TYPE III PROCOLLAGEN IS THE MAJOR COLLAGENEOUS COMPONENT PRODUCED BY A CONTINUOUS RHABDOMYOSARCOMA CELL LINE

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1. Introduction

Collagens are structural glycoproteins of connective tissue that are secreted by various cells in a precursor form [1]. The amino-terminal extension peptides of type I and type III procollagens have been isolated from dermatosparactic or fetal skin and have been used in structural studies [2–4]. These peptides are biologically active in regulating procollagen biosynthesis in normal and diseased fibroblasts [5,6]. They also contain strong antigenic determinants [7]. The carboxy-terminal extension of procollagen type I has been chemically characterized using an organ culture system [8]. So far the carboxy-terminal propeptide of type III procollagen has not been isolated and hence subjected to structural studies.

Continuous cell lines from human tumors provide a means to isolate great amounts of material normally secreted by the respective untransformed cells in vivo; such products include immunoglobulins [9] and interferon [10]. Although the level of procollagen-specific mRNA [11,12] as well as the net production of procollagen [13,14] are often decreased in transformation, this is not invariably the case [15]. We here report production of large amounts of type III procollagen by a continuous embryonal human rhabdomyosarcoma cell line. Unlike normal human skin fibroblasts or smooth muscle cells [16,17], these tumor cells produce also some basement membrane collagen but no type I procollagen. Immunohistological studies are consistent with this and show only type III procollagen and type IV collagen in the endomysium around the individual skeletal muscle fibers.

2. Experimental

Rhabdomyosarcoma cells (strain ATCC-CCL 136, American Type Culture Collection) and normal skin fibroblasts [18] were grown to confluency in minimal essential medium modified according to Dulbecco and supplemented with 10% fetal calf serum, ascorbic acid (50 μg/ml), streptomycin (50 μg/ml) and penicillin (400 U/ml). Cells were labeled for 24 h by adding [^{14}C]glycine (5 μCi/ml) or [^{3}H]proline (10 μCi/ml) in fresh medium containing now β-aminopropionitrile (50 μg/ml) but lacking serum and streptomycin. Radioactivity incorporated into proteins was measured in a β scintillation counter prior to or after separating hydroxyproline and proline in acid-hydrolyzed samples on a Multichrome amino acid analyzer.

Characterization of medium proteins by precipitation with ammonium sulphate (176 mg/ml) or NaCl (2.7 or 4 M), pepsin treatment and chromatography on DEAE cellulose, CM cellulose and agarose followed described procedures [5,19]. Slab gel electrophoresis in sodium dodecylsulfate was performed according to Laemmli [20] using fluorography [21] for the visualization of protein bands. Medium was digested with bacterial collagenase (form III, Advanced Biofacturers Corp., Lynbrook, NY) at 37°C for 60 min using 30 units/ml. Immunoprecipitation was performed with purified antibodies (10–20 μg/ml) and an excess of second antibody against immunoglobulin. Radioimmunoassays for type III procollagen and fibronectin followed published procedures [7]. Indirect immunofluorescence analysis of cell layers and of tissues was carried out as in [18,22].

3. Results and discussion

In a previous immunofluorescence analysis [18] we have found that rhabdomyosarcoma cells contain large amounts of intracellular type III procollagen but no or only little type I procollagen. The biosynthetic capacity of confluent cell cultures was now more thoroughly examined by metabolic labeling (table 1). About 30% of the newly synthesized proteins and usually > 80% of the collageneous proteins (as based on radioactive hydroxyproline) were secreted into the medium. The medium proteins include type III procollagen and fibronectin as judged from radioimmunoassays. Only small amounts of these two proteins could be detected in the cell layer. A comparison with normal skin fibroblasts cultured under the same conditions showed that rhabdomyosarcoma cells produced about 2–4-times as much type III procollagen but 2–5-fold less fibronectin.

Analysis of the medium proteins on DEAE-cellulose showed a complex pattern. However, two peaks which accounted together for ~60% of the radioactivity contained mainly type III procollagen and fibronectin, respectively, as judged from radioimmunoassays (fig.1A). Type I procollagen emerges from the DEAE-cellulose column in front of the type III procollagen peak [16] but only little material was found in this position (fig.1A).

The nature of the collageneous proteins was further examined after treating the medium with pepsin in order to remove most of the non-collageneous protein and to convert procollagen into collagen. About 90% of the pepsin-resistant radioactivity was insoluble in 2.7 M NaCl. This material eluted from agarose mainly in the position of γ components (290 000 mol. wt), but a shift to the position of α chains (95 000 mol. wt) was noted after reduction (fig.1B). Since prior to reduction only little radioactivity was detected in the position of α chains the data indicate that rhabdomyosarcoma cells produce

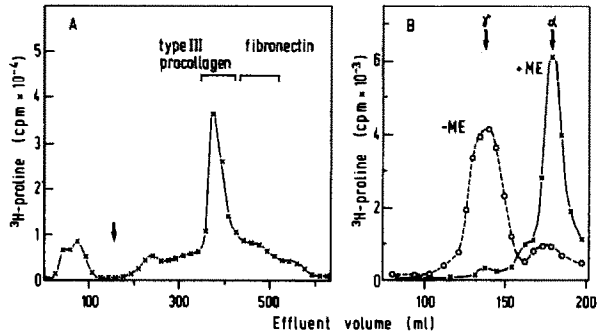

Fig.1. Chromatographic separation of rhabdomyosarcoma cell medium proteins on DEAE-cellulose (A) and characterization of the pepsin-resistent collageneous protein on agarose A5m (B). The DEAE-cellulose column (1.8 × 8 cm) was equilibrated in 0.05 M Tris–HCl (pH 7.4), 2 M urea and eluted with a linear gradient from 0–0.3 M NaCl (300/300 ml). The arrow denotes the start of the gradient. Total recovery from the column was 68%. The fractions containing type III procollagen and fibronectin were identified by radioimmunoassay. The agarose column (1.5 × 120 cm) was equilibrated in 1 M $CaCl_2$, 0.05 M Tris–HCl and samples run prior to (–ME, o——o) and after (+ME, x——x) reduction with 0.4% 2-mercaptoethanol. γ and α denote the elution positions of γ components and α chains determined in a calibration run with denatured type I collagen.

Table 1
Analysis of protein synthesis of rhabdomyosarcoma cells by metabolic labeling and immunochemical assays[a]

	Protein-bound radioact. (cpm/cell)		Radioimmunoassay (pg/cell)	
	Hyp[b]	Pro	Type III procollagen	Fibronectin
Medium	0.34	0.78	6.2	1.9
Cell layer	0.05	2.42	0.4	0.2

[a] Average values from 3 experiments
[b] Based on the hydroxyproline-proline ratio [5] ~10% of the newly synthesized proteins are collagens

⩾ 10-times more type III than type I procollagen. This observation was confirmed by CM cellulose chromatography showing a major peak in the α1(III)/α2 chain region while only little radiocativity was eluted in the position of α1(I) chains.

Sodium dodecyl sulfate gel electrophoresis and specific immunological techniques were used to verify the chromatographic data and to screen for additional proteins. An ammonium sulfate precipitate of the medium containing 85% of the total radioactivity showed 5 major electrophoretic bands (fig.2, lane 1). A doublet of strongly labeled bands was sensitive towards bacterial collagenase (fig.2, lane 2, see also scans a,b) and could be precipitated by antibodies against the amino-terminal peptide of type III procollagen (fig.2, lane 3). The major, slower moving band of the doublet comigrates with intact procollagen chain (150 000 mol. wt) while the faster migrating band may resemble a truncated form of the molecule [23,24]. Collagenase digested also a doublet of slower migrating bands, at the 170 000 and 180 000 mol. wt positions (fig.2, lanes 1, 2) that were precipitated with antibodies to human type IV collagen (fig.2, lane 5). Electrophoretic analysis of pepsin-treated medium (2.7 M NaCl, precipitate) showed a band (fig.2, lane 4) which moved in the position of α chains. In unreduced conditions this major band migrated in the γ position (not shown). Small amounts of type AB_2 collagen were also found in the pepsin-treated medium and could be precipitated between 2.7 M and 4 M NaCl. These polypeptides were identified by the characteristic mobility in slab gel electrophoresis when compared with authentic A and B chains [25]. Together the data demonstrate that type III but not type I procollagen is the major collageneous product synthesized by the cells and the basement membrane collagens (types AB_2 and IV) are minor components only.

As shown above fibronectin represents a major non-collageneous protein secreted into the medium which was confirmed by immunoprecipitation (fig.2, lane 6). A considerable fraction of the labeled non-collageneous proteins, however, are recovered in the cell layer (table 1) and were not identified in the present study. All of the above matrix glycoproteins, type III procollagen, fibronectin and type IV collagen, were detected by indirect immunofluorescence intracellularly but were not deposited in pericellular

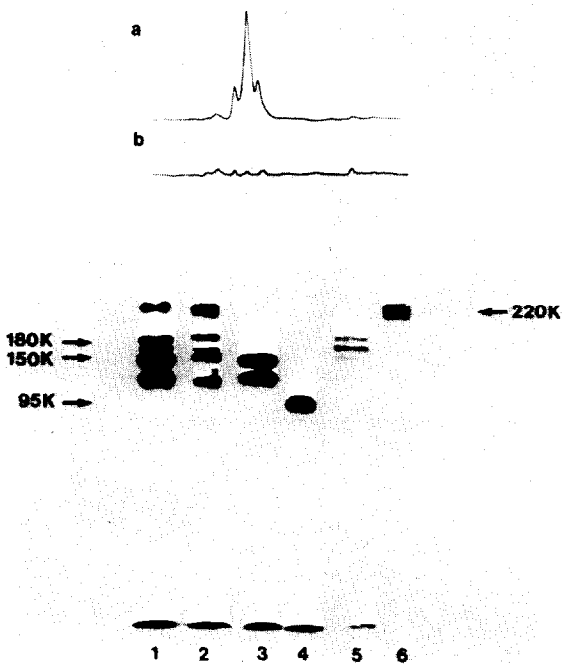

Fig.2. Polyacrylamide gel electrophoresis and fluorography of labeled polypeptides secreted by rhabdomyosarcoma cells. Lane 1, ammonium sulfate precipitation of culture medium; lane 2, collagenase digestion followed by ammonium sulfate precipitation; lane 3, immunoprecipitation with type III procollagen antibodies; lane 4, pepsin digestion of culture medium followed by 2.7 M NaCl precipitation; lane 5, immunoprecipitation with type IV collagen antibodies; lane 6, immunoprecipitation with fibronectin antiserum. Each sample was reduced prior to electrophoresis. The graphs at the top represent densitometric scans of ammonium sulfate precipitated medium before (a) or after (b) collagenase digestion. Molecular weight markers for collageneous (left-hand side) and noncollageneous polypeptides (right-hand side) are indicated by arrows.

matrix structures such as are formed around normal adherent cells in culture [18].

Immunohistological data have indicated that type III collagen and procollagen are major constituents of the loose connective tissue and mainly found in the reticular network [26]. In immunofluorescence of mouse skeletal muscle both type III procollagen (fig.3a), fibronectin (fig.3b) and type IV collagen (fig.3c) but no type I procollagen (fig.3d) were seen in the endomysium. Similar data have been reported on bovine muscle [27]. The biosynthetic

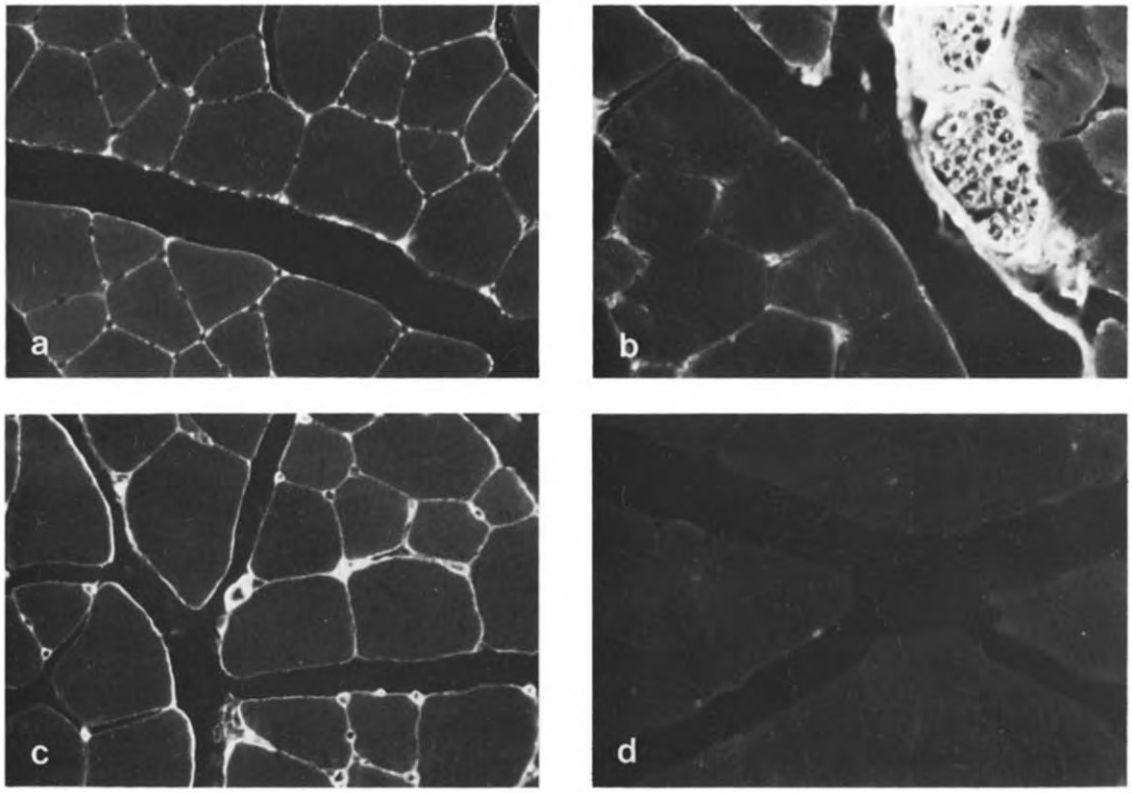

Fig.3. Indirect immunofluorescence micrographs of sectioned mouse muscle obtained with antibodies to: (a) type III procollagen; (b) fibronectin; (c) type IV collagen; (d) type I procollagen. The endomysium stains positive in a–c; fig.3b includes a section through a peripheral nerve. The gaps between the muscle fibers are artifacts due to dehydration in the fixation process. × 220.

data presented here suggests that the muscle cells themselves may produce components of the endomysium.

The rhabdomyosarcoma cell line appears to be a good source for isolating intact type III procollagen in amounts sufficient for chemical studies. Previous attempts to study post-translational modifications of type III procollagen, i.e., formation of interchain disulfide bonds [23] and of feedback inhibition by procollagen peptides [5,6] were hampered by the high level of type I procollagen production in the cultures used. Here again, this cell line may be helpful for circumventing these problems.

Acknowledgements

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