THE INTERCELLULAR SUBSTANCE OF THE CONNECTIVE TISSUE IN MYXEDEMA*

A MORPHOLOGICAL AND HISTOCHEMICAL STUDY

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Until recently, the intercellular substance of the connective tissue of normal skin, the mucinous matrix enveloping fibrils and cells, was regarded as an inert mass with no biological purpose. Recent morphological and chemical investigations as well as experiments involving the enzymatic hydrolysis of the viscous substance have, however, suggested that it plays a rôle in the protective mechanism of the organism (22, 23). In addition, it appears to be of primary significance in tissue regeneration (24).

Chemically, the intercellular substance of cutaneous connective tissue is made up of mucopolysaccharides, chiefly hyaluronic acid and chondroitin sulfuric acid, the former being by far the most important one in human skin (27, 28). It is a mucopolysaccharide containing no sulfuric acid, a compound of glucuronic acid and N-acetyl glucosamine, polymerized into a very large molecule (18). Pure hyaluronic acid is met with in the synovial fluid, in the vitreous humor, and, with equal amounts of chondroitin sulfuric acid, in the jelly of Wharton in the umbilical cord.

Hitherto, its origin has been unknown, but it is presumed to be secreted by young fibroblasts (18).

The large molecule of hyaluronic acid is partially or completely hydrolyzed by hyaluronidase, an enzyme present in certain human tissues and organs (testes, ciliary body, spleen, and, perhaps, the skin). It is formed by bacteria possessing invasive properties, and it is met with in some animal toxins. In addition, it is known that the enzyme plays a rôle in the infiltrative growth of at least some malignant tumors. Depolymerization of hyaluronic acid yields aldobiouronic acid chains which are hydrolyzed to hexoses (15, 16, 18). During this process, the viscosity of hyaluronic acid is reduced.

Judging by the results obtained in skin experiments, the specificity of purified testis hyaluronidase is absolute. (Some workers, have reported that their preparations also hydrolyzed chondroitin sulfuric acid at a slow rate (10, 19)).

Apparently, the amount, state, and composition of the intercellular substance depends on thyroid function (3, 20, 21, 25, 26, 27), perhaps by way of variations in the concentration of hyaluronidase in the tissues.

Hypothyroidism is accompanied by an increase in the amount of the intercellular substance which in some cases may be so marked that a visible swelling of the skin and subcutaneous tissue is formed, resembling edema and known as myxedema.

Exceptional cases of hyperthyroidism have been found to exhibit localized, circumscribed myxedema, similar to the condition attending thyroid hypofunction. This phenomenon is as yet inexplicable.

By hydrolysis experiments, Carol (2), in 1932, proved that the substance is made up of carbohydrates. Later chemical investigations (3, 25, 26) have shown that it consists partly or wholly of hyaluronic acid.

Bloom, Herrmann and Sharlit as well as Grais (1, 5) have reported improvement of myxedematous skin disorders following treatment with hyaluronidase.

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Specimens of myxedematous skin from two patients were examined histologically and histochemically, particularly with a view to the intercellular substance of the connective tissue.

The biopsy specimens were obtained with a punch, 8 or 4 mm. in diameter. The anesthesia consisted of local freezing by ethyl chloride.

The specimens were fixed in absolute alcohol. In parallel experiments, a 4 per cent solution of basic lead acetate and Carnoy's fluid were used.

The histological staining methods were as follows:

(A) **Metachromatic staining with toluidine blue (Lison)** (after Sylvén (24)).

The reaction has previously been considered specific for polysaccharide esters of sulfuric acid. Hyaluronic acid, however, also stains metachromatically. In the corium, the granules of the mast cells and a substance of an intercellular situation stain red, whereas most other structures stain blue.

(B) **Metachromatic staining with Unna's polychrome methylene blue.**

The mast cells are set out distinctly by this staining which, however, is not as specific as (A) in other respects.

(C) **Staining of the tissue polysaccharides by the method of Hotchkiss (9).**

By means of periodic acid, polysaccharides are oxidized to polyaldehydes which latter stain intensely with fuchsin sulfite.

(D) **Staining of acid polysaccharides by the method of Hale (6).**

When biopsy specimens are submitted to the action of an acid solution of ferric hydroxide, the iron will unite with the acid polysaccharides and not with the neutral ones or the proteins. When hydrochloric acid and potassium ferrocyanide are added, the bound iron is demonstrated by means of the Prussian blue reaction. (Examples of acid polysaccharides are hyaluronic acid, chondroitin sulfuric acid, and heparin.)

**Case 1.** A 68-year-old female, T. P., suffering from hypothyroidism (basal metabolic rate without treatment: —35 per cent). When the patient applied to the Clinic of Dermatology in May 1949 because of symmetrical, cushiony myxedematous swellings of the backs of both hands, the B.M.R. was kept normal by constant maintenance treatment with a thyroid hormone preparation, 800 units, corresponding to thyroxin, 0.8 mg. daily.

A biopsy specimen was obtained from the back of the left hand. At the same time, the treatment with thyroid hormone was suspended, and 7 days later another biopsy specimen was obtained from the symmetrical site on the right hand. (At this juncture, the patient exhibited marked general symptoms and had gained 3 kg. Therefore, it was not considered warrantable to restrain the patient from resuming the treatment.)

The histological difference between the two specimens was striking, the second specimen showing a considerably larger quantity of metachromatic intercellular substance (more intense staining) than the first one. In both, the metachromasia was most marked in the subepithelial tissue. In the second specimen the mast cells were more numerous, containing only a few granules.

With polysaccharide staining, the second specimen stained more intensely than the first one, this difference being most marked in method D.
**Case 2.** A 40-year-old female had undergone operation for Graves' disease (subtotal thyroidectomy) 10 years previously. In the course of the past two or three years there had been a recurrence of the hyperthyroidism, and during the same period a localized, circumscribed myxedema had appeared on both legs. The surface of these areas was tuberous, made up of arched, hemispherical, comparatively soft swellings on a firm bed, ranging in color from pink to slightly cyanosed. During the stay in hospital (in June 1949) the patient exhibited thyrotoxicosis, B.M.R. + 64 per cent.

**EXPERIMENTAL**

(1) Repeated injections of a solution of *testis-hyaluronidase* into various sites of the localized myxedema without removal of biopsies.

(2) Control injections of normal saline solution.

(3) Biopsy of untreated myxedematous skin.

(4) Biopsy of untreated normal skin (from right buttock).

(5) Intracutaneous injections of hyaluronidase, 5 turbidity-reducing units (12, 13) dissolved in 1 ml. of normal saline solution into various sites of the myxedematous tissue; punch biopsy followed upon two injections into the same site, received 4 and 2 days prior to the removal of the biopsy.

(6) Control injections of normal saline solution followed by removal of biopsies.

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1Kindly supplied by the Swedish medicinal factory LEO.
(7) Biopsy specimen from untreated myxedematous skin soaked in a solution of hyaluronidase.
(8) Control experiments in vitro with normal saline solution.

RESULTS

After a few days' treatment with hyaluronidase, the tuberous and arched surface of the myxedematous areas showed local flattening, most marked at the site injected with the largest dose (80 T.R.U.). At the outset, this flattening was slightly larger than a finger impression. Gradually, it grew flatter, and two months later it was barely visible. The injected tissue acquired a softer consistency and the color had changed from a reddish cyanosed to a more vivid red hue.

The control sites showed no changes.

Histological Examination

In addition to Heidenhain's iron hematoxylin staining, the following methods were used:

  a. Metachromatic Staining (A and B). Untreated myxedematous skin showed marked and extensive metachromasia of a homogeneous intercellular substance deep in the corium (whereas the papillary layer took a relatively fainter stain). Scattered in this substance, numerous large and highly granular mast cells were seen.

  On the other hand, "normal" skin from the thyrotoxic patient failed to show metachromatic intercellular substance. Comparatively few, small mast cells were seen.

  Myxedematous skin, treated in vivo with hyaluronidase, showed very faint metachromasia, only surrounding the numerous large, but sparsely granular mast cells. Infiltration with lymphocytes, a few plasma cells and polymorphonuclear leukocytes was found, mainly about the vessels. An increased number of histiocytes were scattered more diffusely.

  The histological appearance of myxedematous skin injected with saline solution was, in 48 hours, like that of untreated skin.

  A myxedematous specimen, submitted to the action of hyaluronidase in vitro, showed marked metachromasia on section. A narrow marginal zone, however, failed to take this staining. The control preparation, which had been soaked in normal saline solution, did not present this marginal zone.

  b. Polysaccharide Staining C. The intercellular substance (corresponding to the metachromatic areas), the epithelium of the hair follicles, as well as the cytoplasm of the mast cells, of the polymorphonuclear leukocytes, and of a few luminal cells in the small vessels stained in varying shades from pink to purplish red with fuchsin, indicating the presence of polysaccharides. In the specimens injected with hyaluronidase, the cytoplasm of the basal epidermic cells stained a granular red, whereas the basal membrane was indistinct or invisible. The action of hyaluronidase is, moreover, apparent from a perceptible, paler red staining of the intercellular substance.
c. **Polysaccharide Staining**

D. On the whole, areas corresponding to the metachromatic ones, stain blue, indicating the presence of acid polysaccharides. This blue stain, distinctly less extensive than the red stain in Method C, faded or disappeared entirely following the action of hyaluronidase.

**DISCUSSION**

In the course of 7 days, suspension of thyroid hormone therapy in the case suffering from hypothyroidism caused marked increase in weight, accompanied by an accumulation of metachromatic intercellular substance, an acid polysaccharide in the connective tissue of the skin. If this substance is hyaluronic acid, the experiment confirms the presumption that thyroid hormone stimulates the production or liberation of hyaluronidase in the connective tissue.

Further confirmation is afforded by the absence of metachromatic substance (present in moderate quantities in the skin of normal human subjects) in “normal” skin from the thyrotoxic patient, Case 2. Similar changes have moreover been observed in other cases of Graves’ disease.

Following the action of hyaluronidase, myxedematous connective tissue from Case 2 showed a decrease in the thickness of the integument; at the same time, the marked and extensive metachromasia faded to a great extent, and the character of this substance of an acid polysaccharide became perceptibly less apparent. The comparatively slight alteration in the staining intensity in Hotchkiss’ method is presumed to be due to the fact that glucuronic acid and glucosamine, the hydrolytic products of hyaluronic acid, also take some stain.

Owing to the specificity of the enzyme, the author is now in a position to confirm the results of the chemical investigations mentioned above, viz, that the highly increased intercellular substance of the connective tissue in myxedema is composed predominantly of hyaluronic acid.

Lison (14) as well as Holmgreen (7) insist that the metachromatic staining reaction is specific for sulfuric acid esters of high molecular weight. Wislocki, Bunting and Dempsey (28) are of a different opinion, finding—like the present author—metachromatic staining of the sulfur-free hyaluronic acid.

As stated above, the origin of hyaluronic acid is unknown. It has been suggested that it is produced by fibroblasts, but the theory cannot be confirmed, since the number of these cells is, in the author’s opinion, unrelated to the amount of hyaluronic acid. There is, however, a relationship between hyaluronic acid and Ehrlich’s mast cells, the granules of which are made up of a substance which is closely related to hyaluronic acid, but which contains sulfuric acid radicals and is, perhaps, identical with heparin. In the present paper the author does not propose to contradict the theory that mast cells should contain and perhaps secrete heparin (8, 11, 24) but supported by the following observations from experiments (including more than 200 biopsies) on hospitalized patients (published elsewhere) and considering what has been stated above, he thinks it likely that hyaluronic acid is produced by the mast cells, directly or by way of a preliminary stage (sulfuric mucopolysaccharide? heparin?).

(1) Myxedematous tissue, containing large amounts of hyaluronic acid, ex-
hibited numerous large mast cells with varying degrees of granulation. Only a very small number of fibroblasts were encountered.

(2) On the whole, the number of mast cells was related to the amount of metachromatic intercellular substance in the cutaneous connective tissue, and, under all circumstances, the granules of the mast cells stained like the latter.

(3) Following experimental injection of hyaluronidase into normal corium, histological examination showed, in addition to a fainter or abolished specific staining of the intercellular substance and infiltration with histiocytes and round cells, an accumulation of mast cells which, at the outset, showed merely faint, but gradually increasing granulation. This phenomenon is interpreted as a compensatory measure on the part of the body: The accumulated mast cells empty out their secretion, at first rapidly, later more slowly.

(4) During the experiments in which biopsies were obtained from two symmetrical sites in each subject, one site being injected with hyaluronidase and one with saline solution, it was observed accidentally that the one injected with hyaluronidase had a shorter healing time.

Granulation tissue contains ample metachromatic intercellular substance (first pointed out by Sylvén (24)). Enzymatic hydrolysis of the intercellular substance may be imagined to elicit over-compensatory new-production of that substance on the part of the mast cells, a process which may further the healing.

(5) Examinations of the jelly of Wharton, which consists mostly of hyaluronic acid, have revealed numerous, large mesenchymal cells with a metachromatic cytoplasm containing acid polysaccharide. As early as the fourth month of fetal life, metachromatic granules may be discerned in subamnionic cells.

(6) The subepithelial layer of the synovial membrane contains 5–10 times the number of mast cells present in the cutaneous connective tissue. The mast cells in the lumen are poor in granules and stain faintly, whereas those of a deeper situation are highly granular.

The theory that the metachromatic substance of connective tissue might consist of heparin (24) does not accord with these experimental results, especially as it is known that heparin is not affected by hyaluronidase (10) and that it has an inhibitory effect on the action of this enzyme (17). Moreover, Fischer's demonstration of an inhibitory effect of heparin on the growth of fibroblasts in tissue cultures (4) does not agree with the definite observation that growing tissue has a constant content of ample free metachromatic substance. Finally, it is worth mentioning that neither biopsies of myxedematous tissue nor mast-cell tumors in man (the skin disease known as urticaria pigmentosa) exhibit hemorrhage like that from skin infiltrated with heparin; on the contrary, such specimens show little hemorrhage and rapid coagulation.

CONCLUSIONS

The highly increased intercellular substance of connective tissue in myxedema consists predominantly of hyaluronic acid.

The amount of such hyaluronic acid is governed by thyroid function.

Hyaluronic acid is probably produced by Ehrlich's mast cells, either directly or by way of a (sulfuric?) preliminary stage.
CONNECTIVE TISSUE IN MYXEDEMA

SUMMARY

The amount of intercellular and interfibrillar substance in the cutaneous connective tissue is governed by the function of the thyroid body, perhaps by way of variations in the concentration of hyaluronidase in the tissues.

In myxedema, thyroid hormone therapy is able to detain the otherwise ample metachromatic intercellular substance of the nature of an acid polysaccharide (hyaluronic acid) at a comparatively low level. "Normal" skin from a thyrotoxic patient shows no metachromasia.

The action of hyaluronidase on atypical localized myxedema in a patient with recurrent hyperthyroidism proved capable of reducing the marked and extensive metachromatic intercellular substance. At the same time, its histochemically demonstrable character of acid polysaccharide diminished or subsided.

The author concludes that the substance consists predominantly of hyaluronic acid.

Numerous, large mast cells with varying degrees of granulation are encountered all over the myxedematous tissue.

The histological results from experiments with more than 200 specimens of skin are submitted in support of the theory that hyaluronic acid is secreted by mast cells.

REFERENCES


