THE JOURNAL OF INVESTIGATIVE DERMATOLOGY
Vol. 51, No. 4

Printed in U.S.A.

HYALINE RETICULOXANTHOMA

THE CHEMICAL NATURE OF ITS HYALINE*

RAUL FLEISCHMAJER, M.D.† AND LEWIS SHAPIRO, M.D.‡

We recently studied an unusual mesodermal tumor with distinct clinical and histochemical features (1). The patient is a 65 year old, male Negro who presented a tumor of many years duration located on the side of the neck, midway between the mastoid and the supraclavicular fossa. It consisted of a solid mass, skin color, raised about 1.5 cm, cylindrical in shape, and firmly bound to the underlying structures. Histochemical studies revealed a connective tumor with 3 distinct components: (a) hyaline material (b) an intense net of reticular fibers and (c) xanthoma cells. The hyaline, which represented the bulk of the tumor, consisted of strongly periodic acid-Schiff (PAS) positive material, diastase resistant which also stained blue with Mallory's trichrome. Stains for elastic tissue were negative. The Congo red, crystal violet and phosphotungstic acid-hematoxylin stains were also negative, while the Alcian blue at pH 2.5 was slightly positive. Under the polarizing microscope the material showed mild birefrigence as compared to that seen in normal collagen. The hyaline material was resistant to pepsin and trypsin digestion. Following exposure to these enzymes, the PAS stain remained strongly positive but the trichrome stained the collagen red instead of blue. The purpose of the present study is to report on the chemical nature of the hyaline of this unusual tumor.

MATERIAL AND METHODS

The tumor was surgically excised including a border of 1 cm of apparently normal skin. One half of the tumor was reserved for histochemical analysis and the details of this study are being reported elsewhere (1). The specimen for chemical analysis was divided into two sections: (a) apparently normal adjacent skin, and (b) the tumor itself. Dissection of the overlying dermis from the tumor was easily achieved since the tumor had a deep yellow-orange color while the overlying dermis was white. Specimens of normal skin, obtained at autopsy, were used as controls. In all control skin specimens, the epidermis was separated from the dermis by stretching and scraping.

Analysis of the Total Tumor

Aliquots of the lyophilized tumor were hydrolyzed with 6 N HCl, in vacuum sealed tubes, for 24 hours at 110° C and use to estimate content of hydroxyproline by the method of Stegemann (2). Hexosamines were determined by hydrolyzing the specimens in 4 N HCl for 15 hours according to the method of Boas (3). Hexoses were estimated by hydrolyzing the samples in 2 N HCl in boiling water for 5 hours and using the anthrone method. Similar determinations were performed in all the controls.

Ground Substance

Eighty milligrams, dry weight, of the tumor were ground through a Wiley micro-model mill for 5 seconds and extracted by stirring with 10 ml of 1 M NaCl at 5° C for 24 hours. The extract was centrifuged at 10,000 RPM for 30 minutes and the supernatant decanted and filtered through a fine sintered glass filter. The residue was then extracted with 10 ml of 0.1 M citrate buffer, pH 3.8 in a similar fashion as described above. Aliquots of both extracts were subjected to determination of total proteins by Lowry's method (4), hexoses, hexosamines and hydroxyproline. The rest of the NaCl extract was dialyzed against distilled water for 48 hours, lyophilized, and analyzed by means of acrylamide gel electrophoresis in the vertical slab apparatus of Raymond (5), using the technique described by Ornstein (6). The spacer gel consisted of 3.5% acrylamide, 1 N HCl, Tris (Tris (hydroxymethyl) aminomethane) and TEMED (N,N,N′,N′-tetramethylenediamine) pH 6.7 while the running gel consisted of 7% acrylamide gel in Tris-glycine buffer, pH 8.9. One per cent samples were prepared and 20 microliters were run at 300 volts, 12 milliamperes per sample, for 45 minutes. The gels were stained with amido black and the PAS stain.

Collagen-bound Hexoses and Hexosamines

The tumor was extracted with 1 M NaCl, and 0.1 M citrate buffer as described above. The residue was washed several times with water, placed in dialysis against distilled water and

* From the Section of Dermatology, Department of Medicine, Hahnemann Medical College and Hospital, Philadelphia, Pa.† and the Department of Dermatology, College of Physicians and Surgeons, Columbia University, and the Dermatology Service, The Presbyterian Hospital, New York, N. Y.‡
Supported by USPHS Grant AM 08127.
Received for publication April 25, 1968; accepted for publication May 10, 1968.

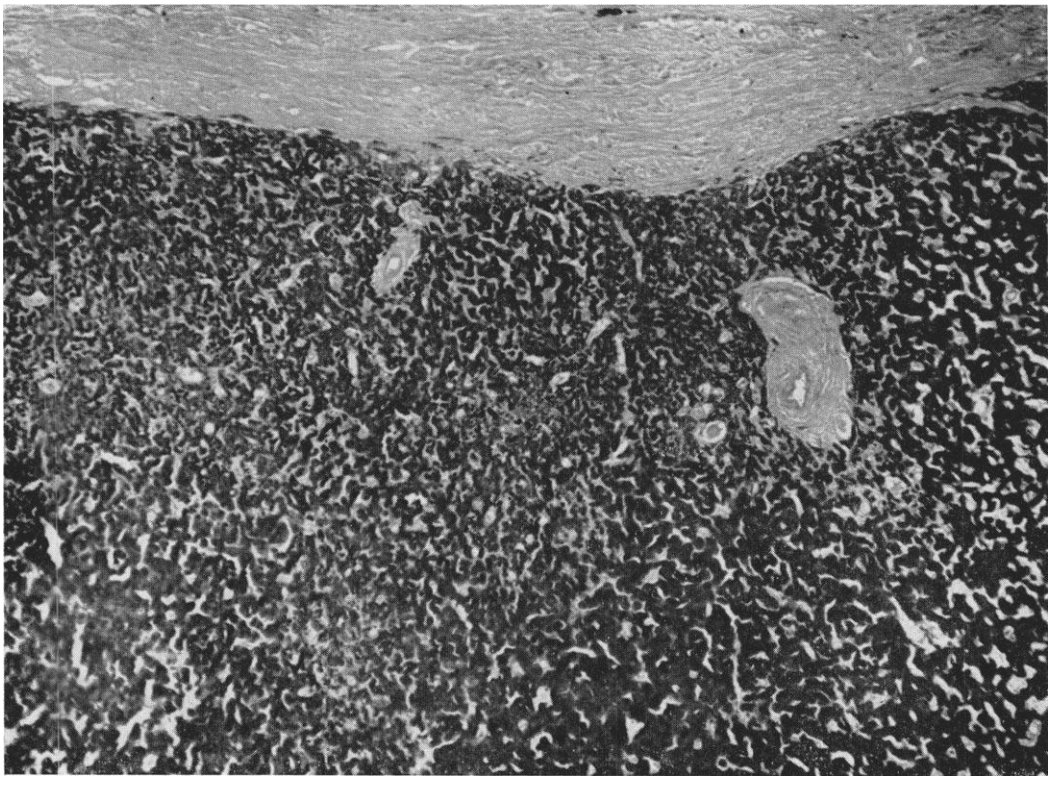

FIG. 1. Note on top the fibrillary structure of the normal dermis and below the massive accumulation of PAS-positive hyaline material. Diastase-PAS 85 ×.

FIG. 2. Higher magnification showing fibrillar arrangement at the periphery of the homogenous masses. Diastase-PAS 600 ×.

TABLE I

Total hydroxyproline, hexoses and hexosamines

	Hydroxy-proline	Hexoses	Hexos-amines
	mg/g dry weight		
Tumor	100.0	25.0	3.2
Dermis (adjacent to the tumor)	83.0	5.0	1.0
Normal dermis*	100.0 ± 3.0	8.5 ± 0.36	2.0 ± 0.29

* Normal and standard deviation of 10 specimens.

lyophilized. Aliquots of the dry material were analyzed for hydroxyproline content and revealed 95% collagen content. The rest of the dry residue was converted into gelatin by autoclaving at 20 pound pressure for 15 hours. After centrifugation, the supernatant was filtered through a fine syntered glass filter, lyophilized and labeled as gelatin. Five milligrams of the gelatin were used to estimate hexoses, and 35 mg were used for the determination of hexosamines. Two milligrams of the gelatin were hydrolyzed with 6 N HCl in vacuum sealed tubes for 24 hours at 110° C and used for amino acid analysis in a Technicon Autoanalyzer according to the technique described by Piez and Morris (7).

Electron microscopy

Due to the unexpected findings of the histochemical study, electron microscopy was performed on the formalin-fixed specimen.* The paraffin was removed with several changes in xylene. The xylene was removed by repeated washes in ethanol. The material was reembedded in Epon by the technique described by Luft (8). The sections were cut on a Porter-Blum microtome at 600 Å and examined in an RCA 3-G electron microscope.

RESULTS

The chemical analysis on the total tumor is reported in Table I. The concentration of hydroxyproline in the tumor was the same as that seen in normal dermis. The increase in hexoses and hexosamines was in good agreement with the intense PAS reactivity of the hyaline material. Obviously the question that immediately arises is whether these sugars represent a neutral polysaccharide of the ground substance or whether they are bound to the collagen. In reference to the latter assumption, one may add that the distribution of the PAS positive material followed the same pattern as seen with the trichrome stain. Analysis of the 1 M NaCl extract, which removed most of the dermal soluble components, revealed no significant changes from the normal controls (Table II). The normal concentration in soluble noncollagenous proteins, hexoses and hexosamines ruled out an increase in neutral or acid mucopolysaccharides. The amount of salt soluble collagen was about the same as that seen in the normal controls, although it was higher than that seen in the apparently normal dermis, adjacent to the tumor. Acrylamide gel electrophoresis of the 1 M NCl extract revealed small amounts of soluble noncollagenous proteins. The tumor extract revealed a band in the haptoglobulin region which was not present in the controls. However, this fraction reacted very faintly with PAS and was rather small (Fig. 3).

The estimation of collagen-bound hexoses and hexosamines revealed a marked increase of these sugars in the tumor specimen (Table III). The amino acid analysis of the purified gelatin from the insoluble collagen of the tumor gave the typical pattern seen in normal collagen (Table IV). Electron microscopy revealed collagen with normal periodicity.

DISCUSSION

Hyaline is a morphologic term that describes the homogenization of normal structures of vascular walls or the connective tissue. Fibrinoid and amyloid are often referred to as forms of hyaline. The chemical nature of

TABLE II

Total proteins, hydroxyproline, hexoses and hexosamines of 1 M NaCl extract

	Protein	Hydro-xyproline	Hexoses	Hexos-amines
	mg/g dry weight			
Tumor	50.0	0.50	2.37	0.32
Dermis (adjacent to the tumor)	43.0	0.13	2.62	0.42
Normal dermis*	51.0 ± 12	0.40 ± 0.20	2.75 ± 0.6	0.34 ± 0.06

* Mean and standard deviation of 6 specimens.

* The electron microscopic study was performed by Jerry M. Brown, M. S., Pathology Branch, Research Laboratories, Edgewood Arsenal, Maryland.

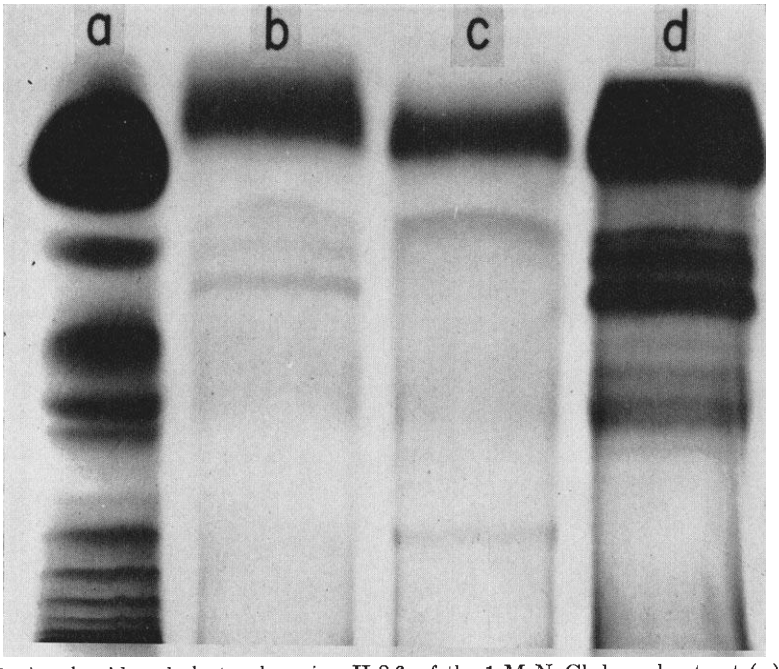

FIG. 3. Acrylamide gel electrophoresis, pH 8.6, of the 1 M NaCl dermal extract (a) serum control (b) normal dermis adjacent to the tumor (c) tumor (d) normal dermis control. Stained with amido black. There are no significant qualitative differences between the tumor extract and the controls, except for the band present in the haptoglobulin zone of the tumor specimens.

TABLE III

Hexoses and hexosamines of gelatins from insoluble collagen

	Hexoses	Hexosamines
	mg/g of gelatin	
Tumor	34.00	3.00
Dermis (adjacent to the tumor)	12.00	1.40
Normal dermis*	6.00 ± 0.6	0.92 ± 0.06

* Mean and standard deviation of 6 specimens.

hyaline is uncertain. It most probably represents a group of substances rather than a specific product since hyaline has been described in a variety of unrelated disorders such as arteriosclerosis, alcoholic cirrhosis, inflammatory and neoplastic processes of connective tissue etc. The current status of the nature of hyaline was recently reviewed by Wagner (9). There are two schools of thought as to the nature of the hyaline in arteriosclerosis, those who regard it as a local degenerative process of the vascular wall, probably involving muscle (10) and those who propose a hematogenous origin, namely, an initial deposition of fibrin followed by conversion of this protein into a collagen-like material (11, 12). Other authors suggested that hyaline may be an altered form of collagen (13, 14). None of the above sug-

TABLE IV

Amino acid composition of gelatin from the hyaline reticuloxanthoma

Amino acid	residues/1000 residues
Hydroxyproline	86.1
Aspartic acid	49.9
Threonine	22.1
Serine	35.8
Glutamic acid	70.3
Proline	118.3
Glycine	309.7
Alanine	128.3
Valine	27.3
Isoleucine	12.8
Leucine	34.6
Tyrosine	7.1
Phenylalanine	15.3
Hydroxylysine	3.5
Lysine	29.4
Histidine	5.3
Arginine	44.3

gestions has been substantiated by purification and chemical analysis of the hyaline in question.

The tumor under discussion gave us an unique opportunity to study the chemical nature of its hyaline, since this material was present in large amounts, as revealed by histologic sections. Histochemically, the most outstanding finding was the presence of a hyalinized material which stained strongly positive with PAS and blue with the trichrome stain. There was also an intense net of reticulum fibers. Since the concentration of salt soluble collagen present was about the same as that seen in normal adult dermis, it is unlikely that this tumor was actively engaged in the synthesis of collagen. Consequently, the intense net of reticulum fibers was most probably of a stromal nature. The analysis of the ground substance did not reveal significant changes in its content of soluble noncollagenous proteins, neutral or acid mucopolysaccharides. The tumor contained about 75 per cent collagen, measured by its hydroxyproline content. In this regard it is noteworthy that Ziff *et al.* (15) were able to detect only traces of collagen in fibrinoid extracted from rheumatic nodules while Calkins *et al.* (16) extracted tissues containing amyloid and estimated the presence of about 10 per cent collagen.

Normal human collagen contains a small amount of bound carbohydrates, (hexoses and hexosamines) which probably account for its mild reactivity with the periodic acid-Schiff stain (17). A significant increase in bound hexoses and hexosamines were noted in the insoluble collagen of the HRX. Thus, we suggest that the hyaline present in this tumor basically consists of collagen which in some way has been altered by its increased content of bound sugars. This would explain why this hyaline behaves like collagen with aniline blue and is strongly positive with the PAS stain. An increase in collagen-bound hexosamines has recently been reported in dermal collagen from scleroderma (18). The function of collagen-bound sugars is not well understood, although hexoses have been suggested as participating in intra- and possible intermolecular cross-linking (19). One may hypothesize that an increase in collagen-bound carbohydrates may in some way alter the chemical and or physical properties of collagen. We believe that this line of investigation should be expanded to other mesenchymal disorders, including rheumatoid arthritis, osteoarthritis, arteriosclerosis, etc.

SUMMARY

This is a report on the chemical nature of the hyaline present in an unusual mesodermal tumor designated as a hyaline reticuloxanthoma. Histochemical studies revealed that this hyaline stains blue with the aniline blue component of a trichrome technique and intensely purple with the periodic acid-Schiff stain. Purification of the hyaline showed that it consists of collagen with a marked increase in collagen-bound hexoses and hexosamines. This collagen revealed a characteristic amino acid composition and normal periodicity.

It is suggested that an increase of bound carbohydrates may in some way alter the physical and chemical properties of collagen with all its possible implications in connective tissue pathology.

REFERENCES

1. Fleischmajer, R. and Shapiro, L.: Hyaline reticuloxanthoma. A new mesodermal tumor. Arch. Derm. In press.
2. Stegemann, H.: Mikrobestimmung von Hydroxyproline mit Chloramin-T und p- Dimethylaminobenzaldehyd. Hoppe Seyler Z Physiol. Chem., *311:* 41, 1958.
3. Boas, N. J.: Method for the determination of hexosamines in tissues. J. Biol. Chem., *204:* 553, 1953.
4. Lowry, O. H., Rosebrough, N., Farr, A. L. and Randall, J. R.: Protein measurement with the folin phenol reagent. J. Biol. Chem., *193:* 265, 1951.
5. Raymond, S.: Acrylamide gel electrophoresis. Ann. N. Y. Acad. Sci., *121:* 350, 1964.
6. Ornstein, L.: Disc electrophoresis. I. Background and theory. Ann. N. Y. Acad. Sci., *121:* 321, 1964.
7. Piez, K. A. and Morris, L.: A modified procedure for the automatic analysis of amino acids. Anal. Biochem., *1:* 187, 1960.
8. Luft, G. H.: Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cyt., *9:* 409, 1961.
9. Wagner, B. M.: Hyaline and fibrinoid: Current status, p. 68, *The Connective Tissue,* International Academy of Pathology, Monograph No. 7. Ed., Wagner B. M., The Williams & Wilkins Co., Baltimore, 1967.
10. Muirhead, E. E., Booth, E. and Montgomery, P. O'B.: Deviation of certain forms of fibrinoid from smooth muscle. Arch. Path., *63:* 213, 1957.
11. Lendrum, A. C., Fraser, D. S., Slidders, W. and Henderson, R.: Studies on the character and staining of fibrin. J. Clin. Path., *15:* 401, 1962.

12. McKinney, B.: The pathogenesis of hyaline arteriosclerosis. J. Path. Bact., *83:* 449, 1962.
13. Gardner, A. F.: Morphologic study of oral connective tissues in lathyrism. J. Dent. Res., *39:* 24, 1960.
14. Van den Hooff, A.: Histological phenomena associated with collagen transformation and breakdown processes. Acta. Morph. Needrl. Scand. 5: 101, 1962.
15. Ziff, M., Kantor, T., Bien, E. and Smith, A.: Studies on the composition of the fibrinoid material of the subcutaneous nodule of rheumatoid arthritis. J. Clin. Invest., *32:* 1252, 1953.
16. Calkins, E., Cohen, A. S. and Larsen, B.: Amyloidosis: Preliminary clinical, chemical and experimental observations. Ann. N. Y. Acad. Sci., *86:* 1033, 1960.
17. Fleischmajer, R.: Hexoses, hexosamines and aldehydes in human dermal gelatins. J. Invest. Derm. In press.
18. Fleischmajer, R. and Krol, S.: Chemical analysis of the dermis in scleroderma. Proc. Soc. Exp. Biol. & Med., *126:* 252, 1967.
19. Hormann, H.: Chemische Untersuchungen uber die Kohlenhydratgruppierung des Kollagens. Das Leder, *11:* 173, 1960.