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Differential Regional Distribution of Mucopolysaccharides in the Human Epiphyseal Cartilage Matrix in Normal and Pathologic Conditions

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Über die unterschiedliche Verteilung von Mucopolysacchariden in der Grundsubstanz des Epiphysenknorpels unter normalen und pathologischen Bedingungen

Zusammenfassung. Im Wachstumsknorpel von menschlichen Tibien wurden histochemische Untersuchungen vorgenommen, und zwar unter normalen wie unter krankhaften Verhältnissen (Hurler und Turner-Syndrom, hypophysärer Zwergwuchs, Fairbank's Krankheit und Morquio's Krankheit, Achondroplasie, Cornelia de Lange — Zwergwuchs, Pseudohyperparathyreoidismus). Die Verteilung der Glucosid-Komponente erscheint bei diesen Krankheiten sehr verschieden von der des normalen Knorpels. Die Verteilung der sauren Mucopolysaccharide im normalen Knorpel und die Veränderungen derselben bei verschiedenen Wachstumsstörungen weisen auf die wichtige Rolle hin, welche die Mucopolysaccharid-Komponente der Knorpelgrundsubstanz bei Wachstum und Verkalkungsvorgängen spielt.

Summary. Histochemical investigations were performed on biopsies of human tibial growing cartilage, in normal and pathologic conditions; (Hurler and Turner's syndrome, pituitary dwarfism, Fairbank's disease and Morquio disease, achondroplasia, Cornelia de Lange dwarfism, pseudoparathyroidism). The distribution of the glucosidic component appears to be different as compared to the normal cartilage. The distribution of acid mucopolysaccharides in normal cartilage and changes occuring in various disorders of growth, indicate the important part that mucopolysaccharidic components of the matrix are playing in the process of growth and calcification.

Previous studies carried out on epiphyseal cartilage noticed significant changes in the composition and distribution of the matrix mucopolysaccharides in subjects displaying Hurler, Turner syndrome, achondroplasia, polyepiphyseal dysplasia, pituitary dwarfism (Bona et al., 1965b, 1966; Stănescu et al., 1965, 1966) as compared to normal subjects (Bona et al., 1965a).

Some authors have already stated that mucopolysaccharides are not equally distributed among the various zones of the epiphyseal and hyaline cartilages. Our observations suggest that the mucopolysaccharide-protein components of the matrix have a certain distribution in relation to the histological zones of the epiphyseal cartilage (resting, proliferating and hypertrophic zones). This differential regional distribution is modified in certain syndromes.

In this study we present the comparative results of the histochemical tests performed in normal and pathologic epiphyseal cartilages.

Material and Methods

Histochemical studies were performed on 18 growing cartilage biopsies as follows:
2 patients with normal growing cartilage (10- and 12-year old);
1 patient with Morquio disease (10-year old);
3 patients with achondroplasia (10, 12 and 7-year old);
4 patients with polyepiphyseal dysplasia (Fairbank's disease) (7, 11.6; 12 and 15 year old);
5 patients with pituitary dwarfism (11.5; 12, 12, 12 and 14 year old);
1 patient with Hurler syndrome (7.9-year old);
1 patient with pseudohypoparathyroidism (9-year old);
1 patient with Cornelia de Lange syndrome (6.6-year old).

The biopsies were obtained from the proximal tibial epiphysis. The antero-medial aspect of the proximal end of the tibia was expressed and a small fragment was removed, centered on the cartilage line. Light general anesthesia was used; procain local anesthesia was used in a few of the older children; in the case of Morquio's disease the biopsy was obtained from the lower femural epiphysis during a surgical arthropaedic intervention.

The fixatives were: 0.5 per cent cetyltrimethylammonium bromide in ethanol-formalin 9:1 (24 hours); 2 per cent calcium acetate in 10 per cent formalin (2 hours) and Carnoy (4 hours). Decalcification was performed in 10 per cent ethylen-diamine-tetra-acetate disodium in buffer — phosphate pH 7, 0.2 M (BALOGH, 1962).

Carbohydrates were studied by means of:

1. PAS reaction (following prior extraction with pyridine, acetylation and saponification), controlled by α amylase digestion, and periodic acid diamine (PAD) reaction (SPICER et al., 1961).

2. Alcian blue (1 per cent) at pH 2.2; Toluidine blue (0.1 per cent) at pH 2.3 and pH 4; basic fuchsine (STEMPIEN, 1962) and barium-rhodizonate (STEMPIEN, 1963). Acriflavine, PAS-Hale and bi-col stainings (WOLMAN, 1961). Controls were performed with a) testicular hyaluronidase (NBC) (1 mg/ml in saline medium) extraction, 3 hours at 37°C and b) following prior methylation: c) trypsin (NBC) (1 mg/ml in phosphate buffer — saline — 0.1 M pH 8.3) over 30 minutes at 37°C; d) sialidase (Welcome) (1:4 v/v), solution in acetate buffer pH 5 0,25 M) over 24 hours, at 37°C.

Collagen component was investigated by the van Gieson method (controlled with collagenase /Mann Res. Lab./ extraction, 1 hour at 37°C) (GREEN, 1960).

Results

The Normal Cartilage. Chondrocytes of all zones contain a PAS reactive material extractible by α amylase digestion. Their cytoplasm was γ metachromatic and stained with Hale, Alcian and barium-rhodizonate. In all zones there are perichondroplastic rims (i.e. hyaluronidase-, sialidase-, and tripsin partially resistant, alcianophylic, γ metachromatic, Hale, and fuchsine positive material). The perichondroplastic rims showed also a moderate PAS reaction and stained brown by the PAD method. The remainder of the matrix in the resting zone faintly stained in the PAS, PAD and Hale reactions.

Within the *proliferating* zone, the remainder of the matrix was (hyaluronidase sensitive but sialidase- and trypsin resistant) γ metachromatic, alcianophylic, Hale and fuchsin positive; its PAS reactivity was very low.

In the upper part of the *hypertrophic* zone, the remainder of the matrix had similar features. In the lower part of this zone the matrix appeared non reactive when stained with toluidine blue at pH 2.1 and 4 and fuchsin, but alcianophylic positive (alcianophylia was hyaluronidase sensitive but sialidase and trypsin resistant). The PAS reaction was here striking positive, while the PAD staining showed weakly positive. Patches of bi-col red-coloured material are evident in this

zone, which could not be demonstrated on testicular hyluronidase extracted sections. A pale colour was obtained in this zone by the van Gieson staining.

Morquio Disease. The epiphyseal cartilage exhibited here important histopathologic changes: disappearance of characteristic regional distribution, presence of fibrous tissue, swelling and degeneration of chondrocytes. The cytoplasm of chondrocytes was not PAS reactive. In the matrix, irregularly disposed fibres-bundles were observed. They stained red in the van Gieson method.

The remainder of the matrix showed a moderate reactivity to the acid mucopolysaccharides detecting tests. In the proximity of the bundles, a bi-col reactive material, extractible with hyaluronidase was observed. The PAS reaction produced a moderate staining in the matrix.

Achondroplasia. The cartilage showed a similar fibrous tissue, but bundles were thiner and ran parallel with the axis of the cartilage. Cells were rare in the hypertrophic zone and directory lines were never found. In this disease the regional distribution is preserved. Chondrocytes of both the proliferating and hypertrophic zones contain no PAS-positive, and very little metachromatic, Alcian- and Hale-reactive material. In the resting zone the matrix appeared Alcian- and fuchsin-reactive, staining as well in the van Gieson method.

In all zones the perichondroplastic rims were hyluronidase-sensitive, but sialidase- and trypsin-resistant. γ metachromatic (pH 2.3), fuchsine and Hale positive. Within the resting and proliferating zones, the rims revealed a PAS-positive, but PAD-negative staining. The remainder of the matrix in the resting zone was also hyaluronidase sensitive but sialidase and trypsin resistant, γ metachromatic, fuchsinophylic, weakly alcianophylic and Hale negative.

In the proliferating and hypertrophic zones all abovementioned reactions were negative. Within the hypertrophic zone hyaluronidase sensitive, bi-col and metachromatic (at pH 4) areas could be observed.

Polyepiphyseal Dysplasia. Columns of the proliferating zone were short in this disease. Chondrocytes revealed little α amylase-sensitive PAS-reactive material. The cytoplasm of these cells, as well as the perichondroplastic rims were intensive γ metachromatic, Alcian and Hale positive. All these reactions were reversed by hyaluronidase-extraction. In the remainder of the proliferating and hypertrophic zones, the same methods failed to stain the tissue, except the Alcian blue and PAD techniques which gave a positive reaction in the hypertrophic zone. The PAS and PAD positive material appeared evenly distributed in all zones. The van Gieson technique failed to stain the matrix.

Turner Syndrome. The cells of the proliferating zone showed here an islet-like distribution. The hypertrophic zone take up about 2/5 of the thickness of the cartilage.

Chondrocytes of all zones were rich in α amylase-sensitive PAS-positive and hyaluronidase-sensitive material, Alcian-, Hale, fuchsine and γ metachromatic-positive.

The perichondroplastic rims showed the same picture as in normal subjects. The matrix of the resting zone appeared hyaluronidase sensitive, but sialidase resistant, γ metachromatic and unevenly Hale positive. The Alcian test was negative. The matrix of the proliferating and hypertrophic zones showed hyaluronidase-completely, and sialidase-partially sensitive, γ metachromatic, alcianophylic,

Table. *The table indicates only the abnormal results. The non-mentioned ones were similar both in normal and pathologic subjects*

Zone		Achondroplasia	Turner syndrome	Pituitary dwarfism	Fairbank's disease	Cornelia de Lange's disease	Hurler syndrome	Pseudohypoparathyroidism
Resting zone	Chondrocytes	±Tb, ±Alc, −αPAS		−αPAS			+Tb	
	Perichondroplastic rims		+TbH		−Alc, +PAS, −F		+TbH, +AlcH	
	Remainder of the matrix	−Ha, −PAS	−Al, −PAS	−Tb, −Alc, −PAD	−Tb	+Alc, +F	+TbH, +Alc, +F, +Tb	
Proliferating Zone	Chondrocytes	±Tb, ±Alc, −αPAS	−Ha	+Alc, +F			+TbH, −PAS	+F
	Perichondroplastic rims	−Tb, −Alc, −PAD, +PAS, +VG	+F, −PAS, −PAD	−Ha	−TbH, −F	−TbH	+TbH, +AlcH, +Bi	−PAS, −AlcH
	Remainder of the matrix	−Tb, −Ha, −PAD, +PAS, +VG	+F, −PAS	−PAS, −Ha, −F	−Alc, +PAS, −Ha, −PAD, −Tb, −F	+F, +Ha	+TbH, +AlcH, +F, +Bi	−TbS, +F
Hypertrophic zone	Chondrocytes	±Alc, −αPAS, ±Ha	−αPAS		−αPAS		+TbH, −PAS	
	Perichondroplastic rims	−PAD, −Ha, −PAS, +VG	+AlcH, +F, −TbH, −PAD	−Ha	+PAS, −TbH	−Alc, −TbH	+TbH, +AlcH, +Bi	−Ha, −AlcH, +F
	Remainder of the matrix	+VG, −Ha	+Tb, +TbS, +F, −PAD, −PAS, −Bi	+Tb, −PAS, −Bi, +Ha	+Alc, −Tb, −Ha	+Tb, +F, +Ha, −PAS, −TbS	+TbH, +AlcH, +F, +Bi, +Alc, +Ha	−TbS, +F
Directory lines		Absent	+TbS, +AlcH		−Tb, −Ha, −F	−PAS, +TbS	+TbH, +AlcH, +F, +Alc	

Tb = Toluidine blue
TbH = Toluidine blue after hyaluronidase extraction
TbS = Toluidine blue after sialidase digestion
Alc = Alcian blue
AlcH = Alcian blue after hyaluronidase digestion
F = Stempien fuchsin
Bi = Bi-col
Ha = Hale
αPAS = PAS material extractible by α amylase digestion
PAD = Diamine Spicer's method
VG = Van Gieson
\+ = increased intensity of stainings or appearance of a staining lacking in normal samples
− = diminution of intensity or disappearance of the staining

fuchsin positive, Hale positive and barium rhodizonate weakly positive. Within the hypertrophic zone, PAS and PAD stainings get positive.

Pituitary Dwarfism. The resting zone occupied here 4/5 of the epiphyseal cartilage thickness. The columns were short and the intercolumnar spaces of the proliferating zone were very broad. In the cytoplasm of chondrocytes an abundant, α amylase-sensitive, PAS reactive and hyaluronidase sensitive, γ metachromatic, Alcian- and Hale positive material was observed. The perichondroplastic rims were deeply intensive stained for mucopolysaccharides tests and moderately reactive to the PAS and PAD methods. The matrix of the resting zone failed to display γ metachromasia (at pH 2.3 and 4), fuchsinophilia and Hale positivity; while hyaluronidase-sensitive, but trypsin- and sialidase resistant alcianophilia was noticed. PAS reaction was also positive. The matrix of the proliferating and hypertrophic zones was highly metachromatic (at pH 2.3), alcianophilic, fuchsin and Hale positive. Metachromasia and alcianophilia were resistant to hyaluronidase (excepting the partial resistant hypertrophic layer) and resistant to sialidase. Trypsin reversed partially the metachromasia of the proliferating zone, but not its alcianophilia. The matrix of these zones were PAS and PAD positive mostly in the hypertrophic zone.

Hurler Syndrome. The cartilage is very narrow. Chondrocytes of the proliferating zone were enlarged and showed an islet-like distribution. The hypertrophic zone was limited to 3—4 ranges of cells. Chondrocytes in all zones contain α amylase-sensitive PAS-positive material and γ metachromatic, partially hyaluronidase-sensitive alcianophilic material. The perichondroplastic rims show metachromatic, hyaluronidase resistant alcianophilic, and Hale-, fuchsin-, PAS and PAD positive. The remainder of the matrix in all zones display a non-homogenous γ metachromasia-, alcianophilia, fuchsinophilia and Hale-positivity. Extraction with hyaluronidase obviously enhanced metachromasia and alcianophilia in both the proliferating and hypertrophic zones, showing besides a more homogenous Hale staining. The PAS and PAD reactions appear weakly positive. The matrix of both, the proliferating and hypertrophic zones reveal the presence of a hyaluronidase-sensitive bi-col positive material.

Cornelia de Lange Syndrome. The growing cartilage exhibits a very narrow hypertrophic layer and very short directory lines. Chondrocytes contain an α amilase sensitive, PAS-reactive material. In their cytoplasm an abundant hyaluronidase-sensitive, γ metachromatic, fuchsinophilic, alcianophilic, Hale- and acriflavine positive material was observed. The matrix of the perichondroplastic rims resembles that of normal cartilages. In the resting zone the matrix appears hyaluronidase-sensitive, γ metachromatic, alcianophilic- and fuchsin-positive; but trypsin- and sialidase resistant. The Hale and PAS reactions were also positive. In the matrix of the proliferating zone, there is a hyaluronidase-sensitive (and partially sialidase-sensitive), γ metachromatic, alcianophilic and fuchsinophilic material. No changes were noticed following trypsin digestion. The Hale- and acriflavine stainings are positive and the PAS reaction weakly positive. The matrix of the hypertrophic zone is similar to that described before, excepting the γ metachromasia which is no longer influenced by sialidase-treatment; and alcianophilia which was only partially hyaluronidase-sensitive. The PAS reaction was positive in this zone.

Pseudohypoparathyroidism. A short columns distribution of chondrocytes in the proliferating zone, and long directory lines were noticed in this disease. The chondrocytes exhibit the same histochemical picture as in normal subjects.

The perichondroplastic rims apperar γ metachromatic, alcianophile-, fuchsine-, acriflavine-, and Hale-positive, but weakly PAS-positive in the resting and proliferating zones; γ metachromasia is hyaluronidase-sensitive in these two zones.

The remainder of the matrix in the *resting zone* exhibits a hyaluronidase-sensitive (but sialidase-resistant) γ metachromatic, alcian-, and fuchsine positive material. This zone showed moderately reactive to the PAS-method. Within the *proliferating zone* γ metachromasia and alcianophilia are reversed by hyaluronidase and trypsin extractions. Intense Hale-positive, hyaluronidase-sensitive, fuchsin-positive and weakly PAS-positive material was noticed here. Within the hypertrophic layer, the matrix is PAS positive and orthochromatic, but there is an intensive alcianophilia. Alcianophilia is only partially sensitive to hyaluronidase treatment and resistant to sialidase extraction.

Discussions

Previous histochemical observations performed in rats growing cartilage (Sylven, 1947; Bona et al., 1964) suggest that the histological zones of the cartilage are in relation to a regional distribution of the glucidic component within the matrix. Similar relations were reported later in human and dog growing cartilages (Bona et al., 1965a; Hjertquist, 1964) and ear elastic cartilages (Conklin, 1963), as well as in cock combs (Szirmai, 1956). Our studies demonstrated evident modifications of the regional distribution of mucopolysaccharides in various diseases of the epiphyseal cartilage (Bona et al., 1965b, 1966; Stănescu et al., 1965, 1966). These modifications may be detected by careful examination of the reactivity of each zone to histochemical stainings.

Thus, in Morquio disease, significant histopathologic changes have been observed in both the chondrocytes and the matrix. The matrix is seen to contain mature collagen fibres. Between the fibres some areas of the matrix have tinctorial affinities characteristic for sulphated acid mucopolysaccharides, whereas others seem to be built up of hyaluronic acid. Glycogen was absent in chondrocytes.

Epiphyseal cartilage which is structurally different in achondroplasia as compared to the Morquio disease, displays however some histochemical similarities with it. Glycogen almost failed in chondrocytes; these cells exhibited a very scarce response to staining-methods for acid mucopolysaccharides. In the matrix these reactions are confined only to the perichondroplastic rims in the proliferating zone and appear almost negative in the hypertrophic zone. This suggests defficient acid mucopolysaccharidic synthesis. Bundles of collagen fibres are disposed in a parallel direction to the axis of growth. Diminution of acid mucopolysaccharides in the proliferating zone, occuring in association with accumulation of collagen, in addition to glycogen spoliation of chondrocytes, may suggest an "ageing" of the cartilage. The absence of the directory lines in the epiphyseal cartilage of the achondroplasic subjects, is largely explained by these modifications.

In both, Morquio disease and achondroplasia an accumulation of hyaluronidase-sensitive, bi-col — positive material (probably hyaluronic acid) is noted in the fibrous zone.

In polyepiphyseal dysplasia, no collagen was found in the matrix; the disappearance of the positive acid mucopolysaccharides reactions are striking in the proliferating zone, as well as glycogen paucity in cells of the hypertrophic zone; this may be indicative for a deficiency in acid mucopolysaccharides secretion. By contrast, the cytoplasm of chondrocytes presents intensive reactions for these components.

Comparison of the lower hypertrophic zone matrix in Turner's disease with normal subjects, demonstrated a decrease of the intensity of the PAS staining. All zones show a very high reactivity to acid mucopolysaccharides stains, indicating thus an accumulation of sulphated acid mucopolysaccharides. Unmasking of acid groups of these components, due to the low proteic content has also to be taken into account.

Somewhat similar patterns were observed in pituitary dwarfism, although in this instance changes were less manifest. The reactions for acid mucopolysaccharides were intensive. In the remainder of the proliferating zone matrix, the PAS reaction failed, whereas the PAD reaction showed intense positive both in the proliferating and hypertrophic zones.

In Hurler's disease the epiphyseal cartilage looked quite distinct from the picture in other troubles. In the matrix, hyaluronidase-resistant acid polysaccharidic material was observed. This observation is valuable in various organs i.e. the presence of B-chondroitin sulphate and heparitin sulphate (Maroteaux, 1962; Meyer, 1963). Results obtained by histochemical reactions, suggest also the existence of hyaluronic acid in high amounts.

In the Cornelia de Lange syndrome, sulphated mucopolysaccharides are obviously predominant, in both the proliferating and hypertrophic layers. Within the proliferating zone, histochemical tests — controlled by specific enzymatic extractions, — indicate the presence of sialic acid contained in glycoproteins, whereas within the hypertrophic zone they reveal the presence of keratosulphate (a PAS positive reaction, besides partially hyaluronidase-resistant metachromasia and alcian stains).

Biochemical data reveal the structural complexity of mucopolysaccharides and glycoproteins within the growing cartilage. These methods can only provide informations about the quantitative part of a chemical component within an organ. Biochemical micromethods investigating different zones of the growing cartilage are very scarce (Hjerquist, 1964). In our opinion histochemical methods, despite the pitfalls they provide, may give valuable informations about the regional distribution of mucopolysaccharides in the normal and abnormal cartilage with active sites. Examination of each zone of the pathologic cartilage as compared to normal ones, may provide informations upon the distribution of the free acid groups of the mucopolysaccharide molecule, and thus may indicate the occurence of chemical alterations which result in a functional disturbance.

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