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# Dermatofibrosarcoma Protuberans: Altered Collagen Metabolism in Cell Culture

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**Dermatofibrosarcoma protuberans is a low-grade malignant tumor that grows invasively but rarely forms metastases. Its origin is still controversial. We characterized the synthesis of collagen in detail in cells which were obtained from dermatofibrosarcoma protuberans tumors by enzymatic tissue disintegration. Similar to fibroblasts, all tumor cell strains produced considerable amounts of collagen. However, the rate was reduced compared to normal skin fibroblasts. Cells grown from the tumors synthesized type I collagen, but no type III could be detected. After serial passaging the cultures started to produce type III collagen, which is probably due to a slow overgrowth by normal fibroblasts.**

Dermatofibrosarcoma protuberans (DP) is a low-grade malignant neoplasm with frequent local recurrence but rare distant metastases. Its origin is still controversial and a histiocytotic, fibroblastic, or neural lineage has been proposed [1–5]. The current interpretations are based mainly on ultrastructural observations in tumor tissues [2,4,5] and also on cell culture studies [3,6]. Connective tissue is composed of several structural glycoproteins including different collagen types [7]. Most of the collagens show a characteristic distribution in the body and are specific for certain cell types and tissues. Expression of collagen types influences the organization of the extracellular matrix molecules, the interaction with other connective tissue glycoproteins (e.g., fibronectin [8]), and determines the biologic function of different connective tissues.

Interaction of cells with components of connective tissue is thought to play a critical role in tumor malignancy such as invasive growth and metastasis [9]. In a large number of established lines of normal or transformed tumor cells an altered synthesis of extracellular matrix proteins has been reported. For example, tumor cells are often characterized by a decreased production of collagen [10,11] and fibronectin [12], which is due to diminished levels of translatable mRNA [12]. However, a switch of the collagen type synthesized has been reported to occur during the process of transformation [13–15].

In DP-tumor tissue, presence of type I collagen as well as a relatively high amount of type III collagen has been found [16]. However, it has remained unclear whether these collagens were actually synthesized by the tumor cells. To address this question directly we attempted to isolate DP-tumor cells and to study synthesis of extracellular matrix proteins in these cells using an in vitro cell culture system. The characteristic biosynthetic capacities of these cells should provide information as to the cellular origin of this tumor in addition to a better understanding of the role connective tissue plays in invasive growth.

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Abbreviations:
DMEM: Dulbecco's modified essential medium
DP: dermatofibrosarcoma protuberans

## MATERIALS AND METHODS

### *Cell Cultures*

Control fibroblasts were obtained from age-matched controls following previously published procedures [17] and were used in the same passages as the investigated tumor cells. DP-tumor cells were derived from 5 patients, in which the diagnosis was established by histologic and electron microscopic techniques [18]. In some instances, both outgrowth from explants and enzymatic digestion of the specimen were used to initiate the cultures. However, when not mentioned otherwise, for all biochemical characterization, cells were used that were obtained by enzymatic digestion of the tumor. For this procedure, the specimen was digested with collagenase (Seromed, Munich) and trypsin in Dulbecco's modified essential medium (DMEM) at 37°C in order to liberate cells from the tissue. Cells were maintained in the same DMEM supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), glutamine (300 μg/ml), sodium ascorbate (50 μg/ml), and 10% fetal calf serum. They were judged by phase contrast and by electronmicroscopy.

### *Determination of Total Protein and Collagen Synthesis*

Confluent cell cultures in various passages were incubated without fetal calf serum in DMEM supplemented with penicillin (400 U/ml), sodium ascorbate (50 μg/ml) and β-aminopropionitril (100 μg/ml) for 24 h in the presence of L-[5-$^3$H]proline [19]. Medium and lysed cells were then separately dialyzed against 1 M $CaCl_2$, 5 mM Tris-HCl, pH 7.5 and subsequently against dilute acetic acid. Radioactivity of protein-bound proline and hydroxyproline was determined as described previously [19]. In parallel cultures, cell numbers were determined using a hemocytometer.

### *Characterization of Newly Synthesized Collagens*

Labeled medium was collected and the cell layer extracted with 1 M NaCl, 50 mM Tris-HCl, pH 7.4 in the presence of protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.1 mM 4-chloromercuribenzoate). The labeled material was dialyzed against 0.2 M NaCl, 50 mM Tris-HCl, 20 mM EDTA pH 7.4. Procollagens I and III were separated by ion exchange chromatography on DEAE cellulose as described earlier [20]. For further characterization the labeled material was dialyzed against 0.5% acetic acid and submitted to limited digestion with pepsin [21]. Collagenous proteins were analyzed and quantitated by electrophoresis on slab gels [22] followed by fluorography and densitometry [23] as well as chromatography on agarose A5 [23,24].

### *Hydroxylation of Collagens*

Isolated collagen molecules from DEAE and agarose A5 columns were used for determination of hydroxyproline and proline using an automated amino acid analyzer [19].

### *Immunofluorescence Studies*

Monolayer cultures were used for indirect immunofluorescence staining as described earlier [25]. Specific antibodies against type I collagen and protype III collagen were kindly provided by Drs. R. Timpl and K. v.d.Mark. Antibodies against fibronectin were purchased from Cappels (Cochranville, Pennsylvania). Rabbit antigoat IgG and goat antirabbit IgG conjugated with fluorescein isothiocyanate were products from Boehringer (Mannheim, F.R.G.).

## RESULTS

In these studies, tumor diagnosis was established by the routine histologic and electron microscopic techniques reported in detail elsewhere [18]. Although a certain heterogeneity was observed among tumors obtained from different patients, all revealed the characteristics of DP tumors. Cells grown from tissue explants of these tumors often displayed a normal fibroblastic phenotype, whereas in cultures obtained by enzymatic digestion of the tumor tissue, cells retained characteristics typical of tumor cells through 3 passages. The cells were less densely aggregated (Fig 1*a*) and a significant number (8–34%) had atypical nuclei. In addition, cytoplasmic vacuoles were found in some of these cells (Fig 1*b*), which were not seen in control cultures. All of the experiments described here were therefore carried out using cells obtained by tissue disintegration.

### *Protein Synthesis*

The growth rate of tumor cell strains was lower than that of normal human skin fibroblasts (not shown). Considerable amounts of peptide-bound hydroxyproline were found in all tumor cells although the amount of collagen synthesis was reduced as compared with that of normal fibroblasts. In contrast, synthesis of noncollagenous proteins did not differ significantly between DP cells and controls (Table I). Similar to control fibroblasts DP cells secreted 80% of the newly synthesized procollagen into the medium, whereas 20% was retained in the cell layer.

### *Characterization of Collagenous Proteins*

When newly synthesized procollagens secreted into the medium were analyzed by chromatography on DEAE cellulose (Fig 2), only a small amount of radioactivity was found to migrate in the position characteristic of type III procollagen. Quantification and statistical analysis of these data compared to controls is given in Table II, where it is shown that this collagen type accounted for 13–20% of total collagen in control cells. These findings were further substantiated by electrophoretic analysis of pepsin-treated collagens pooled from medium and cell layer. In DP-tumor cells no production of type III collagen was noted, which migrates as a $\gamma$-component under nonreducing conditions (Fig 3). The absence of type III collagen was also demonstrated by chromatography on agarose A5. The ratio of $\alpha 1$ and $\alpha 2$ chains of type I collagen was close to 2:1 as is usually found in type I collagen synthesizing fibroblasts. No evidence for increased synthesis of $\alpha 1$(I) trimers was noted in DP-tumor cells. Type V collagen chains accounted for less than 5% of total collagen in both tumor cells and control fibroblasts. Isolated $\alpha 1$ and $\alpha 2$ chains of type I collagen had a normal degree of hydroxylation of prolyl residues (not shown). When cells were studied serially after several passages in culture, a continuous increase in the relative rate of type III collagen synthesis was noted (Table II). A rather normal rate of type III collagen production was observed in cells obtained by out-

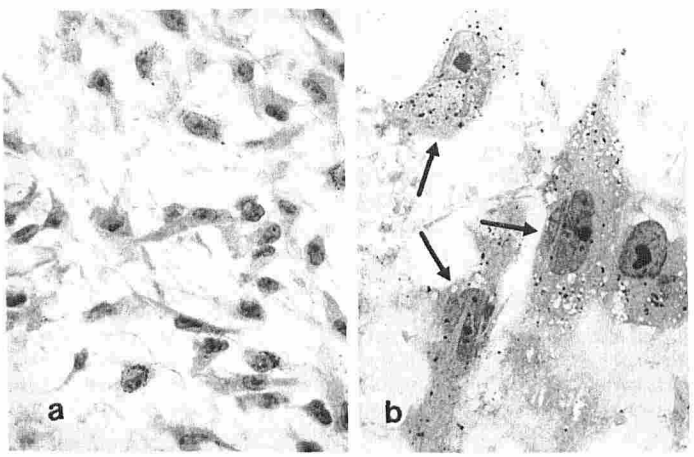


FIG 1. Dermatofibrosarcoma protuberans, cultured fibroblast-like tumor cells, semithin sections. *a*, Low-power magnification showing a loose and irregular arrangement and a reduced length of the cells (× 400). *b*, High-power magnification revealing atypical nuclei with pronounced convolutions (*arrows*) in some of the cells (× 1000).

TABLE I. *Synthesis of collagenous and noncollagenous proteins in cells obtained by disintegration of dermatofibrosarcoma protuberans tumor tissue in early subcultures*

| Cell strain | cpm Hydroxyproline/cell | cpm Proline/cell |
|---|---|---|
| 1 | 0.10 ± 0.01 | 0.85 ± 0.05 |
| 2 | 0.07 ± 0.01 | 1.16 ± 0.04 |
| 3 | 0.13 ± 0.02 | 1.02 ± 0.03 |
| 4 | 0.10 ± 0.01 | 1.12 ± 0.06 |
| 5 | 0.05 ± 0.01 | 0.99 ± 0.02 |
| Control fibroblasts | 0.17 ± 0.01 (n = 6) | 0.98 ± 0.03 (n = 6) |

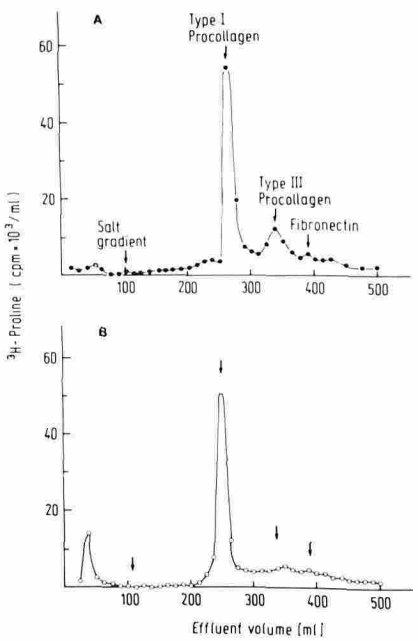


FIG 2. DEAE chromatography of newly synthesized material secreted into the medium of control fibroblasts (*A*) and DP-tumor cells (*B*).

TABLE II. *Synthesis of type III collagen in cells obtained from dermatofibrosarcoma protuberans tumors by disintegration of the tissue and dependence on subcultivation*

| Cell strain | Type III (pro)collagen/total collagen (%) | | |
|---|---|---|---|
| | 2nd–4th passage | 5th–8th passage | 11th–12th passage |
| 1 | n.d. | 7 | 12 |
| 2 | <2 | 5 | 10 |
| 3 | <2 | 6 | n.d. |
| 4 | <2 | 7 | 12 |
| Control fibroblasts | 18 ± 3 (n = 3) | 13 ± 4 (n = 5) | 17 ± 3 (n = 4) |

The relative amount of procollagen type III was calculated from DEAE-chromatograms (2nd–4th and 11th–12th passage). Pepsin-treated material was chromatographed on agarose A5 and the relative amount of type III collagen was calculated as described earlier [23] (5th–8th passage). n.d. = not determined.

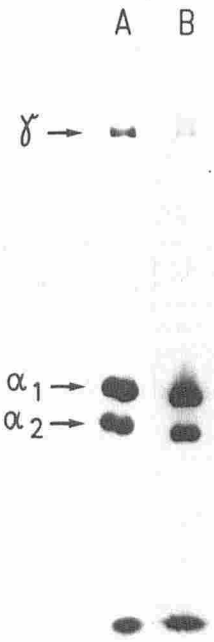


FIG 3. Fluorogram of pepsin-treated newly synthesized material from control fibroblasts (*A*) and cells obtained by disintegration of DP tumors (*B*). The band migrating in γ-position in the control fibroblasts is found migrating as α-chains after reduction.

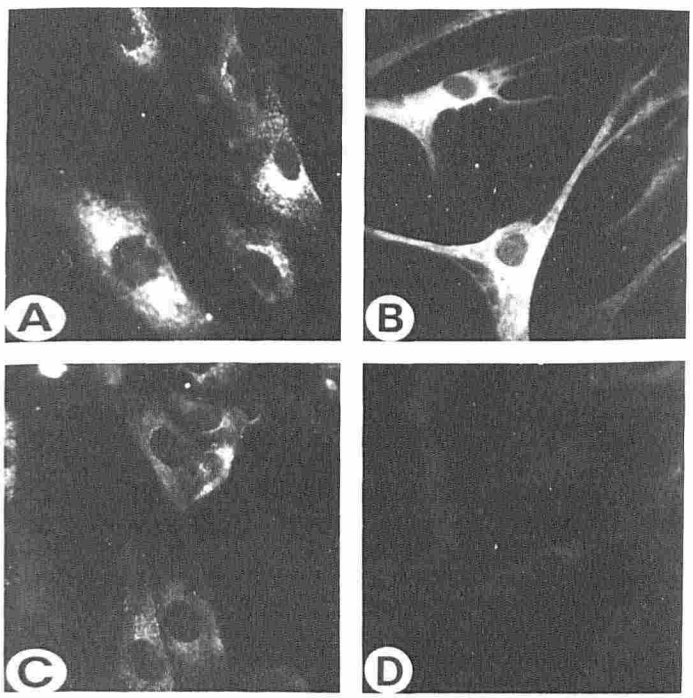


FIG 4. Immunofluorescent localization of type I and type III collagen *A*, Type I collagen in control fibroblasts. *B*, Type I in DP-tumor cells. *C*, Type III collagen in control fibroblasts. *D*, Type III in DP-tumor cells.

growth from explants even in early passages (case 1: 15% type III; case 2: 16%; case 3: 7%).

*Localization of Extracellular Matrix Proteins*

Indirect immunofluorescence techniques were used for studying the distribution of connective tissue matrix proteins in cultured cells. An intracellular staining with antibodies directed against type I collagen was found in all cells derived from DP tumors and controls (Fig 4). When antibodies directed against fibronectin were used, a normal fibrillar distribution in the extracellular matrix was noted. However, more than 95% of cells isolated from DP tumors by tissue disintegration lacked any staining with antibodies directed against type III procollagen in early passages. With continued passaging of cells in culture, the percentage of cells staining positive for type III procollagen steadily increased.

## DISCUSSION

Biochemical and immunologic data show that in vitro cells derived from DP tumors synthesize significant amounts of type I collagen that are qualitatively identical to those of control fibroblasts. Although type III collagen comprises a considerable portion of the collagen pool synthesized by skin fibroblasts, only trace levels of such synthesis could be observed by the tumor cells studied in culture. Furthermore DP cells produced substantally less hydroxyproline than did control cells. Thus since the quantitative decrease in collagen production could not be accounted for by the lack of type III collagen synthesis, it can be concluded that an alteration in the control of type I collagen production has occurred as has been reported for other transformed cells [10,11]. Others have also reported that as a result of transformation, cells may synthesize an altered pattern of collagen types not necessarily resembling the pattern found in the tissue of origin [14,15]. Since low collagen production and lack of type III collagen synthesis have been observed only in early subcultures of DP cells, the onset of type III collagen and the normalization of total collagen synthesis after several passages may be explained by the heterogeneity of cultures. This possibility is further substantiated by immunofluorescence data showing an increasing number of type III collagen-producing cells in later passages and by the observation that large numbers of type III collagen-producing cells are already found in early passage of cells derived by outgrowth from tumor explants. Since the growth potential of the DP-tumor cells is lower than that of normal fibroblasts, it is conceivable that contaminating fibroblasts can progressively overgrow the neoplastic cells in the culture system. However, since clones from single cells could not be established, it is difficult to rule out the possibility that increase of type III collagen synthesis after several passages may be due to a successive dedifferentiation of tumor cells grown in culture, as has been reported for other differentiated cells [26,27].

These data are not in total agreement with observations made by others in analyzing tumor tissue [16]. The differences might be due to the fact that type III collagen present in the tissue can be synthesized by blood vessel cells or by normal connective tissue cells trapped within the tumor [16]. However the difference might also reflect the influence of the culture system used. Even though a certain morphologic heterogeneity was observed both at the light- and electron-microscopic level, biochemical alterations in collagen metabolism were found to be relatively consistent among DP-tumor cell strains studied. It is noteworthy that the fibrillar arrangement of fibronectin and type I collagen in the cell matrix compare well with those of normal fibroblasts. Thus it is questionable whether the low rate of collagen synthesis and lack of type III collagen production representing an altered phenotype of the neoplastic cells plays a role in deficient extracellular matrix formation and hence for the progressive invasion of these cells. Although from the data presented a firm conclusion about the controversial histiogenesis of DP tumors [1–6] cannot be made, the characteristic pattern of proteins synthesized by DP cells is similar to that found in fibroblasts.

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