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Hexamethylene bisacetamide induces morphologic changes and increased synthesis of procollagen in cell line from glioblastoma multiforme

(cell differentiation/collagen synthesis)

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ABSTRACT **Addition of hexamethylene bisacetamide (diacetyldiaminohexane) to cultures of a malignant mesenchymal cell line derived from a human glioblastoma multiforme induces morphological changes and stimulates the synthesis of procollagen. The morphological changes include cell elongation, an increase of extracellular material with staining properties of collagen by light microscopy, and an increase in extracellular 220-Å fibrils by electron microscopy. The rate of procollagen synthesis increased as much as 20-fold, and the ratio of type I: type III procollagen changed, with type I becoming the predominant form. The change in type I:type III ratio is similar to that seen in the maturation of normal fetal to adult connective tissue.**

Reuben and her associates have recently reported that hexamethylene bisacetamide (diacetyldiaminohexane) is a potent inducer of erythroid differentiation in murine erythroleukemia cells (1). In the present report, we have shown that addition of hexamethylene bisacetamide to cultures of a malignant mesenchymal cell line derived from a human glioblastoma multiforme resulted in striking morphological changes and an increased synthesis of procollagen, with a predominance of type I procollagen.

MATERIALS AND METHODS

Cell Line and Culture Methods. The malignant mesenchymal cell line referred to as the CBT cell line was established from tumor tissue obtained at craniotomy from a 49-year-old woman with a glioblastoma multiforme. The tumor tissue was minced into explants and grown in 75-cm^2 plastic flasks in a medium composed of 20% fetal calf serum and 80% RPMI 1640 with penicillin (30 μg/ml), streptomycin (50 μg/ml), and gentamicin (50 μg/ml). The initial cultures were pleomorphic and contained fibroblast-like cells as well as polygonal cells with cytoplasmic processes. The cultures were subcultured with 0.25% trypsin at weekly intervals, and after 2 months they were composed of uniform elongated cells resembling cultures of normal human fibroblasts. After 6 months, however, the cells were noted to be more pleomorphic and cuboidal than normal human fibroblasts, and they have maintained this morphology in the subsequent year.

The CBT cells have cytological features of malignancy in hematoxylin and eosin-stained coverslip preparations (Fig. 1 *Upper*) and histological features of sarcoma in sections of cell pellets. They produced a fibrosarcoma rather than a glioma in one of five NIH *nude* mice after subcutaneous injection of 10^6 cells. They thus appear to be of mesenchymal rather than glial origin.

Cell pellets for light microscopy were fixed in B5 fixative and stained with hematoxylin-eosin and the Masson stain (2). Cell pellets for electron microscopy were prepared and examined as described (3).

Materials. Hexamethylene bisacetamide was obtained from Celia Tabor and Herbert Tabor, and had been prepared by the acetylation of hexamethylenediamine with acetic anhydride. The recrystallized preparation was used in these experiments. Both phenylmethylsulfonyl fluoride and *p*-hydroxymercuribenzoate were purchased from the Sigma Corp. DEAE-cellulose (DE-52) was the product of Whatman, Ltd. [2,3-^{3}H]Proline was purchased from New England Nuclear Corp. specific activity, 22 Ci/mmol. Urea, ultrapure grade, was the product of Schwarz/Mann.

Cell Labeling Conditions. Cultures were grown in 25-cm^2 plastic flasks in the presence or absence of 5 mM hexamethylene bisacetamide for 1–28 days. For labeling, they were rinsed thoroughly with RPMI 1640 without proline, incubated for 30 min, and then rinsed again before the addition of medium containing RPMI 1640 without proline and supplemented with β-aminoproprionitrile-fumarate to prevent collagen cross-linking, 100 μg/ml; ascorbic acid, 100 μg/ml; [2,3-^{3}H]proline, 10 μCi/ml; penicillin, 30 μg/ml; and streptomycin, 50 μg/ml. The hexamethylene bisacetamide was added to the medium at a 5 mM concentration for the labeling period, where indicated. The incubation in a humidified atmosphere with CO_2 at 37° was continued for 18 hr. At the end of that time, the medium containing the secreted procollagen was removed. A 1/40 volume of 0.8 M EDTA in 2M tris, pH 8.1, was added. Then a 1/100 volume of inhibitor mixture was added. This inhibitor mixture, which prevented conversion of procollagen to collagen by nonspecific proteases, contained 1 mM phenylmethylsulfonyl fluoride, 100 mM *p*-hydroxymercuribenzoate, and 25% ethanol in 50 mM tris, pH 11.5.

The medium was centrifuged at 800 × *g* for 10 min to remove cells. After centrifugation, one-tenth of the medium was removed for acid hydrolysis and for proline and hydroxyproline determinations. Before hydrolysis the aliquot was dialyzed against 1 M NaCl, then 0.2 M NaCl, and finally exhaustively against distilled H_2O to remove all traces of free labeled amino acids. Three milligrams of lathyritic rat skin collagen dissolved in 0.1 M acetic acid was then added to the remaining supernatant, as carrier. Ammonium sulfate was added to 20% saturation with constant stirring at 0° for 30 min. The resulting

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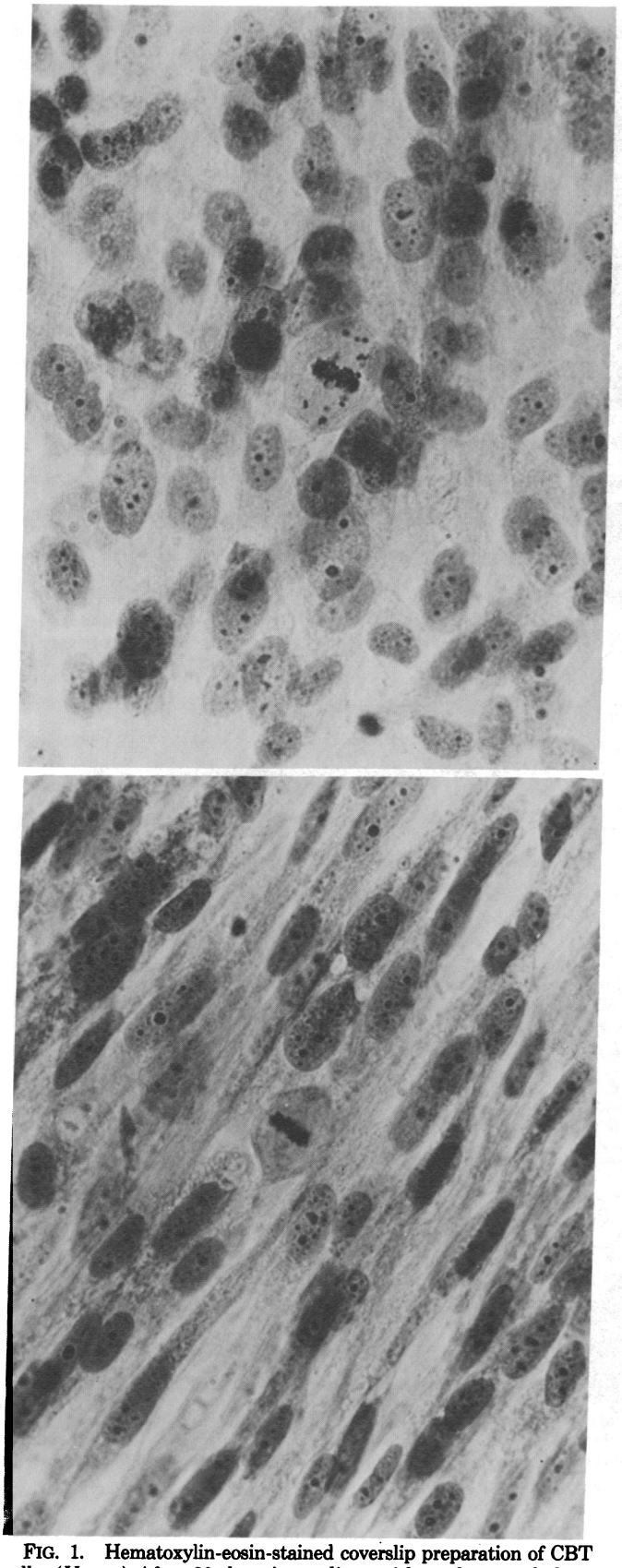

FIG. 1. Hematoxylin-eosin-stained coverslip preparation of CBT cells. (*Upper*) After 20 days in medium with no hexamethylene bisacetamide. Cells are cuboidal with pleomorphic hyperchromatic nuclei, multiple prominent nucleoli, and poorly defined cytoplasm. (Magnification ×380.) (*Lower*) After 20 days in medium containing 5 mM hexamethylene bisacetamide. Cells are elongated with parallel orientation. (Magnification ×380.)

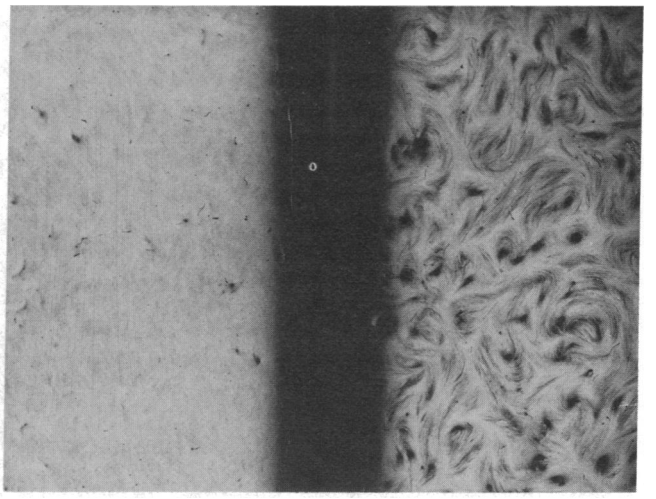

FIG. 2. Unstained flasks of CBT cells grown for 21 days with and without 5 mM hexamethylene bisacetamide. Flask on right was grown with hexamethylene bisacetamide and has a striking whorled pattern. Control flask on left does not have this pattern.

precipitate was collected by centrifugation at 18,000 × *g* for 30 min. This pellet, containing procollagen, was dissolved in a solution of 2 M urea in 50 mM Tris·HCl, pH 7.4. The solution was dialyzed extensively against this initial buffer for the DEAE-cellulose chromatography. Purified lathyritic rat skin collagen used as carrier was obtained as described (4), from animals given a diet containing 0.3% β-aminoproprionitrile.

Cell layers were rinsed several times with phosphate-buffered saline to remove free labeled amino acids. Five milliliters of 0.5 M HCl was added and the mixture was incubated with continuous stirring at 4° for 24 hr. The suspension was then dialyzed exhaustively against 0.5 M acetic acid and centrifuged at 18,000 × *g* for 30 min and the resulting supernatant was hydrolyzed for amino acid determinations.

Column Chromatography. Chromatography of the procollagen was carried out as described by Smith *et al.* (5). Samples were applied to a column of DEAE-cellulose (1.7 cm inner diameter × 7 cm) that had been equilibrated with 50 mM Tris·HCl (pH 7.5) and 2 M urea, and washed with 100 ml of this same buffer. A linear gradient of 400 ml, from 0 to 0.2 M NaCl, was then applied to the column. Fractions of 10 ml were collected at a flow rate of 1 ml/min. One-milliliter aliquots of each fraction were placed in 9 ml of Hydromix and radioactivity was measured in a Beckman scintillation counter, LS-233.

Dowex Column Chromatography. Labeled proline was separated from hydroxyproline in acid hydrolysates of the cell layer and culture media proteins by chromatography on Dowex 50W-X8, as described by Cutroneo *et al.* (6).

RESULTS

Morphologic Studies. Confluent cultures of CBT cells were placed in medium containing 5 mM hexamethylene bisacetamide, a concentration that is highly effective in inducing hemoglobin production in murine erythroleukemia cells. No effects were noted for 1–2 days, but subsequently the cells became more elongated with a parallel orientiation (Fig. 1 *Lower*). After 10–14 days, the treated cultures developed a whorled pattern that was evident when the living cultures were examined with no magnification (Fig. 2). This whorled pattern progressively increased in cultures maintained in 5 mM hexamethylene bisacetamide for 21–28 days. No morphological effects were seen in cultures treated with 0.5 mM hexamethyl-

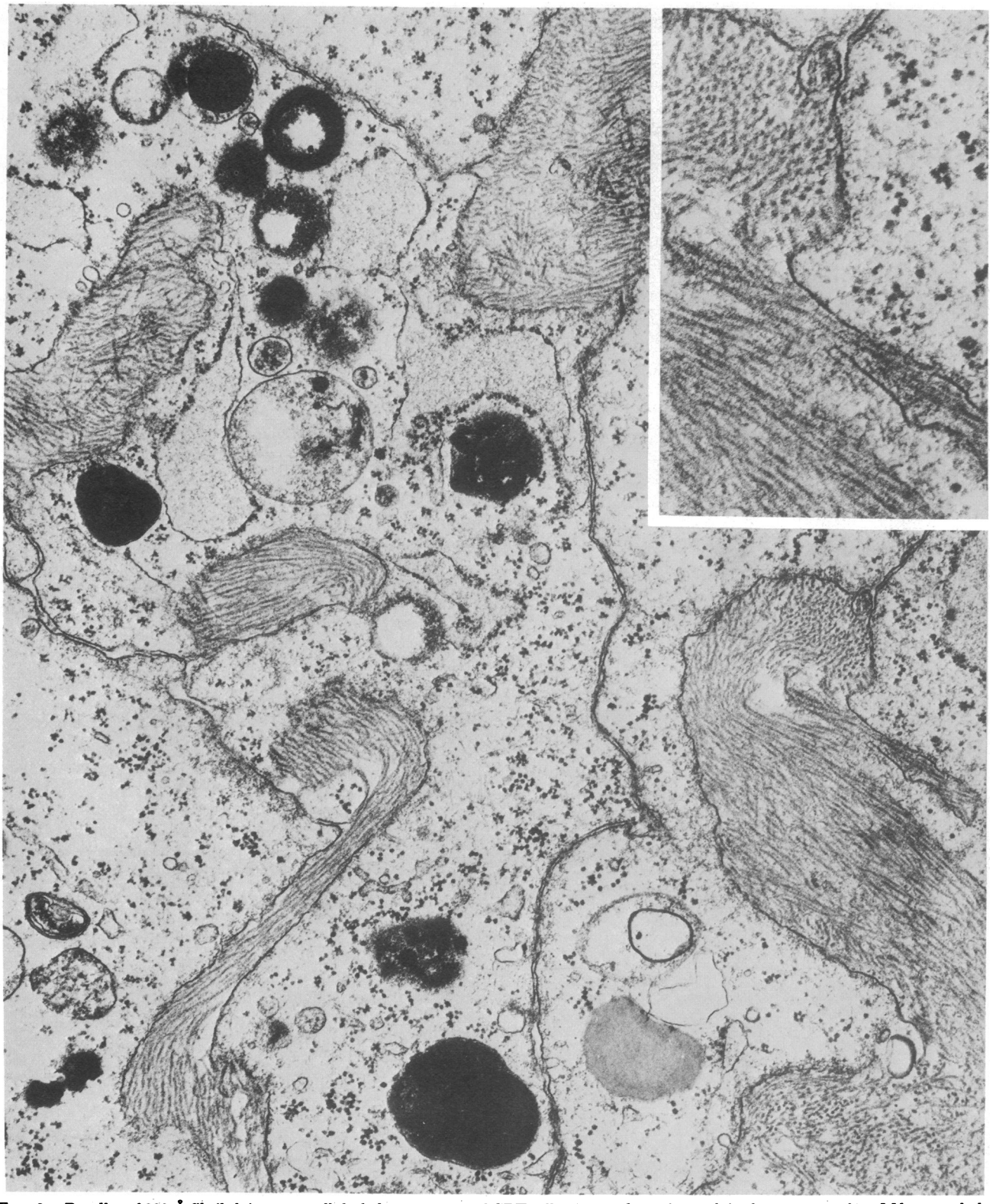

FIG. 3. Bundles of 220-Å fibrils lying extracellularly between several CBT cells after 21 days of growth in the presence of 5 mM hexamethylene bisacetamide. (Uranyl acetate and lead citrate stain. Magnification ×25,900; *inset* ×51,870.)

ene bisacetamide, while 50 mM hexamethylene bisacetamide killed the cells within 24 hr.

Cell pellets from cultures treated with 5 mM hexamethylene bisacetamide for 21 days and control cultures maintained in medium without hexamethylene bisacetamide were examined by light and electron microscopy. Hematoxylin- and eosin-stained sections of control cell pellets contained sheets of pleomorphic polygonal and elongated cells with hyperchromatic nuclei. The histologic appearance was that of a sarcoma, and small amounts of extracellular fibrillar material with the staining properties of collagen were seen in sections prepared with the Masson stain. In the hexamethylene bisacetamide-

Table 1. Synthesis of collagen by CBT cells

Culture conditions	Fraction	Counts incorporated Proline	Hydroxyproline	Collagen/noncollagen protein synthesis*
Control cells	Cell layer	216,800	7,700	0.012
	Medium	21,500	8,300	0.26
Cells grown for 21 days in 5 mM HMB†	Cell layer	566,100	12,400	0.008
	Medium	105,400	55,000	0.47

* In these calculations, it is assumed that all nondialyzable hydroxyproline is in collagen or procollagen. Collagen contains 1.2 proline residues for each hydroxyproline. Therefore, 1.2 × hydroxyproline = proline content of collagen. Hydroxyproline represents 12.2% of the amino acids in collagen, while in the average protein, proline represents 4.1% of the total number of residues (7, 8). From this information, the ratio of collagen to total protein synthesis can be calculated: Collagen/noncollagen protein = (4.1 × hydroxyproline)/(12.2 × noncollagen proline)

† HMB, hexamethylene bisacetamide.

treated cultures, the cells were more elongated with less nuclear pleomorphism. In Masson-stained sections, there was a marked increase in fibers with the staining properties of collagen.

Ultrastructurally, CBT cells were pleomorphic with large elongated irregular nuclei and abundant cytoplasm containing variable mixtures of organelles and fibrils. Extracellularly, fibrils 220 Å in diameter were seen around the cells; however,

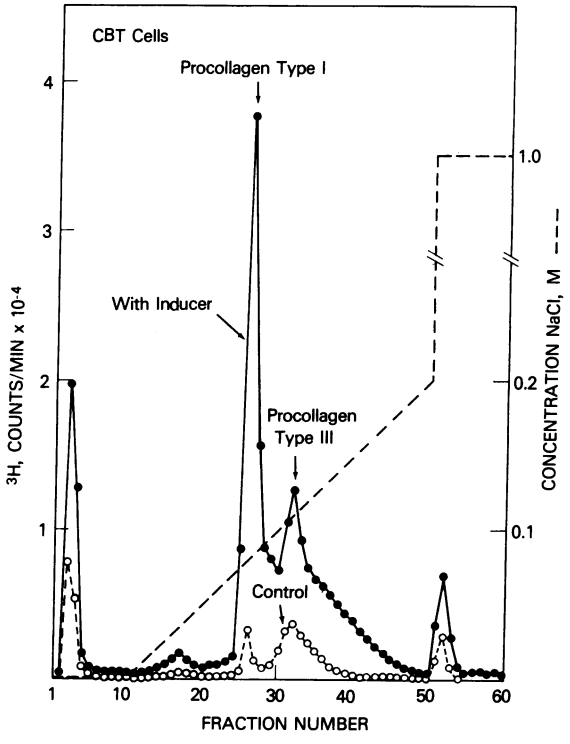

FIG. 4. Separation of proteins of CBT cell culture supernate labeled with [^{3}H]proline (10 μCi/ml). The DEAE-cellulose chromatography was performed as described under *Materials and Methods*. Cells grown in the absence and presence of hexamethylene bisacetamide for 21 days were labeled for 20 hr in the continued absence or presence of the drug. The arrows indicate the elution position of procollagen types I and III from normal human fibroblast cultures from parallel chromatographic runs performed under the identical conditions.

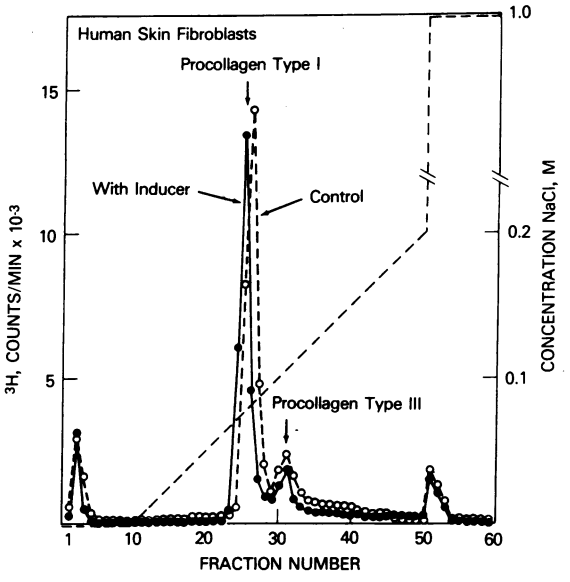

FIG. 5. Separation by DEAE-cellulose chromatography of cell supernate proteins from cultured normal human skin fibroblasts labeled with [^{3}H]proline. The chromatography was identical to that described under Fig. 4.

no large collagen bands with periodicity were found. The most striking ultrastructural effect of hexamethylene bisacetamide treatment was a marked increase in the extracellular 220-Å fibrils (Fig. 3). No periodicity could be detected in these fibrils. The cell nuclei were generally more ovoid and less irregular, with more homogeneous chromatin. There were also more membranous lysosomes and lipid granules. There was a decrease in intracellular fibrils, microfilaments, and microtubules, and single cilia were seen projecting from the surface of several cells.

Biochemical Studies. CBT cultures that had been treated with 5 mM hexamethylene bisacetamide for 21 days had a 500% increase in proline incorporation into proteins of the cell supernatants (Table 1 and Fig. 4). These cultures had the morphologic changes shown in Figs. 2 and 3 at the time the labeled proline studies were initiated. The effect of hexamethylene bisacetamide on procollagen synthesis in CBT cultures could be observed within 24–40 hr of initiation of treatment. The maximum effect was seen after 7 days of treatment. At that time, the rate of procollagen synthesis was 4–5 times that seen at 21 days, and 20-fold greater than control cultures. When the medium proteins labeled with [^{3}H]proline were examined with DEAE-cellulose chromatography, an increase in procollagen in the hexamethylene bisacetamide-treated cultures was observed. This increase was primarily type I procollagen (Fig. 4).

Confluent cultures of normal human fibroblasts derived from skin punch biopsies [CRL 1295, third to fourth passage, obtained from the American Type Culture Association] were also exposed to hexamethylene bisacetamide for 5 days. Profiles of labeled proteins from the tissue culture medium on DEAE-cellulose column chromatography showed no difference with cells grown in the presence and absence of hexamethylene bisacetamide (Fig. 5). A distribution of type I:type III procollagens of 75:25, which is a distribution seen with normal adult fibroblasts, was observed. The profile of procollagens from the CBT cells after induction with hexamethylene bisacetamide (Fig. 4) tended to resemble that from normal skin fibroblasts.

In the presence of hexamethylene bisacetamide, the overall

rate of protein synthesis was increased, as reflected in the level of [^{3}H]proline incorporation into cell proteins (Table 1). Procollagen is a major protein elaborated by mesenchymal cells into tissue culture medium. Normally, *in vivo* the procollagen secreted by cells is rapidly converted to collagen. However, in tissue culture, the enzymes that catalyze these cleavage reactions are not as effective, perhaps because of dilution effects. Most of the procollagen synthesized by cells in culture is secreted and is found unchanged in the culture medium. Therefore the analyses of procollagen synthesis are good approximations of overall collagen production.

The rate of procollagen production was also stimulated by hexamethylene bisacetamide treatment of the CBT cells, as can be seen by the level of [^{3}H]hydroxyproline incorporated into the proteins of the culture medium. In addition, the ratio of collagen to noncollagen protein synthesis increased from 0.26 to 0.47. Under these conditions, the proportion of synthesis devoted to collagen in the cell layer decreased slightly from 0.012 to 0.008.

DISCUSSION

This paper reports that hexamethylene bisacetamide, a potent inducer of erythroid differentiation in murine erythroleukemia cells, also induces increased procollagen synthesis in a human cell line derived from a glioblastoma multiforme. Although the cell line was established from explants of a malignant glioma, the cells produced a fibrosarcoma rather than a glioma when transplanted to *nude* mice, indicating that the CBT cells are mesenchymal rather than glial. The cell line may have arisen from *in vitro* transformation of mesenchymal cells in the stroma of the tumor; however, it is also possible that the original tumor contained malignant mesenchymal cells in addition to a major component of malignant glia.

The morphological changes we have observed are similar to those described by Johnson and his associates with dibutyryl cyclic AMP in cultures of transformed cells (9). Evans and Peterkofsky have demonstrated that dibutyryl cyclic AMP treatment of Kirsten virus-transformed 3T3 murine cells causes them to assume a more normal morphology and increases their relative rate of collagen synthesis (10).

Collagen is not a single unique protein but a family of proteins, each a separate gene product comparable in complexity to the various hemoglobin chains (11). Types I and III collagen are produced simultaneously by tissues of mesenchymal origin throughout the body, except bone and tendon, which synthesize only type I. Recently it has also been demonstrated that a single fibroblast can produce both types I and III collagen simultaneously (12). Type III is generally observed in rapidly growing tissues and is the most abundant collagen in fetal connective tissue. The content of type III collagen in human skin falls in a linear fashion from the tenth week of gestation to age 80 as the amount of type I increases (13, 14). In adult fibroblast cultures, the proportion of types I:III collagen is approximately 80:20.

Type III procollagen was the major component in the profile from the media of our uninduced CBT cell cultures. With induction, the profile reverted to a more differentiated adult-like state, and the DEAE-cellulose profile of the hexamethylene bisacetamide-treated cell procollagen was indistinguishable from the profile of the procollagens from the normal adult skin fibroblast. It is also of interest that normal adult fibroblast cultures were not stimulated nor could a change in profile be induced by hexamethylene bisacetamide, as would be expected of fully differentiated cells.

Nudel and associates have described further studies on the induction of hemoglobin synthesis by hexamethylene bisacetamide and related compounds in murine erythroleukemia cells (15). They find differences in the relative amounts of β major and β minor globins with different inducing agents. Their results suggest that the inducers act directly on control rates of synthesis of globin by affecting either the transcription or the processing of mRNA. It will be of interest to determine whether similar mechanisms are operative in the stimulation of type I procollagen in CBT cells, whether types I and III procollagen are under separate controls, and if differential gene expressions can be achieved.

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