Molecular and Cellular Biochemistry **120**: 25–32, 1993.

Neoplastic association of enhanced type V collagen production in rat fibrosarcoma

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Received 2 March 1992; accepted 8 September 1992

Abstract

Collagens present in the connective tissues of the extracellular matrix of fibrosarcoma were isolated and characterized. The fibrosarcoma was induced in rats by the administration of 3-methylcholanthrene. The results obtained were compared with normal muscle. An excess amount of type V collagen was found to be produced by the fibrosarcoma tissue compared to the normal muscle. Type V collagen from fibrosarcoma was characterized on the basis of solubility behavior in sodium chloride solutions, electrophoretic mobility on SDS-polyacrylamide gels, elution pattern of phosphocellulose chromatography and amino acid composition. (Mol Cell Biochem **120:** 25–32, 1993)

Key words: extracellular matrix, collagen, fibrosarcoma

Introduction

The extracellular matrix of connective tissue is composed of collagen, elastin, proteoglycans and glycoproteins and form a three-dimensional supporting scaffold that isolates tissue compartments, mediates cell attachment and determines the tissue architecture [1]. The matrix acts as a selective macromolecular filter and influences cytodifferentiation as well as mitogenesis and morphogenesis [2]. The expansion of knowledge regarding the phenotypic variability of collagens in the extracellular matrix and identification of at least 15 distinct collagen types [3, 4] have emphasized the role that collagens may play in human pathology. It has been well established that the molecular composition and distribution of the extracellular matrix are tissue specific, and the types of collagen present in each tissue differ depending on the source of the material. An interstitial matrix associated with stromal cells contains type I and type III collagens, fibronectin and specific types of proteoglycans and glycoproteins [5]. The collagenous components of the extracellular matrix are supposed to be involved in the mediation of regulatory signals to the epidermal cells [5, 6]. In particular, type III and type V collagens are reported to participate in cell development, tissue damage and repair mechanisms [7, 8]. Type V collagen has been identified to be present in association with basement membranes, chondrocytes, smooth muscle cells and matrices [9–11].

The role of the extracellular matrix in the control of normal cell growth and development has been recognized for several years [12], but its potential influence

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on neoplastic cell behavior only recently has gained attention [13]. The interactions between tumor cells and the extracellular matrix are multiple and complex. Normal cell matrix interactions may be altered in neoplasia, and this may influence tumor proliferation and invasion [14]. Tumor cells can degrade matrices via the production of several enzymes such as collagenase, cathepsin B, cathepsin L and elastase [15, 17], but they also may synthesize matrix components [18–20] or influence the synthesis of matrix constituents by stromal cells [21, 22]. The origin of the matrix in tumor tissue remain uncertain, but tumor cells may synthesize their own matrix. Several studies have shown the production of various collagens and glycoproteins by human tumor cells [18–20]. The matrix in a neoplastic tissue may also be contributed by host stromal cells, and some tumor cells produce factors that stimulate collagen synthesis by normal cells [23]. The mechanisms involved in the production of tumor matrix collagen are therefore complex, and observations with highly selected cell lines may be deficient in showing how collagen expression is controlled in neoplasmas.

It has been reported that accumulation of type V collagen is associated with several pathological tissues such as hypertrophic scar [9], atherosclerotic plaques [23] and diseased human gingiva [24]. An increase in the concentration of type V collagen also has been found in human breast cancer [20]. Similarly, Peltonen *et al.* [25] have reported that the expression of type VI collagen genes is increased in patients with systemic sclerosis. Furthermore, recent studies have found the increased levels of type VIII collagen in human brain tumors compared to the normal brain [26]. By contrast, little is known about the effect of fibrosarcoma on the content and distribution of type V collagen. Previously, we have reported altered metabolism of glycosaminoglycans in fibrosarcoma tissue [27]. In the present study, an attempt has been made to investigate the changes in the type V collagen content in rat fibrosarcoma induced by 3-methylcholanthrene, and the results were compared with normal muscle tissue.

Materials and methods

Reagents

Acrylamide, methylene bis acrylamide, pepsin and 3-methylcholanthrene were obtained from Sigma Chemical Co., St. Louis, U.S.A. Phosphocellulose p-11 was obtained from Whatman, England, and cyanogen bromide was purchased from CSIR Center for Biochemicals, New Delhi, India. All other chemicals used were of analytical grade.

Tumor induction and transplantation

Fibrosarcoma was induced in Wistar strain female albino rats according to the established procedure, and the fibrosarcoma was maintained by subcutaneous transplantation as described earlier [27]. Subcutaneous skeletal muscle tissue from the thigh region of the same age group of rats was taken as control.

Measurement of total collagen content in tissue specimens

To determine the total collagen content present in normal muscle and fibrosarcoma tissues, the samples were freeze dried, and the concentration of hydroxyproline was measured by the method of Neuman and Logan [28]. Briefly, the dried tissue specimens were hydrolyzed in 6 N HCl for 20 hr at 110° C. The acid hydrolysate was evaporated in a vacuum desiccator, and the amount of hydroxyproline was determined. The amount of collagen was then calculated by multiplying the hydroxyproline content by the factor 7.46 [28].

Extraction and fractionation of matrix collagens

The collagen was extracted from normal skeletal muscle and fibrosarcoma tissues according to the method of Miller and Rhodes [10]. Briefly, tissue samples were minced, washed with cold saline, suspended in 10 volumes of 0.05 M Tris-HCl buffer, pH 7.5, containing 1.0 M NaCl and homogenized with a polytron homogenizer at 4° C. The homogenate was stirred gently for 24 hr at 4° C and centrifuged at 40,000 × g for 30 min. The residue was suspended in the same buffer and re-extracted twice as described above. The supernatants were combined and used for analysis. The insoluble residue from the above extractions was homogenized in 0.5 M acetic acid and acid soluble material was extracted. After acid extraction, the residue was subjected to pepsin digestion as described below. The residue was suspended in 5 volumes of 0.5 M acetic acid, pepsin (100 μg/ml) was added, and extracted thrice by stirring gently at 4° C for 24 hr. The pepsin solubilized material was separated by centrifugation, supernatants from each pepsin extraction were combined and used for further studies. Type I collagen was then isolated from both neutral salt soluble and acid soluble extractions by precipitating with 4.0 M and 2.0 M NaCl, respectively, as described by Miller and Rhodes [10].

Collagens extracted in the pepsin digest were precipitated by adding NaCl to 2.0 M concentration, and the precipitated protein was separated by centrifugation. The precipitated proteins were dissolved in 0.05 M Tris-HCl buffer, pH 7.5, containing 1.0 M NaCl and subjected to selective salt precipitation by dialyzing against the same buffer containing various concentrations of NaCl, to isolate different types of collagens. Type III collagen was isolated by precipitating at 1.8 M NaCl concentration, and type I and type V collagens were precipitated at 3.0 M and 4.0 M NaCl concentrations, respectively. Type I trimer collagen was isolated by precipitating at 3.5 M NaCl concentrations in dialysis buffer. The precipitated collagen was removed each time by spinning at 35,000 × g for 30 min and redissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid and lyophilized. The amount of collagen present in the lyophilyzed material was determined by estimating the hydroxyproline content as described earlier.

Electrophoretic analysis

The collagen samples obtained from different extractions were analyzed on SDS-polyacrylamide gels by the procedure of Laemmli [29], and the protein bands were located by staining with Coomassie blue.

Phosphocellulose chromatography of collagen sample obtained from 4.0 M NaCl precipitation

Type V collagen comprised of α1(V), α2(V) and α3(V) chains was chromatographed on phosphocellulose p-11 column according to the method described by Sage and Bornstein [11]. Prior to the application, the sample was dissolved in 0.1 M acetic acid at a concentration of 0.5 mg/ml and dialyzed against the column buffer (30 mM dibasic sodium phosphate, pH 6.3) at room temperature. The sample was heated at 42° C for 30 min and applied onto the column. The unbound proteins were washed and the collagen chains were then eluted with a linear gradient of 0-0.2 M NaCl in the column buffer.

Cyanogen bromide peptide mapping of collagen sample obtained from 4.0 M NaCl precipitation

Cyanogen bromide peptide mapping was carried out according to the method of Black *et al.* [30]. Collagen sample was dissolved in 5 ml of 70% formic acid (w/v). Cyanogen bromide was added in excess and incubated for 3 hr at 30° C [31]. The reaction was arrested by adding 10-fold distilled water, and the material was lyophilyzed. The CNBr cleaved peptides were analyzed on SDS-polyacrylamide gels. In a separate experiment, the eluted fractions representing peak 1 (Fig. 2) were pooled, dialyzed against distilled water and lyophilyzed. The lyophylized material was then subjected to CNBr treatment as described above. The resulting peptide fragments obtained by CNBr treatment were then separated on CM-cellulose column chromatography. The unbound materials were washed with 0.02 M citrate buffer, pH 3.6, containing 0.04 M NaCl. The bound peptides were eluted with linear gradient using 0.04 M NaCl to 0.14 M NaCl in the elution buffer.

Amino acid analysis

The amino acid composition of various collagen samples were determined according to the method described by Heinrikson and Meredith [32]. About 5 mg of sample was hydrolyzed in 6 N HCl for 18 hr at 110° C and the acid was removed by evaporating in a rotary evaporator. The samples were then prederivatized with phenyl isothiocynate and analyzed according to the PICO-TAG method using HPLC (Waters Associates, USA).

Results

Biochemical alterations in the connective tissue matrix are a common feature of many diseases including tumor invasion and metastasis. Previously we observed that the turnover of glycosaminoglycans is increased in rat fibrosarcoma induced by 3-methylcholantherene. In this investigation we showed characteristic alterations in the relative amounts of various collagen types in fibrosarcoma tissue. As indicated in Table 1, the content of type V collagen is increased 26-fold in fibrosarcoma tissue compared to the normal muscle. Accord-

Table 1. Effect of 3-methylcholantherene induced fibrosarcoma on the content of various collagens

Collagen type	Normal muscle tissue	Fibrosarcoma tissue	Increase/ decrease (fold)
Total	84.50 ± 5.20	74.35 ± 4.95	− 0.88
Type I	75.20 ± 4.72	53.80 ± 3.84	− 0.71
Type III	0.62 ± 0.05	3.20 ± 0.18	+ 5.20
Type V	0.17 ± 0.02	4.40 ± 0.20	+ 26.00
Type V/total collagen ratio	0.002	0.06	+ 30.00

Values are mean ± SD.
Results were expressed as mg/100 mg of dry weight of tissue.

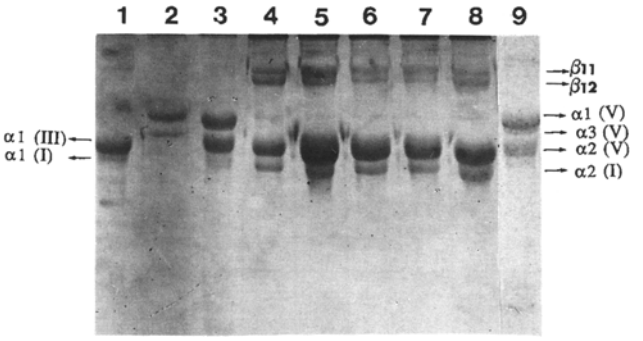

Fig. 1. SDS-polyacrylamide gel electrophoresis of collagens. Fibrosarcoma tissue was subjected for the extraction of various collagens as described in the text. Purified collagens were then dissolved in sample buffer containing SDS and heated at 95° C in boiling water bath for 5 min. Approximately 5–6 μg of sample was loaded on 7.5% polyacrylamide gel. Lane 1, collagen precipitated at 1.8 M NaCl concentration from pepsin digested material; Lanes 2 and 3, collagen precipitated at 4.0 M NaCl concentration from pepsin digested material; Lane 4, collagen extracted in neutral salt buffer; Lane 5, collagen extracted in 0.5 M acetic acid; Lanes 6 and 7, collagen precipitated at 3.5 M NaCl concentration from pepsin digested material; Lane 8, type I collagen from rat skin; Lane 9, standard type V collagen from human placenta.

ingly, the ratio of type V to the total collagen was found to be enhanced 30-fold in fibrosarcoma tissue. On the other hand, the effect of fibrosarcoma on the contents of total collagen and type I collagen was found to be very minimal (Table 1). However, considerable alterations in the amount of type III collagen (5-fold increase) were observed in fibrosarcoma tissue compare to the normal muscle.

In order to study the alterations in relative amounts of type V collagen in rat fibrosarcoma, we isolated type V collagen from normal and diseased tissues and characterized. Extractions of the homogenized normal muscle and fibrosarcoma tissues in 0.05 M Tris-HCl buffer, pH 7.5, containing 1 M NaCl, and 0.5 M acetic acid containing pepsin, yielded 82% and 86%, respectively. On SDS-polyacrylamide gel electrophoresis the purified collagen samples showed different characteristic pattern of collagen chains when compared to the type I collagen of rat skin (Fig. 1). However, the collagen samples extracted in neutral buffer and 0.5 M acetic acid showed a typical pattern of type I collagen of rat skin (Fig. 1, lanes 4 and 5). Also, collagen precipitated at 3.5 M NaCl concentration in the pepsin digest showed a pattern of type I collagen (Fig. 1, lanes 6 and 7). The ratios of α1 and α2 chains of these collagen molecules extracted in neutral buffer, 0.5 M acetic acid and 3.5 M NaCl precipitate of pepsin digest were apparently found to be 2 : 1. The collagen samples obtained from 1.8 M NaCl precipitation of pepsin digest showed a different pattern on SDS-polyacrylamide gel and contains only α1 chains (Fig. 1, Lane 1). The characteristic mobility of the bands on SDS-polyacrylamide gel indicates that these chains are derived from type III collagen in the material precipitated at 1.8 M NaCl concentration. However, the material precipitated at 4.0 M NaCl concentration showed bands above the α chains of type I collagen on SDS-polyacrylamide gel electrophoresis (Fig. 1, lanes 2 and 3). This material was then chromatographed on phosphocellulose column, and the results were compared with the elution profile obtained for gingival type V collagen reported by Narayanan *et al.* [33] to establish the identity of the type of collagen and separation of different subunits. A close examination of these results from the elution profile of phosphocellulose column revealed that type V collagen is extracted in 4.0 M NaCl precipitation of pepsin extract. By phosphocellulose chromatography, the material was separated into three peaks which were corresponding to the α1, α2 and α3 of type V collagen (Fig. 2). The fractions corresponding to each peak were pooled separately, dialyzed against distilled water, lyophilyzed and further characterization was made by amino acid analysis and cynogen bromide fragmentation.

Amino acid composition analysis of chain A, chain B and chain C of collagen which had been isolated from fibrosarcoma tissue and purified by phosphocellulose column chromatography are shown in Table 2, and the results were compared with the various chains of type V collagen reported earlier [11, 34]. The results on amino acid composition analysis reveal very marginal differences between the amino acid compositions of different tumor collagen chains and those of human collagen chains except that lysine and proline were less hydroxylated. Cyanogen bromide cleavage was made on the collagen sample obtained from 4 M NaCl precipitate of pepsin extract, and resulting peptide fragments were analyzed by SDS-polyacrylamide gel electrophoresis. A typical pattern of CNBr peptides of type V collagen was obtained (Fig. 3). The results from CM-cellulose chromatography of cyanogen bromide peptide fragments of the sample obtained from phosphocellulose column of peak 1 showed also a characteristic elution pattern of type V collagen (Fig. 4). On the basis of solubility behavior, electrophoretic migration, elution profile pattern on chromatography, cyanogen bromide analysis and amino acid composition, this collagen was confirmed to be type V collagen.

Discussion

Alterations in the amounts and relative proportions of various collagens constitute an important aspect of many diseases affecting the connective tissue matrices [35]. Increased proportion of type V collagen has been observed in chronically inflamed human gingiva [24, 36], and atherosclerotic plaques [8] and during hyper proliferation of fibroblasts such as hypertrophic scars [9]. Moreover, characteristic accumulation of type V collagen has been reported in certain carcinomas [37] and in desmoplasia associated with stroma of human breast cancer [20]. These data suggest that type V collagen is synthesized in the vicinity of proliferating cells. Barksy *et al.* [20] have also showed that the source for elevated type V collagen content is not by the host stromal cells. Recently, Marian and Danner [38] have shown that type V collagen content is increased during skin tumor promotion by 12-O-tetradecanoyl phorbol-13-acetate (TPA). They also showed that when TPA treatment was discontinued after a period that was sufficient for a transient reduction for the dermal collagen content but insufficient for tumor formation, type V collagen remained normal.

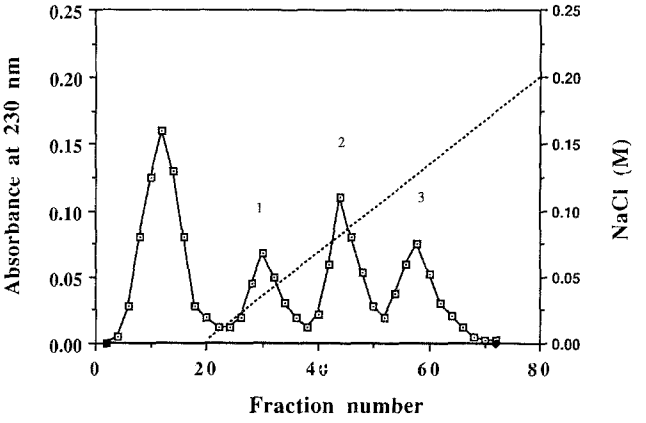

Fig. 2. Phosphocellulose chromatographic profile of collagen extracted from fibrosarcoma tissue. The collagens precipitated with 4.0 M NaCl concentration from pepsin digest were dissolved in acetic acid and dialyzed against in column buffer (30 mM sodium phosphate buffer, pH 6.3) at room temperature and applied on to the column. The column (0.7 × 9 cm) was equilibrated in column buffer prior to the application of the sample. Unbound proteins were washed, and the bound proteins were eluted under a linear gradient of 0–0.2 M NaCl in the column buffer. Fractions of 4 ml were collected.

Previously, it has been reported that type V collagen promote cell attachment and migration [6], and specifically, a fragment of cynogen bromide peptide (CB6) of

Table 2. Amino acid composition of different chains of type V collahen obtained from fibrosarcoma. (Rsidues/1000 residues)

Amino acid	Human Chain A* α1 (V)	Tumor Chain A α1 (V)	Human Chain B* α2 (V)	Tumor Chain B α2 (V)	Human Chain C** α3 (V)	Tumor Chain C α3 (V)
Alanine	54	53	41	43	62	49
Arginine	48	47	40	38	42	33
Aspartic acid	50	53	49	54	42	58
1/2 Cystine	0	0	0	0	1	1
Glycine	331	343	332	324	330	321
Glutamic acid	89	82	100	94	97	97
Histidine	10	9	10	8	14	12
Hydroxylysine	23	23	36	32	43	34
3-Hydroxyproline	3	1	0	2	1	1
4-Hydroxyproline	106	92	110	96	91	91
Isolucine	15	16	17	18	20	18
Lucine	37	35	36	35	56	38
Lysine	13	12	18	18	15	20
Methionine	11	11	9	10	8	7
Phenylalanine	11	14	12	12	9	17
Proline	107	102	130	136	98	112
Serine	34	46	23	32	34	41
Threonine	29	24	21	22	19	23
Tyrosine	2	2	4	4	2	3
Valine	27	25	17	24	29	22

* Data taken from Rhodes and Miller [30].

** Data taken from Sage and Bornstein [9].

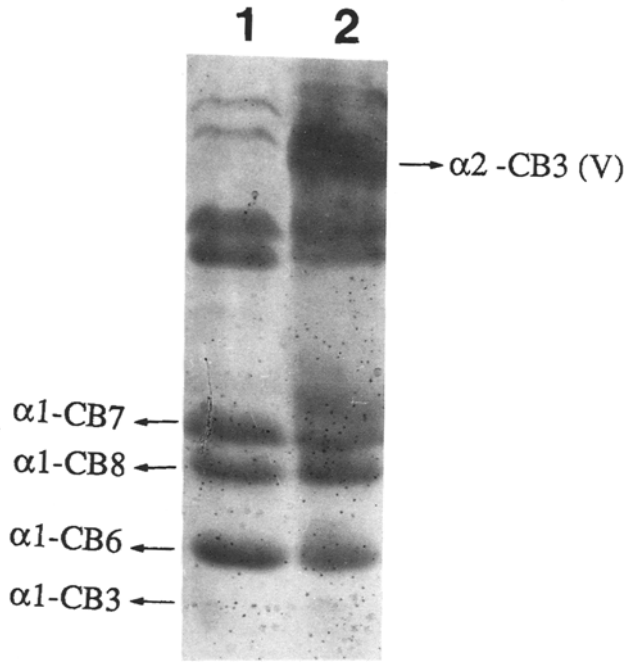

Fig. 3. SDS-polyacrylamide gel electrophoresis of collagen fragments. Purified collagen from fibrosarcoma tissue was subjected to the cynogen bromide treatment as described in the text. The resulting peptide fragments were dissolved in sample buffer containing SDS and heated at 95° C in boiling water bath for 5 min and then analyzed on 12% SDS-polyacrylamide gels. Lane 1, peptides obtained from type I collagen of rat skin; Lane 2, peptides obtained from collagen precipitated at 4.0 M NaCl concentration of pepsin digest.

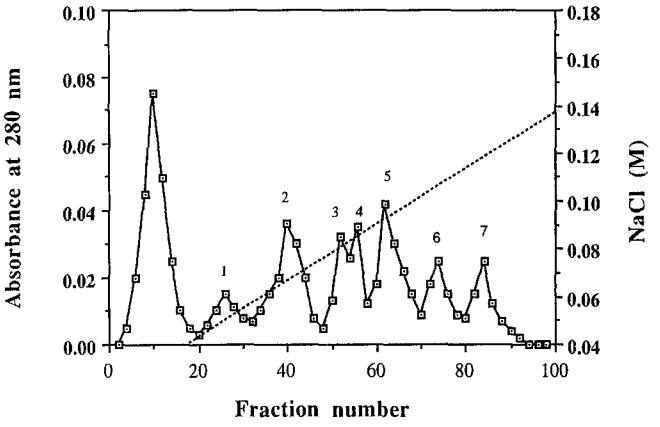

Fig. 4. CM-cellulose chromatography of collagen-peptides obtained by cyanogen bromide treatment. The sample obtained from phosphocellulose column (peak 1) was treated with cyanogen bromide as described in the text. The resulting peptide fragments were then chromatographed on CM-cellulose column. The column (0.7 × 9 cm) was equilibrated in column buffer (0.02 M citrate buffer, pH 3.6), containing 0.04 M NaCl. The unbound materials were washed, and the bound fragments were then eluted with linear gradient of 0.04–0.14 M NaCl in column buffer. Fractions of 2.6 ml were collected.

α2 (V) chain serves as a binding site to some plasma membrane glycoprotein [39]. Type V collagen also interacts with preferentially other collagens and with thrombospodin [40]. Our results indicate that type V collagen is present in fibrosarcoma tissue and that it is increased in relative amounts, compared with normal skeletal muscle. The increase in type V collagen in fibrosarcoma tissue may have some influence on the tumor cell migration and attachment which is essential future for the cancer cells. Similarly, other investigators also have reported the increased amounts of type V collagen in capillary blood vessels denuded of their endothelial lining [41] and in atherosclerotic plaques [8].

Although presently it is not clear how type V collagen content is increased in fibrosarcoma tissue, evidence suggests that it may be produced by a small sub-population of cells as diseased gingiva [21], and the size of this population may undergo expansion in pathological conditions. Alternatively, all the resident cells in both normal and diseased tissues may produce this type of collagen, but the amounts may be regulated by environmental ligands uniquely present at different concentrations in diseased tissues compared to normal tissues. Finally, the diseased tissues may contain abnormally large numbers of cells other than fibroblasts, such as smooth muscle cells [42] or epithelial cells [5] which may produce type V collagen.

The precise localization of type V collagen and its exact role are not well understood, although it appears that this molecule may be localized in pericellular spaces and near the basement membrane [43]. However, Martinez-Hernandez *et al.* [44] showed that this collagen plays an important role in binding collagen fibrils to associated structures; thus any alterations occurring in the type V collagen may influence the accessibility to degradative enzymes of the other collagens. In addition, the type V collagen also has been shown to promote the cell attachment of some cell types and their chemotaxis in the absence of glycoproteins such as fibronectin [45]; thus, any variation in the content and distribution of this collagen under pathological conditions is likely to have important consequences.

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