes 1–5) and intracellular fractions (lanes 6–10) by late logarithmic fibroblast cultures. Cultures were labeled with [^{3}H]proline, and the fractions were subjected to SDS–PAGE and fluorography. Lanes 1 and 6 (control); lanes 2 and 7 (5 μg/mL); lanes 3 and 8 (10 μg/mL); lanes 4 and 9 (15 μg/mL); and lanes 5 and 10 (20 μg/mL).

nevertheless, these bands showed a slight increase between 5 and 15 μg/mL of mebendazole and a significant increase (224.18%) at the highest concentration. The low molecular weight radioactive bands represented 68.5% (control) and 64.5% (20 μg/mL) of the total radioactive bands (Table 2).

Experiments were also done using postconfluent fibroblast cultures, since Breul *et al.* [23] showed that fetal lung derived human fibroblast (HFL-1) synthesized more collagen upon reaching confluence. The values for total protein and collagen biosynthesis are presented in Fig. 3. The extracellular matrix fractions showed a gradual decrease of incorporated TCA-precipitable radioactivity into total proteins, with increasing mebendazole concentrations, that corresponded to 50.0% ($P < 0.05$) at 30 μg/mL (Fig. 3A). The same pattern was observed when measuring collagen biosynthesis. In this case, the decrease of incorporated radioactivity was 53.85% ($P < 0.05$) at 30 μg/mL (Fig. 3B). These results were similar to those obtained when using late logarithmic phase cultures. Also, the intracellular fractions showed a gradual increase of [^{3}H]proline incorporation for both total proteins (Fig. 3A) and collagen (Fig. 3B) that reached a maximum at 20 μg/mL of mebendazole and corresponded to 152.84% ($P < 0.05$) for total proteins and 136.36% ($P < 0.05$) for collagen, respectively.

These data showed that secretion of total proteins, as well as collagen, is inhibited by mebendazole. To confirm this observation, pulse-chase analysis was performed. The results are presented in Fig. 4 and revealed that mebendazole caused an increase of intracellular TCA-precipitable radioactivity during the pulse (zero time). Chases determined at 30, 60, and 90 min showed an increase of secreted TCA-precipitable radioactivity in the medium (Fig. 4A) that was correlated with a corresponding decrease in the intracellular fraction (Fig. 4B). An interesting observation was that the levels of secreted radioactive proteins determined at 90 min were similar to those measured in the

TABLE 2. Densitometric analysis of intracellular and extracellular fractions*

Mebendazole concentration (μg/mL)	Extracellular matrix fraction		Intracellular fraction		
	Total area†	α_2	Total area‡	pro α_2	<pro α_2§
0	1.8946	0.3512	0.5160	0.0916	0.3539
5	2.1599	0.3445	0.5818	0.0975	0.4053
10	2.0794	0.3354	0.5705	0.0855	0.4328
15	1.1214	0.2171	0.6470	0.0846	0.4928
20	0.6994	0.0854	1.2300	0.1880	0.7934

* Densitometric analysis was performed from the fluorographs presented in Fig. 2.

† Total area comprised the radioactive material including from β chains to α_2(I) chains.

‡ Total area comprised the radioactive material including from pro α chains to the last radioactive band before the front of the gel.

§ These values correspond to the radioactive material with molecular weights <pro α_2(I).

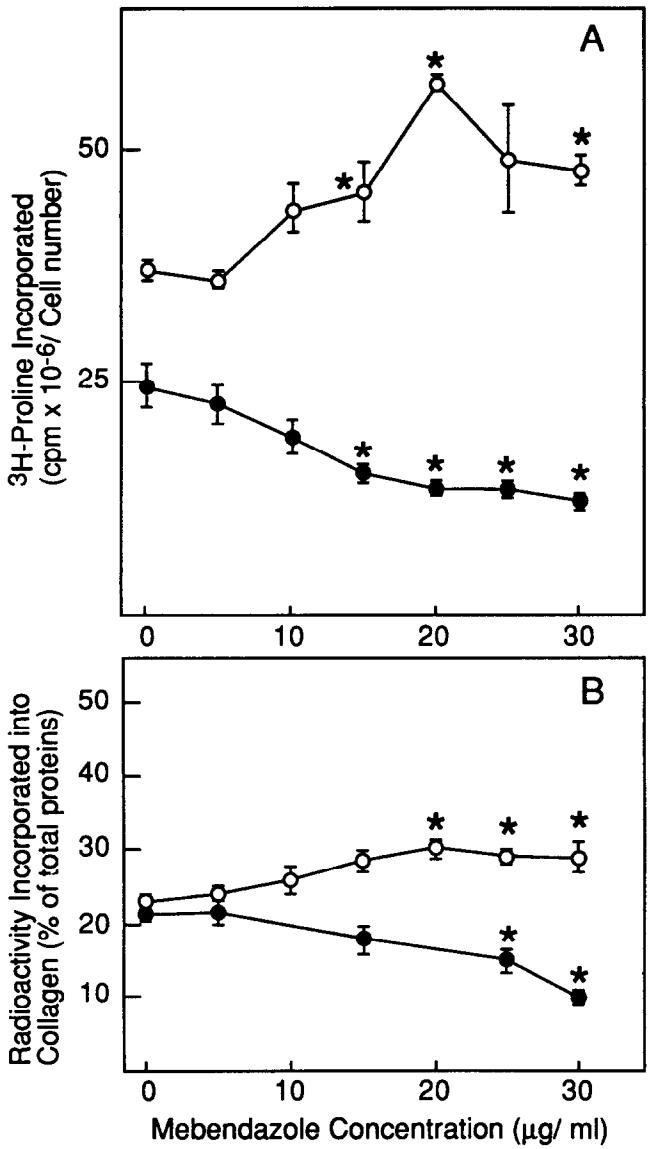

FIG. 3. Effect of mebendazole on total protein (A) and collagen (B) biosynthesis by postconfluent fibroblast cultures. Cultures were treated with different concentrations of mebendazole (5–30 µg/mL) and labeled with [^{3}H]proline. Isotope incorporation was measured in extracellular matrix (●—●) and intracellular fractions (○—○) by 10% TCA precipitation. Key: (*) $P < 0.05$. Each point represents the mean ± SD from three separate experiments, each done in duplicate.

intracellular fractions at zero time. Experiments conducted by labeling for 40 min and chasing at 30 and 60 min showed results similar to those from long-term labeling (results not shown).

To determine the nature of the proteins accumulated in the intracellular fractions, the electrophoretic gel patterns corresponding to these fractions were transferred to nitrocellulose paper and were incubated with polyclonal antibodies against type I collagen. The results, presented in Fig. 5, showed an increase of collagenous material at 30 µg/mL of mebendazole. Although the major reactive bands corresponded to collagen precursors (pro-α chains and pro-β components), lower molecular weight material, reacting with anti-type I collagen antibodies, was also present.

These data indicated that mebendazole caused an accumulation of collagen within the cells and that it could induce arresting in the biosynthesis of collagen.

Morphological and Immunofluorescence Studies

Light microscopy studies were done. They revealed that mebendazole treatment of the cultures induced morpho-

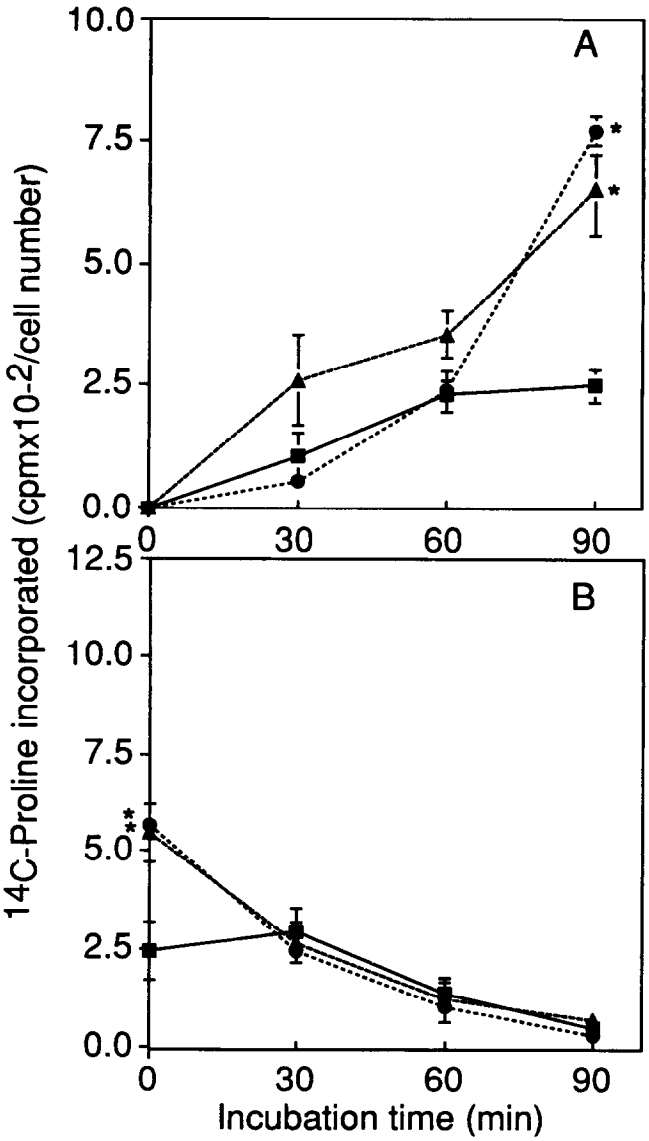

FIG. 4. Effect of mebendazole on intracellular radioactive protein secretion by pulse-chase analysis, using late logarithmic fibroblast cultures. Fibroblasts were seeded in 35 mm 6-well culture plates (150,000 cells/well). Cultures were labeled with [^{14}C]proline (3 µCi/assay) as described under Materials and Methods. Chasing was performed at 0, 30, 60, and 90 min. Isotope incorporation was measured in medium (A) and intracellular (B) fractions by 10% TCA precipitation. Key: control (■—■), 20 µg/mL of mebendazole (▲---▲), and 30 µg/mL (●=●). (*) Significantly different from control (0 µg/mL) at t = 0, $P < 0.05$. Each point represents the mean ± SD (N = 3).

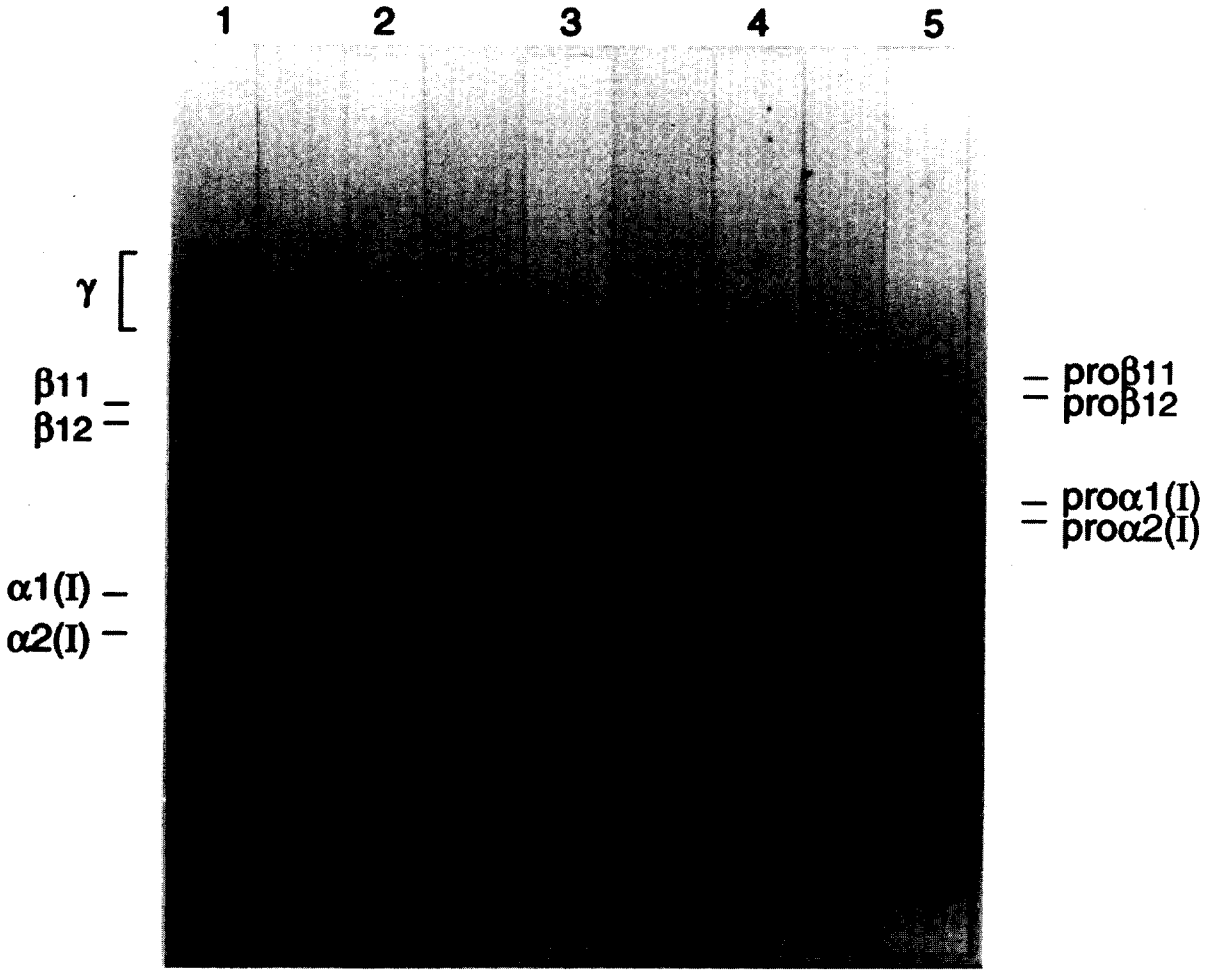

FIG. 5. Characterization of the accumulated intracellular proteins in postconfluent fibroblast cultures treated with mebendazole. Cultures were treated with mebendazole (5–30 μg/mL) and subjected to SDS–PAGE and immunotransfer analysis. Electrophoretic patterns were assayed with a polyclonal anti-type I collagen antibody. Lane 1 (type I collagen); lane 2 (control); lane 3 (10 μg/mL); lane 4 (20 μg/mL); and lane 5 (30 μg/mL).

logical changes in the fibroblasts that were characterized by alterations in shape and size, and nuclei were larger, especially at higher concentrations (Fig. 6). This was suggestive of polyploid nuclei, and was supported by earlier observations of these alterations, when *Plasmodium* was treated *in vitro* with methylbenzimidazol-2-carbamate [24]. Intracellular collagen was detected with anti-type I collagen antibodies, using an indirect immunofluorescence technique. The results showed an accumulation of collagen that was dependent upon increasing concentrations of mebendazole (Fig. 6). The same behavior was obtained when using anti-fibronectin antibodies (Fig. 7); the localization of the label was in the perinuclear region, which might be correlated with alterations in the microtubular system.

Effect of Mebendazole on Collagenase Activity

To measure the effect of mebendazole on collagenolytic activity, conditioned media from confluent fibroblast cultures exposed to different concentrations of mebendazole were used. Samples of dialyzed media, previously activated with trypsin, were assayed for collagenase activity in the presence or absence of 20 mM EDTA. The results are presented in Fig. 8. No detectable collagenase activity was determined at all mebendazole concentrations tested, when 20 mM EDTA was present in the incubation mixture. In the absence of 20 mM EDTA, collagenolytic activity was only observed in samples derived from fibroblast cultures treated with 30–40 μg/mL of mebendazole.

DISCUSSION

Fibroblasts and other mesenchyme-derived cells, such as chondroblasts, osteoblasts, odontoblasts, and reticular cells, are differentiated for the synthesis and secretion of extracellular matrix components in most connective tissues [25]. In skin, lung, and periodontal tissues, fibroblasts comprise 40–60% of the total cell population, and it has been suggested that they are responsible for most of the collagenous components present in these tissues. Also, fibroblast cultures derived from tissue biopsies present functional and biological properties similar to those observed *in vivo*.

Nevertheless, several reports in the literature are contro-

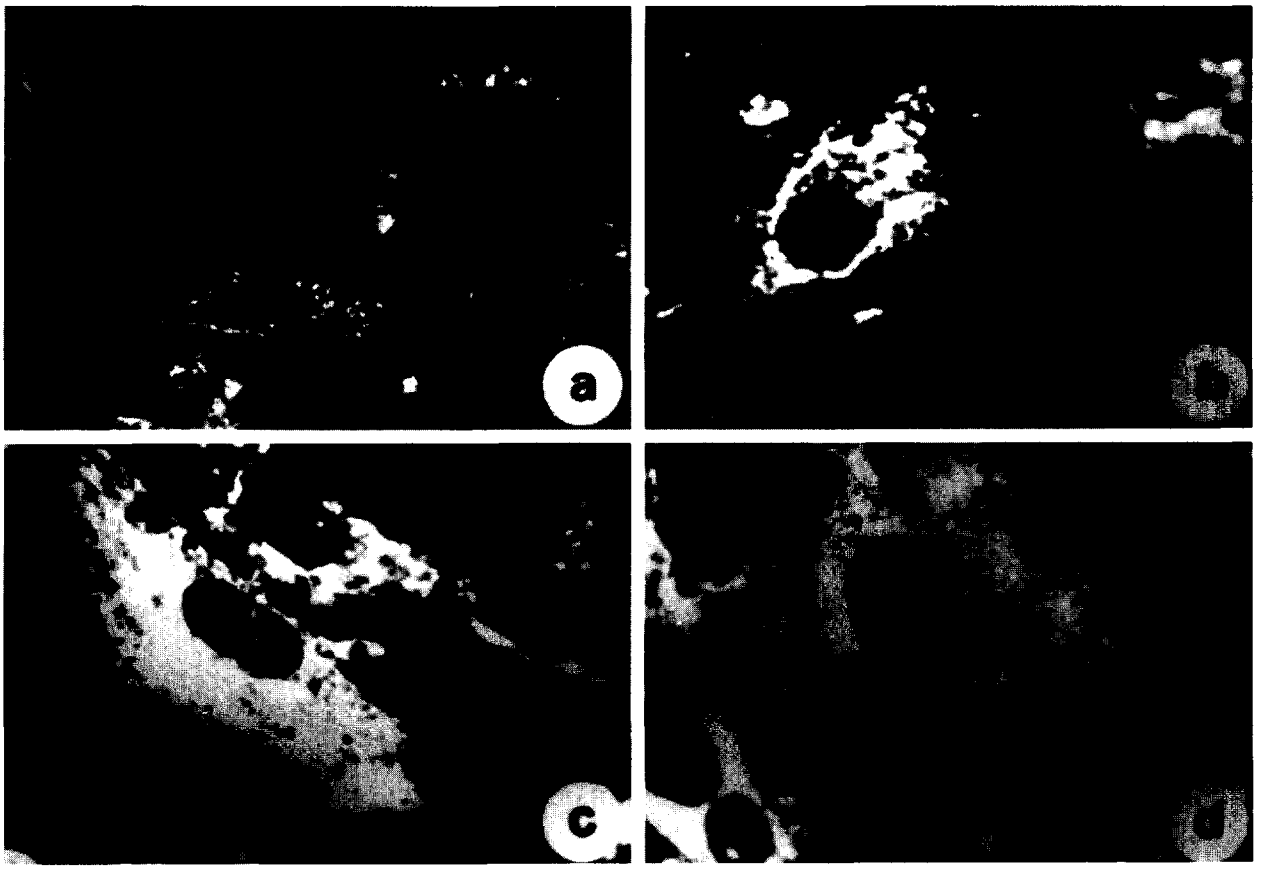

FIG. 6. Identification of the accumulated intracellular type I collagen in late logarithmic fibroblast cultures treated with mebendazole. Cultures were treated with mebendazole (5–30 μg/mL), and cells were fixed and processed for indirect immunofluorescence analysis. Fibroblasts were assayed with a polyclonal anti-type I collagen antibody. Key: (a) control; (b) 10 μg/mL; (c) 20 μg/mL; and (d) 30 μg/mL.

versial in regard to collagen production by fibroblast cultures, when measured at different stages of the growth curve. Breul *et al.* [23] reported that fetal lung derived human fibroblasts (HFL-1) synthesized more collagen upon reaching confluence. On the other hand, Tolstoshev *et al.* [26] demonstrated that collagen biosynthesis is constant during all phases of the growth curve, in spite of a 2-fold increase in the levels of type I procollagen mRNAs, determined at confluence. Also, Voss and Bornstein [27] reported that there are no changes in the levels of procollagen mRNAs during the growth curve. More recent data suggest that human fibroblast cultures secrete more collagen during the logarithmic phase than at stationary and/or confluence stages [28].

The results obtained in this work were similar to those reported by Bruel *et al.* [23], indicating an increase of collagen production in the cultures when reaching confluence (results not shown).

The studies performed to determine the effect of mebendazole on cell number and [^{3}H]thymidine incorporation suggested that mebendazole exhibits antiproliferative activity. Several studies performed *in vivo* and *in vitro*, using various antimicrotubular drugs (colchicine, vinblastine, mebendazole, and nocodazole), demonstrated that these compounds inhibit tubulin polymerization and induce microtubule disassembly [29], as well as a general disruption of the Golgi complex and cisterns throughout the cytoplasm [30]. These changes were reflected in alterations to the intracellular transport and all secretory cell processes [31]. In fact, the accumulation of fibronectin (Fig. 7) in the perinuclear area might be the result of disorganization of microtubules.

The increase of intracellular incorporated radioactivity in both logarithmic and confluent cell cultures treated with mebendazole (Table 1, Figs. 2 and 3) could be explained by its antimicrotubular activity. The collagen appears to accumulate intracellularly in drug-treated cells; nevertheless, the relative percentages of radioactivity in the low molecular weight intracellular material presented similar values when compared with the control (68.5 vs 64.5%). Although we did not observe a concentration-dependent accumulation of intact chains, the variations in the relative percentages of low molecular weight material were in the range of 20%. These results are suggestive of a decreased secretion due to mebendazole rather than an increased turnover, as was corroborated by pulse-chase analysis (Fig. 4). In addition, it was reported that colchicine, another antimicrotubular drug, inhibits the intracellular degrada-

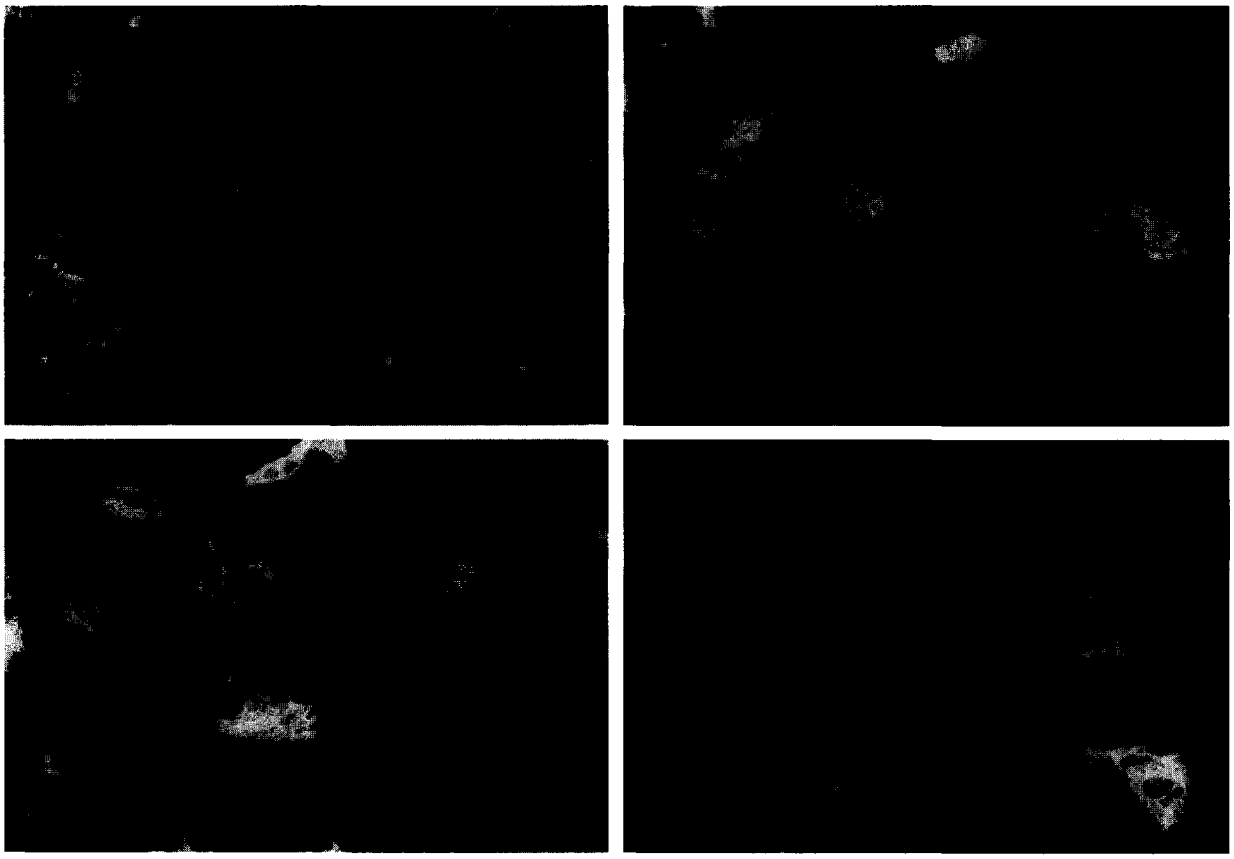

FIG. 7. Effect of mebendazole on fibronectin. Late logarithmic fibroblasts were treated with mebendazole (5–30 μg/mL), and cells were fixed and processed for indirect immunofluorescence analysis, using anti-fibronectin antibodies. Key: (a) control; (b) 10 μg/mL; (c) 20 μg/mL; and (d) 30 μg/mL.

tion of newly synthesized collagen [32]; therefore, this mechanism could be present in mebendazole-treated cultures.

The decrease in total protein biosynthesis determined in logarithmic cultures (Table 1), as well as in confluent cultures (results not shown), can be explained by mebendazole affecting the role of cytoskeleton in the regulation of protein synthesis, at the levels of translation [33, 34], and also by a feedback mechanism on the processes of translation and secretion, caused by accumulation of intracellular proteins [35, 36].

An interesting observation was the marked decrease in the deposit of radioactive total proteins into the ECM (Table 1 and Fig. 3), as well as a reduction in the percentage of collagen (Figs. 2 and 3B). The extracellular matrix of skin-derived fibroblast cultures is composed of collagen (types I, III, V, and VI), fibronectin, non-collagenous proteins, and proteoglycans [37]. A possible explanation for the observed decrease of total proteins in ECM could be that a diminution in collagen deposit might affect the proper assembly of other matrix components, since collagen is the major protein synthesized by fibroblast cultures [38].

According to Bissell and Barcellos-Hoff [39], there is a dynamic reciprocity between cytoskeleton and ECM. By the disruption of the cytoskeleton and the decrease in the secretion and deposit of proteins into ECM, caused by the administration of mebendazole, this reciprocity could be altered and result in the diminution or redistribution of membrane receptors for ECM proteins. This could explain, in part, the morphological changes observed in mebendazole-treated cultures.

The slight increase of collagenolytic activity, detected by SDS–PAGE, of samples derived from fibroblast cultures treated with mebendazole (30–40 μg/mL) was correlated with the decrease in cell viability determined at the same concentrations. This increased collagenolytic activity could be the result of cell death, without any active role of mebendazole in the increase or secretion of enzyme activity, even though it was reported that colchicine is a powerful inducer of collagenase activity by cultured synovial cells [40].

Several reports suggest that the integrity of microtubules is necessary for the intracellular transport and secretion of proteins *in vivo* [41, 42] and *in vitro* [38, 39, 42, 43]. Also, other studies suggest that the secretion of proteins could be differentially affected by antimicrotubular agents [44]. In addition, other reports have demonstrated that the intracellular transport of some membrane glycoproteins is independent of the integrity of the microtubular system [45, 46].

In the present study, no differences in the percentage of

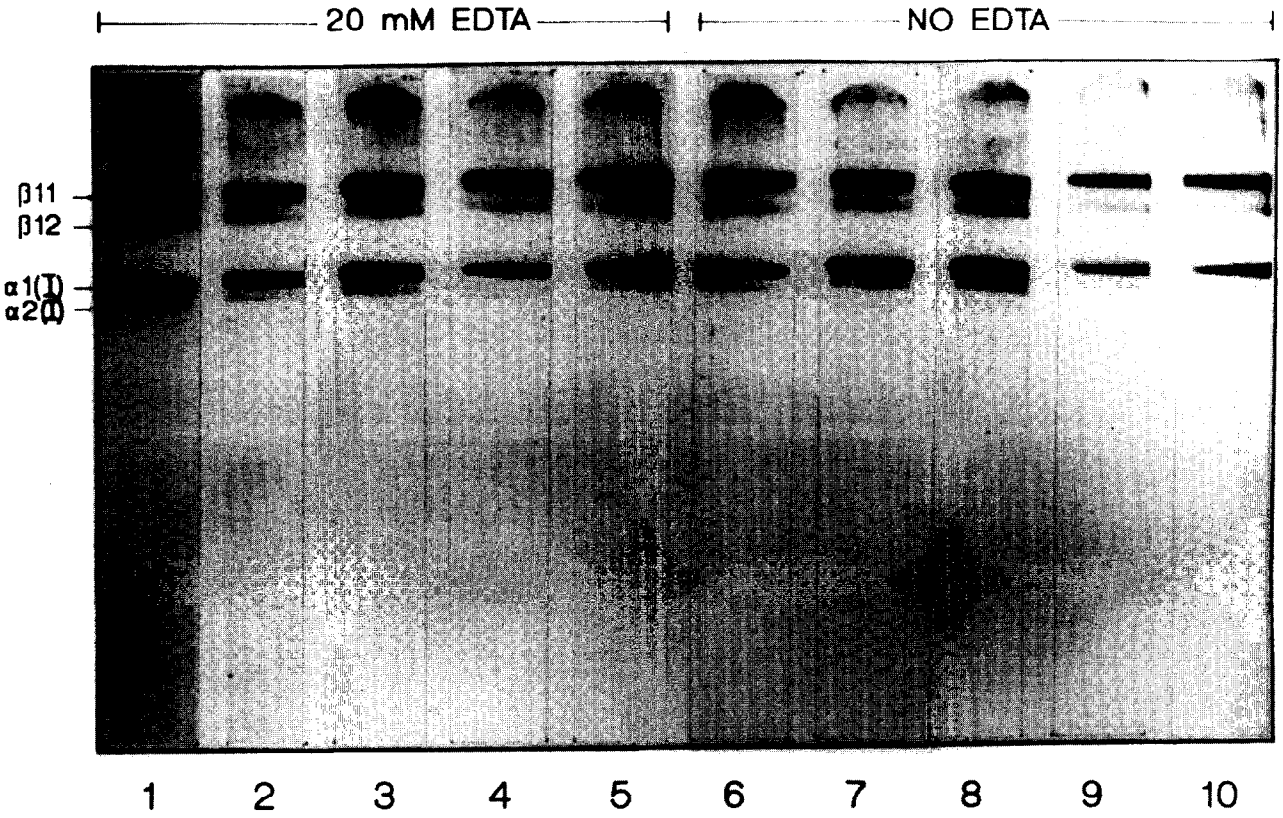

FIG. 8. Effect of different concentrations of mebendazole on collagenolytic activity secreted by confluent fibroblast cultures. Cultures were treated with mebendazole (5–40 μg/mL), and conditioned media were assayed for collagenolytic activity as described under Materials and Methods. Following incubation, samples were subjected to SDS–PAGE and fluorography. Assays were performed in the presence of 20 mM EDTA (lanes 1–5) and in the absence of EDTA (lanes 6–10). Lanes 1 and 6 (controls); lanes 2 and 7 (10 μg/mL); lanes 3 and 8 (20 μg/mL); lanes 4 and 9 (30 μg/mL); and lanes 5 and 10 (40 μg/mL).

radioactive proteins secreted to the medium were determined in the cultures with different concentrations of mebendazole. Most of the reports in the literature do not mention or have not measured changes in the proportions of intracellular proteins and in proteins deposited in ECMs. This constitutes a particular feature of this work because upon treatment with mebendazole, the major changes determined in the cultures affected these parameters.

Finally, the main conclusion in this study is that mebendazole administration to fibroblast cultures affects protein synthesis and secretion. These changes are reflected in an intracellular accumulation of total proteins and collagen and result in a marked decrease of its deposit in the extracellular matrix. Also, the intracellular accumulation of newly synthesized proteins could function as a feedback mechanism in fibroblast cultures.

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References

1. van der Rest M and Garrone R, Collagen family of proteins. *FASEB J* **5:** 2814–2823, 1991.
2. Kivirikko K, Collagens and their abnormalities in a wide spectrum of diseases. *Ann Med* **25:** 113–126, 1993.
3. Rojkind M, Fibrogenesis in cirrhosis. Potential for therapeutic intervention. *Pharmacol Ther* **53:** 81–104, 1992.
4. Takase S, Takada A, Yasuhara M, Sato H and Matsuda Y, Effects of malotilate treatment on the serum markers of hepatic fibrogenesis in liver cirrhosis. *Jpn Soc Gastroenterol* **23:** 639–645, 1988.
5. Ferenci P, Dragosics B, Dittrich HF, Benda L, Lochs H, Meryn S, Base W and Schneider B, Randomized controlled trial of silymarin treatment in patients with cirrhosis of the liver. *J Hepatol* **9:** 105–113, 1989.
6. Van Den Bosche H, The molecular basis of anthelmintic action. In: *Biochemistry of Parasites and Host-Parasite Relationships*, (Ed. Van Den Bosche H), pp. 553–572. Elsevier-North Holland Publishing, Amsterdam, 1976.
7. Díaz de León L, Santamaria P, Morales BL, Barrios R and Soto H, La administración crónica del mebendazole y su aplicación en la cirrosis hepática experimental. In: *Advances en el Mecanismo de Acción de Fármacos* (Eds. Contreras M, Cortinas de Nava C and Barragan LA), pp. 27–33. Masson, Barcelona-México, 1984.
8. Laclette JP, Guerra G and Zetina C, Inhibition of tubulin polymerization by mebendazole. *Biochem Biophys Res Commun* **92:** 417–423, 1980.
9. Freshney RI, *Culture of Animal Cells. A Manual of Basic Techniques*. Alan R. Liss, New York, 1983.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
11. Woessner JF Jr, The determination of hydroxyproline in tissue

and protein samples containing small proportions of this imino acid. *Arch Biochem Biophys* **93:** 440–447, 1961.
12. Laclette JP, Merchant MT, Willms K and Cañedo L, Paracrystalline bundles of large tubules, induced *in vitro* by mebendazole in *Cysticercus cellulosae*. *Parasitology* **83:** 513–518, 1981.
13. Gosporadowicz D, Delgado D and Vlodavsky I, Permissive effect of the extracellular matrix on cell proliferation *in vitro*. *Proc Natl Acad Sci USA* **77:** 4091–4098, 1980.
14. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685, 1970.
15. Laskey RA and Mills AD, Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur J Biochem* **56:** 335–341, 1975.
16. Timpl R, Wich G and Gay S, Antibodies to distinct types of collagens and procollagens and their application in immunohistology. *J Immunol Methods* **18:** 165–182, 1977.
17. Paglia L, Wilczek J, Díaz de León L, Martin GR, Hörlein D and Müller P, Inhibition of procollagen cell-free synthesis by amino-terminal extension peptides. *Biochemistry* **18:** 5030–5034, 1979.
18. Díaz de León L, Greene RM and Paglia LM, Histological and biochemical characterization of murine sarcomas. *Connect Tissue Res* **211:** 1–10, 1983.
19. Osborn M and Weber K, Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: Tubulin-containing structures. *Methods Cell Biol* **24:** 97–132, 1982.
20. Burnette WN, "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* **112:** 195–203, 1981.
21. Murawaki Y, Yamada S, Koda M and Hirayama C, Collagenase and collagenolytic cathepsin in normal and fibrotic rat liver. *J Biochem (Tokyo)* **108:** 241–244, 1990.
22. Zar JH, *Biostatistical Analysis*. Prentice-Hall, Englewood Cliffs, NJ, 1984.
23. Breul SD, Bradley KH, Hance AJ, Schafer MP, Berg RA and Crystal RG, Control of collagen production by human diploid lung fibroblasts. *J Biol Chem* **255:** 5250–5260, 1980.
24. Wright M, Moisand A, Tollon Y and Oustrin ML, Demonstration of the action of methyl benzimidazole 2 yl carbamate (MBC) and methyl (5(2-thienyl carbonyl) 1 H benzimidazole 2 yl carbamate) (R17934) on the nucleus of *Physarum polycephalum* (Myxomycetes). *C R Acad Sci Hebd Seances Acad Sci D* **283:** 1361–1364, 1976.
25. Sollberg S, Mauch C, Eckes B and Krieg T, The fibroblast in systemic sclerosis. *Clin Dermatol* **12:** 379–385, 1994.
26. Tolstoshev P, Berg RA, Rennard SI, Bradley KH, Trapnell BC and Crystal RG, Procollagen production and procollagen messenger RNA levels and activity in human lung fibroblasts during periods of rapid and stationary growth. *J Biol Chem* **256:** 3135–3140, 1981.
27. Voss T and Bornstein P, Regulation of type I collagen mRNA levels in fibroblasts. *Eur J Biochem* **157:** 433–439, 1986.
28. Berman AE and Morozevich GE, Secretion and intracellular degradation of collagen in cultures of normal and SV-40 transformed human fibroblasts. *FEBS Lett* **263:** 285–288, 1990.
29. Lee JC, Field DJ and Lee LY, Effects of nocodazole on structures of calf brain tubulin. *Biochemistry* **19:** 6209–6215, 1980.
30. Thyberg J and Moskalewki S, Microtubules and the organization of the Golgi complex. *Exp Cell Res* **159:** 1–16, 1985.
31. Gelfand VI and Bershadsky AD, Microtubule dynamics: Mechanism, regulation and function. *Annu Rev Cell Biol* **7:** 93–116, 1991.
32. Bienkowski RS, Intracellular degradation of newly synthesized collagen. *Collagen Relat Res* **4:** 399–412, 1984.
33. Lenk R and Penman S, The cytoskeletal framework and poliovirus metabolism. *Cell* **16:** 289–301, 1979.
34. Hesketh JE and Pryme IF, Interaction between mRNA ribosomes and the cytoskeleton. *Biochem J* **277:** 1–10, 1991.
35. Dehm P and Prockop DJ, Time lag in the secretion of collagen by matrix-free tendon cells and inhibition of the secretory process by colchicine and vinblastine. *Biochim Biophys Acta* **264:** 375–382, 1972.
36. Diegelmann RF and Peterkosfky B, Inhibition of collagen secretion from bone and cultured fibroblasts by microtubular disruptive drugs. *Proc Natl Acad Sci USA* **69:** 892–896, 1972.
37. Hatamochi A, Aumailley M, Mauch C, Chu M-L, Timpl R and Krieg T, Regulation of collagen VI expression in fibroblasts. Effects of cell density, cell–matrix interactions, and chemical transformation. *J Biol Chem* **264:** 3494–3499, 1989.
38. Bateman JF, Cole WG, Pillow JJ and Ramshaw JAM, Induction of procollagen processing in fibroblast cultures by neutral polymers. *J Biol Chem* **261:** 4198–4203, 1986.
39. Bissell MJ and Barcellos-Hoff MH, The influence of extracellular matrix on gene expression: Is structure the message? *J Cell Sci Suppl* **8:** 327–343, 1987.
40. Harris ED Jr and Krane SM, Effects of colchicine on collagenase in cultures of rheumatoid synovium. *Arthritis Rheum* **14:** 669–684, 1971.
41. Busson-Mabillot S, Chambaut-Guerin AM, Ovtracht L, Muller P and Rossignol B, Microtubules and protein secretion in rat lacrimal glands: Localization of short-term effect of colchicine on the secretory process. *J Cell Biol* **95:** 105–117, 1982.
42. Cho M-I and Garant PR, An electron microscopic radioautographic study of collagen secretion in periodontal ligament fibroblasts of the mouse: II. Colchicine-treated fibroblasts. *Anat Rec* **201:** 587–598, 1981.
43. Birkett CR, Coulson C, Pogson CI and Gull K, Inhibition of secretion of proteins and triacylglycerol from isolated rat hepatocytes mediated by benzimidazole carbamate antimicrotubule agents. *Biochem Pharmacol* **30:** 1629–1633, 1981.
44. Eilers U, Klumperman J and Hauri H-P, Nocodazole, a microtubule-active drug, interferes with apical protein delivery in cultured intestinal epithelial cells (Caco-2). *J Cell Biol* **108:** 13–22, 1989.
45. Connolly JA, Microtubules, microfilaments and the transport of acetylcholine receptors in embryonic myotubes. *Exp Cell Res* **159:** 430–440, 1985.
46. Virtanen I, Microtubule disruption does not prevent intracellular transport and secretory processes of cultured fibroblasts. *Eur J Cell Biol* **42:** 281–287, 1986.