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COLLAGEN αA AND αB CHAINS CONSTITUTE TWO SEPARATE MOLECULAR SPECIES

Zdenek Deyl, Karel Macek and Milan Adam

Institute of Physiology, Czechoslovak Academy of Sciences, Prague-Krc and Research Institute for Rheumatic Diseases, Prague-2, Czechoslovakia

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SUMMARY

Rat skin contains αA and αB collagen the proportion of which changes independently with age. The proportion of αA chains alters more rapidly when compared to αB. It has been also shown that both these collagen categories are closely associated with tissue vasculature. It is suggested that αA and αB constitute two different collagen molecules of the composition $(\alpha A)_3$ and $(\alpha B)_3$ respectively.

INTRODUCTION

It has been well established that there is a genetically determined polymorphism in the molecular structure of collagen. At least five different types of this protein were proven to be present in different tissues. As shown previously, in skin the proportion of individual collagen types changes with age (1,2). As long as the production of all collagenous types is likely to occur in the same type of cells, it has been suggested that there should be a programming mechanism which determines what type of collagen is produced at a particular stage. Such changes appear to have a strong impact upon the physiological function of the organ as shown in the case of increased basement membrane collagen (type IV) in kidneys with age (3). There are, however, also other two types of collagen specific for basement membranes (4). Consequently αA and αB chains were described in (5), which differ from type IV collagen in the absence of cysteine. It is feasible to anticipate that the structural differences of these proteins will be reflected in their biological function. Besides Brown et al. (4) were able to separate the so called (αB) chain into two bands (αB) and (αC) upon discontinuous SDS polyacrylamide gel electrophoresis. These components are very soluble in quite high salt concentrations (3.2 M). While at the beginning of their history several descriptions of such components appeared relating them to tissues with a high basement membrane content, recent reports revealed

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their presence in a number of other tissues as well (4). It is likely that these collagen chains derive from capillaries or small blood vessels present in the respective tissues.

The present study was devoted to the age dependent occurence of this type of basement membrane collagen in tissues and to further information about its molecular organization.

MATERIAL AND METHODS

Animals and tissue:

Male Wistar rats of appropriate age were used (foetal, newborn, 1 week, 1 year).

Collagen preparation: The skin was freed from fat, hair and muscle, cut into small pieces and homogenized. Samples were then extraced twice with 1M sodium chloride - 0.05 M TRIS at pH 7.5 and then four times with 0.5 M acetic acid. The residue was then cleaved with pepsin according to (1). Twice re-crystallized pepsin (Worthington 100 mg) was dissolved in 200 ml of 0.4 M acetic acid, 1 g of insoluble collagen added and the suspension stirred at 15° C for 22 hours. The insoluble residue was redigested under the same collagen - enzyme ratio, but the digestion temperature was raised to 37° C. The supernatant fluid was filtered and precipitated twice by dialysis against 0.02 M sodium phosphate. Then the supernatant material was dialyzed against 0.5 M acetic acid containing 0.86 M sodium chloride. The precipitates were dissolved in 0.1 M acetic acid.

Sodium chloride precipitation (5): The collagen solution in 0.1 M acetic acid was adjusted to pH 7.5 by dialysis against 0.5 M sodium chloride-0.05 M TRIS (pH 7.5) and successively dialyzed against 2.6 M and 4.0 M sodium chloride containing 0.05 M TRIS (pH 7.5). The precipitates obtained at these sodium chloride concentrations were harvested by centrifugation and dissolved in 0.5 M acetic acid and lyophilized.

Analytical methods:

Gel electrophoresis (6): Gels containing 3 per cent (stacking gel), 8.0 per cent or 10 per cent acrylamide were prepared from a stock solution of 30 per cent by weight of acrylamide and 0.8 per cent by weight of N,N'-bis-methylene acrylamide. The final concentrations in the separation gel were as follows: 0.375 M TRIS-HCl (pH 8.8) and 0.1 per cent SDS. The gels were polymerized by the addition of 0.025 per cent by volume of tetramethyl-ethylenediamine (TEMED) and ammonium persulphate. The electrode buffer (pH 8.3) contained 0.025 M TRIS and 0.192 M glycine and 0.1 per cent SDS. The samples (0.2-0.3 ml) contained the final concentrations ("final sample buffer"): 0.0625 M TRIS-HCl (pH 6.8), 2 per cent SDS, 10 per cent glycerol, 5 per cent 2-mercaptoethanol and 0.001 per cent bromophenol blue as the dye. The gels were diffusion-destained by repeated washing in 7 per cent acetic acid.

Densitometric scans of polyacrylamide gel slabs were done on PHI 5 densitometer (Wissenschaftlich Technische Werkstätten GmBH, Weilheim B.D.R.).

CM cellulose chromatography of collagen chains was performed on a 2x30 cm column. Equilibration buffer consisted of 0.04 M sodium acetate at pH 4.8 and 6.0 M urea. The column was developed with a superimposed linear gradient from 0-80 mM sodium chloride at 42° C (500 ml). Absorbance at 230 nm was recorded in the effluent.

Amino acid analysis: This was done with the Automated Amino acid Analyser Microtechna Prague. Columns were packed with Ostion KS and pH 4.26 citrate buffer (column temperature 40° C, flow rate 0.6 ml per min) was used for elution.

The amount of collagen fractions in balance studies was asayed by hydroxyproline determination according to (7).

Immunofluorescence staining: For immunofluorescence staining frozen sections were freed from proteoglycans with 4 M guanidinium hydrochloride. Further, sections were labeled with antibodies to the αA and αB chains (0.05-0.15 mg/ml) for thirty minutes at room temperature, washed with phosphate buffer, saline and counterstained with fluorescein conjugated with goat anti rabbit γ-globulin, diluted 1:10 (8).

RESULTS

Fraction precipitation of the pepsin solubilized insoluble rat skin collagen allowed the identification and quantitation of αA and αB chains. An enriched fraction of these α-chains was obtained by treating the insoluble skin collagen with pepsin and by precipitating the digest with 1.7, 2.5 and 4.0 M NaCl. While the ratio of the 1.7 and 2.5 M NaCl precipitable fractions changes with the age (foetal, newborn and 1 week old rats) only slightly (avarage values being 56% and 40% resp.), the 4 M NaCl precipitable fraction decreased within this period of time quite drastically (Table I) and no material precipitable at this concentration was recovered in pepsin treated insoluble rat skin collagen from individuals of one year of age.

CM cellulose chromatography of the precipitated material resulted into two dominant peaks (fig. 1), containing αA and αB fractions as indicated by amino acid analyses (Table II) and SDS-polyacrylamide gel electrophoresis (not shown).

Polyacrylamide gel electrophoretic separation of the crude 4 M NaCl precipitate showed distinct peak with a slower mobility than α_1(I) chain, containing αB chain. It was possible to quantitate this material and to determine its proportion in the mixture. As shown in Table III, a distinct drop in the proportion of this material occured during the first week of life.

Reprecipitation of the crude 4 M precipitate removed most of contaminating α_1 and α_2 chains; thereby the conditions for SDS polyacrylamide gel electrophoresis were improved in such a way that the determination of the proportion of αA and αB chains was possible (Table IV). As indicated, the relative proportion of αA drops much faster than the proportion of αB; the decrease of the high molecular weight α-chains was shown not to occur in parallel and the relative proportion of αA and αB chains was proved to change during early ontogeny.

TABLE I. The amount of 4 M NaCl precipitable fraction of pepsin treated rat skin collagens in relation to the age of the animal

Age of the animal	I. pepsin extract (15°C)			II. pepsin extract (37°C)			Percentage of total insoluble collagen solubilized by pepsin in the first step			Percentage of total insoluble collagen solubilized in the first and second step		
	% of total pepsin soluble collagen											
Foetal	2.0	2.5	1.9	7.2	8.7	7.6	80	92	90	95	98	98
Newborn	2.0	2.3	2.6	8.4	8.6	8.2	90	90	83	98	97	88
1 week	0.54	0.70	0.70	0.40	0.40	0.60	75	73	69	80	85	80
1 year		none			none		60	58	63	75	67	78

Note: individual data refer always to three independent analyses of pooled material

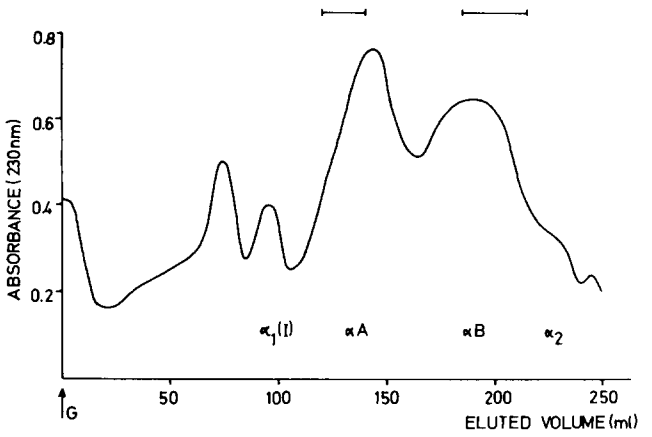

Fig. 1 CM-cellulose elution pattern of the 4.0 M NaCl precipitable collagen fraction. Pooled fractions (indicated by horizontal bars) were used in further study.

TABLE II. The amino acid composition of αA and αB chains (in residues per 1000 amino acids)
(Isolated from skin of newborn rats)

	αA	αB
4-Hyp [x)]	120	115
Asp	50	45
Thr	30	15
Ser	42	25
Glu	100	100
Pro	111	146
Gly	323	334
Ala	50	47
Cys	-	-
Val	30	25
Met	9	5
Ile	11	17
Leu	29	35
Tyr	1	2
Phe	13	13
Hyl	28	32
Lys	12	10
His	6	4
Arg	35	30

x) 3-Hyp was not detected

TABLE III. The variation of αB collagen fraction with age

Age of animal	Content of αB fraction in pepsin solubilized material		
Foetal	0.80	0.80	0.75
Newborn	0.80	0.75	0.85
1 week	0.38	0.45	0.40
1 year	not detected		

Note: individual data refer always to three independent analyses of pooled material

TABLE IV. Relative proportion of αB : αA fraction with age

Age	αB : αA ratios
Foetal	1 : 0.80
Newborn	1 : 0.75
1 week	1 : 0.55

In our hands both αA and αB bands were PAS positive in polyacrylamide gel electrophoresis indicating thus presence of sugars in a rather high amount. Immunofluorescence staining of granulation tissue from arthritic joint showed the presence of αA and αB collagen in the walls of small blood vessels (Fig. 2).

DISCUSSION

Three main conclusions could be derived from our data:

1) The αA and αB chains appear to constitute separate collagen molecules. This conclusion is based on the observation that αA and αB chains change their proportion independently during ontogeny. This observation can be further supported by data published by Rodes and Miller (9) who have reported the different ratios of both αA and αB chains in placenta and bone and morover they state that αA chain is absent in cartilage collagen preparations.

2) The ratio of αA and αB chains changes during ontogeny in favour of the αB chain. This appears to be true not only for skin as shown in this paper,

Fig. 2 Synovial granulation tissue - immunofluorescent staining with antibodies to αAB collagen pretreated with guanidinium chloride.

but also for other tissues like bone and cartilage (9). In skin of adult animals both αA and αB chains were not accessible to analysis by methods available to us.

3) The observed age changes of the αA and αB chains may arise from the changes in tissue vasculature. As shown in this paper, both these chains are related to small blood vessels the number of which is known to decrease in tissue with age.

Our results indicate not only that the high molecular weight α-chains disappear from the tissue rather rapidly after birth, but mainly that the speed of disappearance is different which is strongly in favor of the idea about the existence of two kinds of this type of collagen in tissues in early ontogeny, reflecting thus analogous age dependent changes in the proportion of other collagen types, c.f. type I and III (3).

The reason for the higher persistence of αB chain in tissues can be seen in its higher stability as indicated by higher melting point resulting very likely from the higher proportion of proline and hydroxyproline in

this structure (αB 261, αA 231). These differences in chemical properties of the αA and αB are likely to influence the properties of tissues involved but at present this is hard to evaluate.

Brown and coworkers (4) came to analogous conclusions with regard to the occurence of high molecular weight α-chains and their assembly at the embryonic stage of development with the exception that they were able to detect three different high molecular weight α-chains in inflamed rheumatoid synovial membrane, inflamed gingiva and foetal skin. In spite of using identical procedure of SDS electrophoresis, in our hands no more than two different high molecular weight α-chains were detected in rat collagen preparations. The reason for this difference can be seen in mainly two facts. Our experiments were not done with inflamed tissues and were done with rats, a source material not investigated with respect to high molecular weight α-chains so far. On the other hand other authors working with high molecular weight α-chains under similar conditions as in our case were also able to detect only two types of high molecular weight α-chains.

The differences in amino acid composition observed by us, Burgeson et al. (5), Rhodes and Miller (9) and Brown et al. (4) do not exceed expected experimental variations.

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