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THE VARIABILITY IN THE HYALURONIC ACID CONTENT OF THE DERMAL CONNECTIVE TISSUE UNDER THE INFLUENCE OF THYROID HORMONE

Mast Cells — the Peripheral Transmitters of Hormonal Action[1]

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The intercellular and interfibrillar ground substance of the skin is made up of a viscous mass, consisting, in human skin, mainly of hyaluronic acid, but also of chondroitin sulphuric acid, both of which are mucopolysaccharides containing uronic acid. It is particularly hyaluronic acid which lends the ground substance its slimy, viscous character.

Hyaluronic acid is a polymeric compound which is broken down into glucuronic acid and N-acetyl glucosamine by the enzyme hyaluronidase — derived *inter alia* from the testes, certain bacteria, animal toxins, and malignant tumours.

The degree of this enzymatic process is expressed in and measurable by a "spreading effect", *i. e.* by the altered permeability of the connective tissue, and in this way the occurrence and amount of hyaluronic acid in connective tissue on the whole may be ascertained.

In certain pathological conditions, sometimes accompanied by cutaneous manifestations, there is an accumulation of hyaluronic acid in the corium. Among such conditions is myxoedema which is rarely met with in its fully developed form — *i. e.* with visible cutaneous swelling resembling oedema. Accumulation of hyaluronic acid may, however, also be demonstrated by various methods in hypothyroidism unaccompanied by visible cutaneous manifestations.

In 1860 Rollett demonstrated "mucin" in connective tissue by precipitating a limewater extract of this tissue with acetic acid. In 1909 van Lier succeeded in isolating a viscous substance from the skin of various animals. In addition, he demonstrated that mucin contained partly reducing hexoses, partly uronic acid. In the course of histological studies, Bensley (1934) found mucinous substances in the skin. By chemical methods Chain & Duthie, Claude, and Meyer & Chaffee identified the "mucin" in the skin of various animals as hyaluronic

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acid, a finding which has been confirmed by Watson & Pearce in the case of human skin.

— Accumulation of "mucin" in *myxoedematous skin* was mentioned as early as 1885 by Horsley and confirmed in 1893 by Halliburton. — Reuter demonstrated mucin by means of mucicarmine staining of tissue sections. In a case of atypical, localized myxoedema associated with thyrotoxicosis, Carol found "mucin" in the skin by chemical methods; after hydrolysis, it reduced Fehling's solution. Accumulation of hyaluronic acid in the skin has been found by Watson in two cases of pretibial thyrotoxic myxoedema. He has, moreover, found an increase in the amount of chondroitin sulphuric acid.

Bloom et alii, Grais, and Asboe-Hansen have observed flattening of localized, myxoedematous cutaneous affections during treatment with hyaluronidase.

Trotter & Eden have suggested the possibility of a reduced activity of hyaluronidase in the connective tissue in cases of myxoedema, but this hypothesis has not been definitely confirmed.

The source of hyaluronic acid is unknown. The presumption that the hyaluronic acid of the connective tissue is formed by young fibroblasts, the humours of the eye by the ciliary epithelium, and the synovial mucin by the synovial membrane is based only on probability.

Writer's Investigations.

The following experiments and investigations were designed to study

(1) histochemically the variations in the amount of the mesenchymal mucopolysaccharide, hyaluronic acid, in patients with hypo- and hyperthyroidism,
(2) the relation between the histological appearances of the dermal connective tissue in hypothyroidism and in thyrotoxic subjects with localized myxoedema,
(3) the origin of hyaluronic acid.

The first step was to demonstrate hyaluronic acid histochemically in a way as elective as possible and standing out in colour contrast as distinctly as possible.

Polysaccharides containing uronic acid are precipitated with basic lead acetate, and the writer used a 4 per cent. solution of basic lead acetate as advocated by Holmgren (1938). Some specimens were fixed in absolute alcohol and Carnoy's solution. Since hyaluronic acid is soluble in water, aqueous fixatives, such as formalin, are inapplicable.

Hyaluronic acid, which is a polysaccharide of high molecular weight, stains red with Schiff's reagent by the method of McManus or Hotchkiss after having been oxidized to polyaldehydes by means of periodic acid.

In addition, hyaluronic acid stains as an acid polysaccharide by the following method as used by Hale.

The tissue section is submitted to the action of a solution of ferric hydroxide the iron of which is bound to the acid polysaccharides and not to the neutral ones or to proteins. When potassium ferrocyanide in hydrochloric acid is added, the bound iron is demonstrated as Prussian blue.

Hyaluronic acid also stains metachromatically by aniline dyes, such as

toluidine blue. True, Lison has described this staining reaction as specific for sulphuric acid esters of high molecular weight (e. g. chondroitin sulphuric acid, mucoitin sulphuric acid, and heparin), but recent investigations (Altschuler & Angevine, Asboe-Hansen, Meyer, Wislocki, Bunting & Dempsey) have shown that it includes also the non-sulphuric, but otherwise kindred hyaluronic acid (and a few non-sulphuric substances of minor interest, *i. e.* desoxyribonucleic acid and hexametaphosphate (Wiame)).

In cutaneous specimens, metachromatic staining with toluidine blue indicates the presence of the mucopolysaccharides hyaluronic acid and/or chondroitin sulphuric acid, whereas this staining of e. g. the humours of the eye and synovia indicates hyaluronic acid which constitutes the mucinous substance in these fluids.

In order to approach hyaluronic acid even further, the writer used the specific enzyme hyaluronidase which does not break down chondroitin sulphuric acid in the dermal connective tissue (Meyer). (A sterile preparation of hyaluronidase derived from bovine testes was kindly supplied by the Swedish factory LEO.)

Metachromatic staining with toluidine blue disappearing after the action of hyaluronidase may be regarded as unquestionable evidence of hyaluronic acid in the connective tissue.

The material (consisting of patients suffering from hypo- and hyperthyroidism) is derived from four medical and two dermatological departments in Copenhagen.[2]

Since patients with myxoedema are rare, the writer suspended thyroidin medication in certain cases of compensated myxoedema, *experimentatis causa*, in order to procure experimental subjects.

A total of 48 specimens from 26 patients with hypothyroidism and 37 specimens from 37 patients with thyrotoxicosis were studied. Two cases of thyrotoxic, circumscribed myxoedema were included.

In 24 of the 26 patients with hypothyroidism, accumulation of metachromatic ground substance was seen in the corium, particularly in the papillary layer, where the substance is usually diffuse, whereas somewhat deeper it is disposed in irregular strands, often of a very intense purplish red colour. Constant, diffuse metachromasia was observed around the vessels, the lumina of which were more or less open. In and around the vessels — be they capillaries, arterioles, or venules — and free in the connective tissue, wherever there is metachromatic ground substance, and in particularly large numbers where the metachromasia is most intense, there is a very striking type of cells, *Ehrlich's mast cells*. Their size is extremely varied, but in myxoedematous connective tissue they are usually large, with ample cytoplasm, round, oval or polyhedric, and, when in a perivascular situation, usually spindle-shaped all more or less saturated with *granules taking an intense metachromatic stain with toluidine blue.*

In the deeper layers of the corium, fibres showing an intense blue stain are as if split apart in a very irregular and loose arrangement. Compared with normal dermal connective tissue stained in the same way, in which the fibrillar structure

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is very indistinct, the fibres taking only a faint stain, the phenomenon is striking and pathognomonic of myxoedema.

In the two cases of circumscribed myxoedema associated with thyrotoxicosis, a 40-year-old female and a 48-year-old male with a B. M. R. of + 64 and + 26 respectively and pronounced exophthalmos as well as localized, pretibial myxoedema, microscopical examination of biopsy specimens from the skin showed myxoedematous changes of exactly the same nature as described above, only far more marked. Metachromasia predominates, most marked deep in the corium, but slight in the papillary layer. The "splitting of the fibres" was also pronounced and the accumulation of mast cells in the tissue striking (in the specimen from Case I about 30 times that of normal corium).

The "normal" skin (right buttock) from Case I failed to show any traces of metachromasia in the corium, and there were only a few mast cells scattered along the vessels.

In addition, a rather marked *atrophy of fatty tissue* is noted in myxoedematous subjects.

The skin from 37 patients with thyrotoxicosis did not in any case exhibit accumulation of metachromatic ground substance. Mast cells, often in quite large numbers, are seen around the blood vessels, but they are small, spindle-shaped, and hold few granules. Outside the vessels there were only occasional mast cells. In eight cases there was some metachromatic substance — without there being a question of accumulation — and free mast cells. All eight patients were affected with pronounced exophthalmos.

Normal skin shows only very faint metachromasia, as a rule only in the papillary layer, in the basal membrane, in the vessel walls, around hair follicles and around sebaceous glands.

If a myxoedematous subject is treated with thyroid hormone, both metachromasia and the phenomenon of split fibres subside rapidly.

Experiments with Hyaluronidase.

Intracutaneous injection of e. g. 0.2 ml. of physiological saline solution into a patient with myxoedema induces a papule which stands out sharply and persists for a long time. If, however, hyaluronidase is added to the saline solution, the fluid will quickly spread and not become demarcated. — In thyrotoxic subjects, on the other hand, the difference in spread is slight, even slighter than in normal skin.

Injection of hyaluronidase into myxoedematous skin produces a perceptible flattening.

A biopsy specimen removed a few hours after the intracutaneous injection of hyaluronidase shows practically no metachromatic ground substance; a small amount, however, persists in the vessel walls and around the hair follicles and sebaceous glands (the chrondroitin sulphuric acid?).

Twenty-four hours after the injection, the metachromasia is still very slight, and the predominant feature is now the presence of perivascular infiltrates of round cells, some of which can be identified as small, faintly granular mast cells. Free in the tissue, the specimen shows only an occasional, faintly granular mast cell. In a few cases there are faint traces of free metachromatic ground substance at this juncture.

Perivascular round-cell infiltrates may still be seen 48—96 hours after the injection, but a large proportion of the cells now exhibit metachromatic granulated cytoplasm, and the cells are on the whole larger, a few even 15—20 μ, but all stages of size and granulation are met with. At this time, mast cells may also be observed outside the perivascular areas, but they are not all equally well-developed and deeply stained. Around the perivascular infiltrates and around the single free mast cells, a free metachromatic substance may be detected; at times it appears to be granular, but usually homogeneous.

If biopsy specimens are removed later, the process of restitution will be seen to have advanced further.

In the present experiments, the writer used from 5 to 100 T. R. U.[3] of hyaluronidase per ml. of saline solution. The most concentrated preparations, which at the same time were the purest, gave the best results.

The same results, only less marked and at a slower rate, are obtainable in experiments on normal skin.

Since mast cells form a striking cellular element in the areas of connective tissue containing a metachromatic ground substance which has been identified histochemically with almost complete accuracy as hyaluronic acid and since hyaluronic acid appears to be formed by the mast cells in the above-mentioned processes of restitution, the writer has sought confirmation of this finding in preparations from eyes the humours of which contain hyaluronic acid without admixture of other mucopolysaccharides and from the synovial membrane, since the mucinous substance of the synovia also consists of hyaluronic acid. (Meyer et al.)

In 39 specimens from *eyes* (enucleated, diseased eyeballs),[4] the writer found, on the whole, an ample content of mast cells in the membranes. In the ciliary body, the iris, and the iritic angle there are always, except in certain pathological conditions, rather numerous large, granular mast cells. In the retina, the choriocapillaris, the sclera, and the subconjunctival tissue, mast cells are found in relation to the vessels, and in the subconjunctival tissue also free. Six preparations confirmed Holmgren & Steenbeck's finding that mast cells are increased in number in glaucoma.

Since the preparations at the writer's disposal were fixed in an aqueous solution of formalin, the staining of the free metachromatic ground substance was faint and unreliable and the size and granulation of the mast cells were affected too.

The synovial membrane (46 preparations from cadavers and from amputated extremities)[5] showed in all cases large numbers of mast cells, most numerous in young individuals, but even in the old 5 to 10 times the number found in normal corium. Moreover, the cells are larger, and more granular. The mast cells are grouped along the surface, facing the joint cavity, apparently independent of the blood vessels. Deeper down, they are localized chiefly around the blood vessels as ordinarily in connective tissue. The granulation of the mast cells

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[3] Turbidity reducing units (Kass & Seastone).

[4] From the Eye Department of the University Hospital (Chief: H. Ehlers, M. D.).

[5] From the Pathological Institutes at the Bispebjerg Hospital (Chief: B. Vimtrup, M. D.), at the Copenhagen City Hospital (Chief: Sv. Petri, M. D.), from the University Institute of Pathological Anatomy (Chief: Professor J. Engelbreth-Holm, M. D.), and from the Copenhagen County Hospital, Surg. Dept. (Chief: O. Kapel, M. D.).

decreases the closer they are to the joint cavity, and in the cells lying among the lining cells or in those free in the intervillous spaces (which cells were not lost in the process of preparation), there is frequently no granulation, but only a very faint metachromasia of the cytoplasm. (They can, therefore, be identified only when the technique of preparation and staining is ideal).

Whereas the granulation of the mast cells decreases towards the joint cavity, the amount of free metachromatic substance increases, and in the superficial area it stands out bright red. The synovial fluid proper shows metachromatic staining like this substance and the granules of the mast cells.

Discussion

The striking number of mast cells wherever hyaluronic acid occurs in the connective tissue (identified histochemically, chemically, and by means of the specific enzyme),

the extremely pronounced accumulation in tissues which form hyaluronic acid,

the conformity between the staining quality of the mast-cell granules and hyaluronic acid,

the evident concourse of mast cells from the vessels and their emission of granules, wherever restitution is taking place following enzymatic break-down of the surrounding intercellular ground substance,

the gradual new-formation of the metachromatic ground substance from areas rich in mast cells,

and the occurrence of various "secretory phases" go to show that an interrelationship exists between hyaluronic acid and mast cells; they belong to the same histological system.

So far, the function of the mast cells in the connective tissue has been unknown. They have been attributed with a multitude of tasks, but no proof has been advanced of any of them.

Sylvén has mentioned the possibility that mast cells might form the "chromotropic substance" in granulation tissue during epithelial regeneration.

It is evident from the studies of Holmgren & Wilander that the mast cells contain an anticoagulant, an ester of sulphuric acid of high molecular weight, which is presumably heparin. — It must be contemplated now, whether the metachromatic substance in the connective tissue around the mast cells could be heparin. Heparin, however, is not affected by hyaluronidase (Humphrey), and tissue containing ample amounts of this substance has no anticoagulative properties.

Nevertheless, heparin might be imagined to be a precursor of hyaluronic acid. Chemically, these two mucopolysaccharides are closely related, both containing glucuronic acid and glucosamine, but, unlike hyaluronic acid, heparin contains a few radicals of sulphuric acid. The conversion of heparin into hyaluronic acid must be presumed to be effected by tissue sulphatases. In this connection it should be mentioned that recent Swedish experiments on sulphonation of hyaluronic acid have resulted in a strong anticoagulant (Högberg).

The increased content of water in connective tissue in cases of myxoedema is presumably a result of the strong osmotic action of hyaluronic acid (Bauer et alii).

It appears to be beyond doubt that *thyroid hormone* exercises a regulating influence on the hyaluronic-acid content of dermal connective tissue. It is not out of place to mention here that Ropes et alii found increased amounts of hyaluronic acid in the joint fluid in myxoedema.

Circumscribed, thyrotoxic myxoedema, which is always accompanied by exophthalmos, has been ascribed to increased production of the *thyrotropic hormone of the pituitary* and the direct action of the latter on the connective tissue, not by way of the thyroid gland (Trotter and Eden, Vilanova & Canadell and others).

The accumulation of hyaluronic acid in hypothyroidism is perhaps due to increased activity of thyrotropin, which is presumed to result from decreased inhibition on the part of the thyroid gland. This hypothesis is supported by increased excretion of thyrotropin in the urine in myxoedema, whereas this pituitary hormone is reduced or inactivated in thyrotoxicosis (van Caulert et alii, Hertz & Oestler, Rawson et alii).

According to these considerations, the rôle played by thyroid hormone is confined to its inhibitory effect on the hypophysis or its ability to inactivate the thyrotropic hormone.

Perhaps, *oestrogenic hormones* also influence the variations in the hyaluronic acid content of the connective tissue (Bachmann et alii, Ogston et alii).

The *adrenocorticotropic hormone* of the pituitary (ACTH) and *cortisone* also act upon the hyaluronic acid of the mesenchymal tissues. In biopsies from the skin of patients suffering from rheumatic fever, the writer observed a subsidence of the metachromatic ground substance in the connective tissue of the corium and a decrease in the number of mast cells in the course of treatment with ACTH and cortisone. At the same time it has been noted that the ability of the organism to form granulation tissue is reduced and the power of wound healing is inhibited or lost. Schmidt & Faber have reported a decreased hyaluronidase inhibition of blood serum during ACTH treatment of rheumatoid arthritis.

It is reasonable to assume that the mast cells play a rôle as the peripheral transmitters of hormonal action, and their localization in the vascular walls may support this interpretation.

Conclusion

The thyroid hormone exercises a regulating influence on the amount of hyaluronic acid in the dermal connective tissue (possibly by inhibiting the actual regulator, the thyrotropic hormone of the pituitary).

Hypofunction of the thyroid gland is accompanied by an accumulation of hyaluronic acid in the dermal connective tissue, and in rare cases, there is localized accumulation of hyaluronic acid in hyperthyroidism too. The histopathological appearance of both conditions is identical.

Influenced by hormones, the mast cells secrete the mesenchymal mucopolysaccharide, hyaluronic acid, perhaps by way of a precursor resembling heparin.

The last-mentioned *hypothesis* unites and elucidates two hitherto unsolved problems:

(1) The origin of hyaluronic acid and
(2) the function of the mast cells in the connective tissue.

SUMMARY

Hyaluronic acid is demonstrable in the connective tissue histochemically and by means of the specific enzyme hyaluronidase.

In myxoedema and in the rare lesion called circumscribed, thyrotoxic myxoedema there is an accumulation of hyaluronic acid and large mast cells. The fibres appear to be split apart and the fatty tissue is atrophied.

In thyrotoxicosis there is usually no demonstrable hyaluronic acid in the dermal connective tissue and only few and small mast cells.

In the course of new-formation of hyaluronic acid following enzymatic breakdown, there will be perivascular accumulation of mast cells of a widely different degree of granulation.

Numerous large mast cells have been found in eyes and in synovial membranes, the fluids of which contain hyaluronic acid. The variations in the cytoplasmatic load of granules are interpreted as a sign of various functional phases.

In addition to thyroid hormone, a number of other hormones influence the variations in the hyaluronic acid content of the connective tissue.

The writer advances the hypothesis that *mast cells, under hormonal influence, secrete the mesenchymal mucopolysaccharide hyaluronic acid, perhaps by way of a precursor resembling heparin.*

RÉSUMÉ

L'acide hyaluronique peut se déceler dans les tissus conjonctifs par la méthode histo-chimique et au moyen de l'enzyme hyaluronidase spécifique.

Dans le myxœdème et dans la rare lésion appelée myxœdème thyrotoxique circonscrit, il y a accumulation d'acide hyaluronique et de grandes mastocytes. Les fibres apparaissent comme fractionnées et le tissu adipeux est atrophié.

Dans la thyrotoxicose on ne peut en général pas déceler d'acide hyaluronique dans le tissu conjonctif du derme et les cellules sont seulement en petit nombre et petites.

Au cours de la formation nouvelle d'acide hyaluronique consécutive à une perturbation enzymatique, il se produit une accumulation périvasculaire de mastocytes dont le degré de granulation est fortement différent.

De nombreuses grandes mastocytes ont été trouvées dans les yeux et dans les membranes synoviales, dont les humeurs contiennent de l'acide hyaluronique. Les variations de la charge cytoplasmatique des granulations sont interprêtées comme un signe de phases fonctionnelles variées.

Outre l'hormone thyroidienne, un certain nombre d'autres hormones influent sur les variations de teneur en acide hyaluronique des tissus conjonctifs.

L'auteur émet l'hypothèse que, *sous l'influence des hormones, les mastocytes secrètent l'acide hyaluronique mucopolysaccharide mesenchymal, peut-être au moyen d'un précurseur semblable à l'héparine.*

ZUSAMMENFASSUNG

Hyaluronsäure lässt sich im Bindegewebe histochemisch sowie mittels des spezifischen Enzyms Hyaluronidase nachweisen.

Bei Myxödem und der seltenen Affektion, welche als umschriebenes thyreotoxisches Myxödem bezeichnet wird, besteht eine Anhäufung von Hyaluronsäure und grossen Mastzellen. Die Bindegewebsfasern erscheinen auseinandergedrängt, und das Fettgewebe ist atrophisch.

Bei Thyreotoxikose findet sich im allgemeinen keine nachweisbare Hyaluronsäure im Bindegewebe der Haut, und man sieht nur wenige sowie kleine Mastzellen.

Die durch die Enzymwirkung abgebaute Hyaluronsäure wird durch Neubildung ersetzt, und dieser Prozess wird von einer Anhäufung von Mastzellen rings um die Gefässe begleitet; der Granulagehalt dieser Mastzellen schwankt in weitesten Grenzen.

In Augen und Synovialhäuten, wo Hyaluronsäure vorhanden ist, wurden zahlreiche grosse Mastzellen gefunden. Die Schwankungen des Granulagehalts werden als durch verschiedene Funktionsstadien bedingt gedeutet.

Noch mehrere andere Hormone als das der Schilddrüse beeinflussen den Hyaluronsäuregehalt des Bindegewebes.

Verf. stellt die Hypothese auf, dass *Mastzellen unter hormonalem Einfluss das mesenchymale Mukopolysaccharid Hyaluronsäure absondern, welches vielleicht als ein Vorläufer dem Heparin entspricht.*

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