ELECTRON-MICROSCOPIC INVESTIGATION OF ISOLATED PROTEOGLYCANS

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Chemically isolated individual preparations of the unaggregated fraction of protein-chondroitin-keratan sulfate (PCKS) from hyaline cartilage and of hyaluronic acid (HUA) from the vitreous body of the eye and umbilical cord were investigated electron-microscopically. PCKS and HUA in films without cytochrome c consisted of granules and differed in their structural organization. In films with cytochrome c, proteoglycans appeared as a network of thin fibrils and they differed more clearly in their macromolecular organization. Complexes formed in mixtures of the two proteoglycans as a result of noncovalent interaction. Uranyl acetate stains proteoglycans well, especially PCKS without cytochrome c.

KEY WORDS: proteoglycans; protein-chondroitin-keratan sulfate; hyaluronic acid.

The various proteoglycans which are components of the structural elements of animal cells and tissues form complexes, aggregates, and associates with each other and with proteins and lipids [5,10]. The discovery of free and structurally bound individual proteoglycans is an important but difficult task. Electron-histochemical methods reveal only aggregates consisting of sulfated proteoglycans, binding protein, and hyaluronic acid in individual tissues, such as hyaline cartilage [6, 7]. In most other tissues histochemical methods have shown the presence or absence of proteoglycans. Their identification with the aid of polysaccharidases cannot be regarded as sufficiently reliable because of the presence of proteases in preparations of these enzymes.

The object of the present investigation was an electron-microscopic study of the macromolecular organization of chemically individual preparations of the unaggregated fraction of protein-chondroitin-keratan sulfate (PCKS) and hyaluronic acid (HUA), which are most widespread in animal tissues, and their interaction products.

EXPERIMENTAL METHOD

PCKS was isolated from bovine tracheal cartilage [1]; HUA was obtained from human umbilical cords and from bovine vitreous bodies in the form of the calcium salts [2].

The electron-microscopic investigations were conducted by the method of Rosenberg et al. [9] with certain modifications. The proteoglycans were dissolved in phosphate buffer, pH 7.0, to give concentrations of PCKS and HUA of 3 and 2 mg/ml respectively. The solutions were mixed for 18 h at 4°C and their pH was adjusted to 5.0 with acetic acid. Mixtures of PCKS and HUA consisted of equal volumes of the final solutions of the proteoglycans. One drop of each test solution was applied to grids sprayed on both sides with gold and covered with formvar film, and dried in air without admission of dust at room temperature until a film had formed. In another series of experiments cytochrome c was added to the solutions of proteoglycans to a final concentration of 0.01%. Into a Teflon tray filled with 0.3 M potassium acetate 0.1 ml of the proteoglycan solution was added and the film which formed on the surface was transferred to a grid, stained for 30-60 sec with 10^{-4} M uranyl acetate solution in acetone, and examined in the IEM-100S electron microscope.

EXPERIMENTAL RESULTS

Solutions of PCKS and HUA without cytochrome c were able to form a strong electron-translucent film, nonuniform in thickness but sufficiently thin, in which structured proteoglycans could be detected.

The PCKS macromolecules were represented as electron-dense granules, round or oval in shape, with

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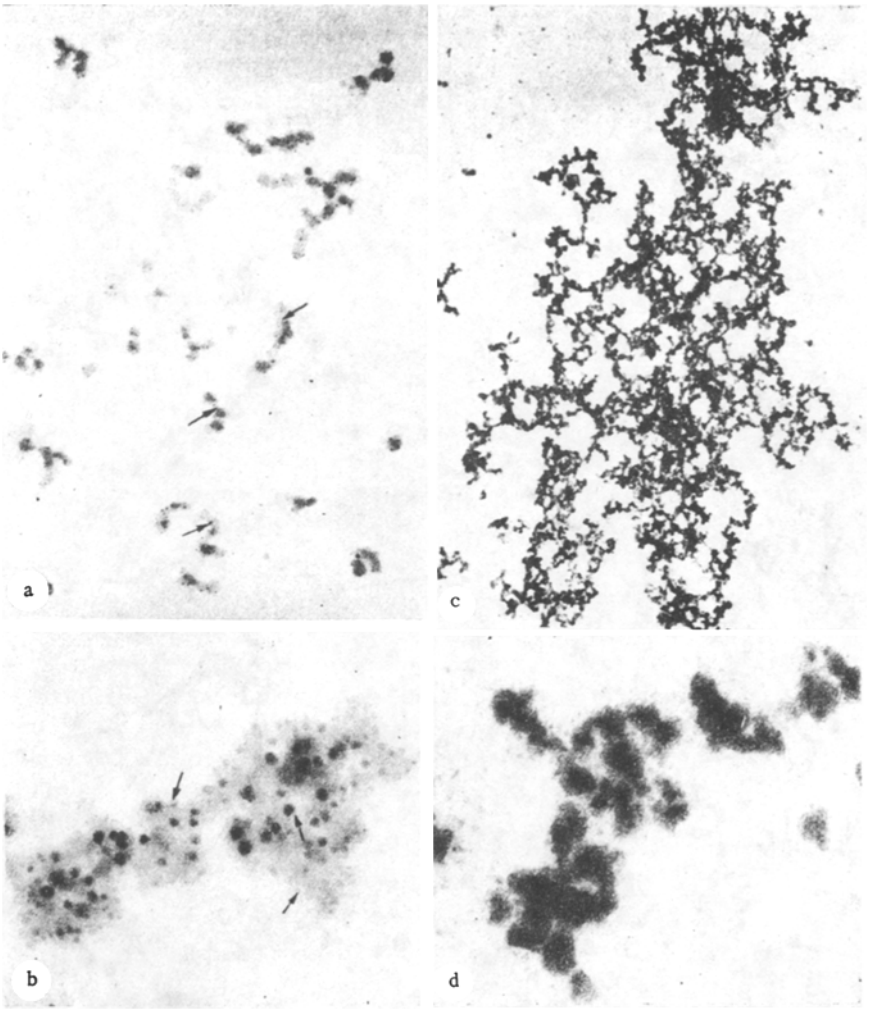

Fig. 1. Preparations of PCKS. Single granules, chains (a, b), and reticular formations of PCKS (c) with grains of uranyl acetate (arrows) in film preparations without cytochrome c. Cellular structure of PCKS in preparations with cytochrome c (d). a) 100,000 ×, b) 200,000 ×, c) 20,000 ×, d) 200,000 ×.

irregular outlines measuring 23-30 nm and with a homogeneous internal structure. They stained well with uranyl acetate which, besides a diffuse distribution, was visualized as relatively large granules. In thin parts of the film PCKS granules were distributed singly, or as chains, whorls, and rosettes. Zones of increased electron density were present in the center of these formations, probably as a result of the vertical orientation of the chain (Fig. 1a, b). In thicker parts of the film the number of long chains, running in different directions and interweaving with each other to form reticular aggregations was considerably increased (Fig. 1c).

In preparations with cytochrome c, granules of PCKS measuring 30-40 nm could be either round or polygonal in shape, finely granular in structure, and they contained no grains of uranyl acetate (Fig. 1d). They were joined into chains of different shapes and 100-200 nm long. A structureless halo of lower electron density was often observed around individual clusters of granules.

Macromolecules of umbilical HUA consisted of circular granules measuring 8-10 nm. Unlike the PCKS, they had more clearly defined outlines and a finely granular structure, they stained less intensely with uranyl acetate, and they were distributed in thin parts of the film separately or as in the form of chains consisting of 5-12 granules 40-90 nm long or of small clusters. In chains formed by 2 or 3 granules they were joined together by a homogeneous electron-dense strip (Fig. 2a). HUA isolated from the vitreous body of the eye had a similar ultrastructural organization (Fig. 2b).

In preparations with cytochrome c, a thin fibrillary, honeycombed structure of HUA was visible. No granular elements were observed (Fig. 2c, d).

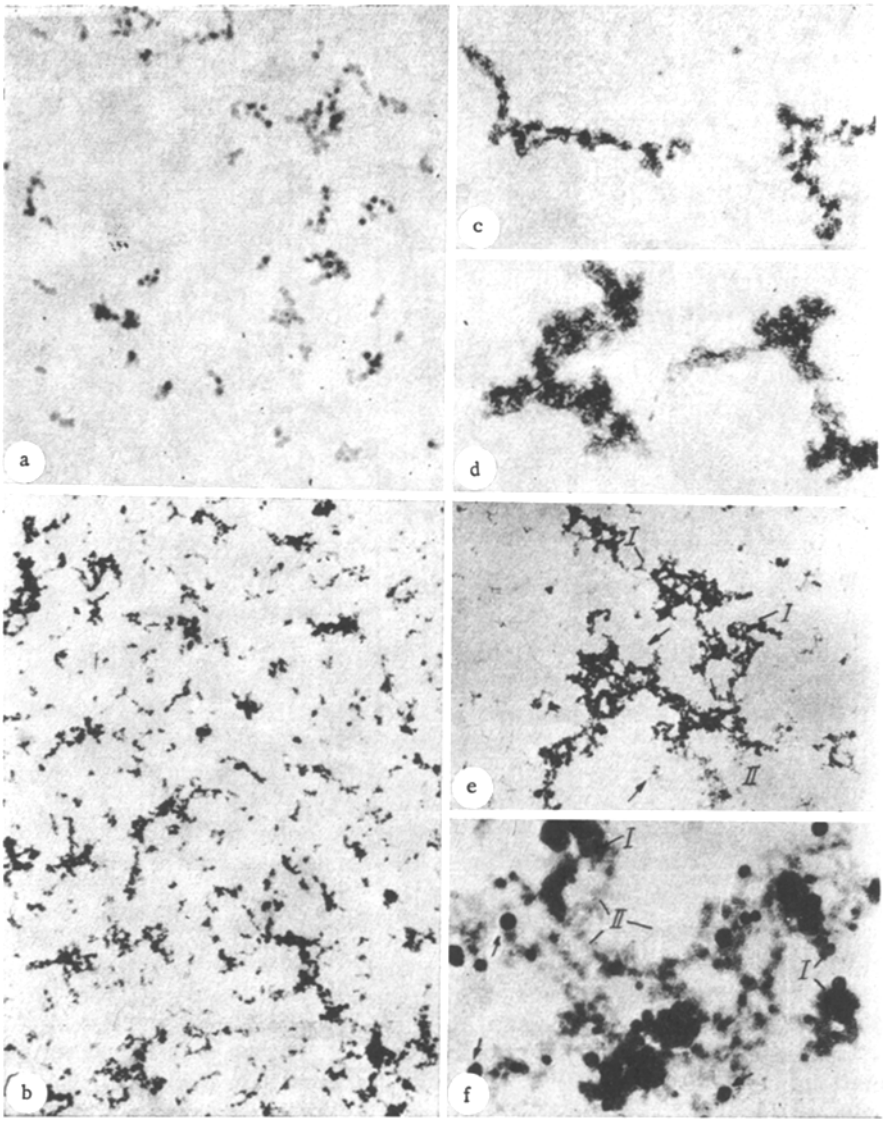

Fig. 2. Preparations of HUA and mixture of HUA with PCKS. Single granules and short chains of umbilical HUA (a). Characteristic interweaving chains of granules of vitreous HUA (b). Thin fibrillary structure of HUA in presence of cytochrome c (c, d). Contrasted reticular formations of PCKS (I), associated with thin-granular chains of HUA (II), contain grains (arrows) of uranyl acetate (e, f). a) 100,000 ×, b) 30,000 ×, c) 100,000 ×, d) 200,000 ×, e) 30,000 ×), f) 200,000 ×.

In films obtained from a mixture of HUA and PCKS reticular structures of various shapes and sizes could be detected. The underlying thin network of HUA fibrils was readily distinguishable among the coarsely granular chains of PCKS, but was particularly clearly visible at the periphery of these combined films. Free macromolecules of HUA and PCKS were present in very small numbers and could be easily identified because of their structural peculiarities (Fig. 2e, f).

Macromolecules of PCKS and HUA in films without cytochrome c thus differed significantly in their structure. The ultrastructure of the proteoglycans in films with cytochrome c was appreciably modified; the change was observed from granular to honeycombed with thin fibrils, and the differences in the macromolecular organization of the PCKS and HUA became more clearly defined. A mixture of the two proteoglycans formed structurally connected complexes in which macromolecules of PCKS and HUA could be differentiated. Free proteoglycans, especially PCKS without cytochrome c, and mixtures of proteoglycans stain well with uranyl acetate.

The extensive use of uranyl acetate for staining in electron microscopy is based on its ability to react

with the acid groups of all biopolymers [4]. In the proteoglycans now investigated, these acid groups are carboxyl groups of glucuronic and sialic acids, dicarboxylic amino acids, and sulfate groups of PCKS. The intense staining of PCKS with uranyl acetate was due to its higher content of anionic groups, both in the glycosaminoglycan and the protein components, than in HUA, in which there are no sulfuric acid residues and the content of the protein component is extremely small. This conclusion is confirmed by comparison of data obtained by the study of film preparations of PCKS and HUA containing and not containing cytochrome c. In the first case proteoglycans, interacting with some of their anionic groups with the cationic groups of cytochrome c, formed complexes with it. As a result, the number of free acid groups of proteoglycan was reduced and the remainder were oriented rather differently in space. In PCKS this led to a decrease in the number of grains of uranyl acetate and to the appearance of structureless halos around the granules. In HUA the decrease in the number of free carboxyl groups reduced the binding of uranyl acetate and revealed its thin fibrillary-honeycombed structure, which was masked in the absence of cytochrome c by the strongly bound uranyl acetate.

The polydisperse nature of the chemically individual preparations of PCKS and HUA, revealed in the electron microscope, reflects the natural physical heterogeneity of these proteoglycans. However, the possibility of an increase in their polydispersion in the course of isolation from the tissues cannot be completely ruled out.

In thicker parts of the film with an increased concentration of proteoglycans aggregations consisting of interweaving chains were observed as three-dimensional structures formed from separate macromolecules with different degrees of dispersion. This natural polydisperse nature of the proteoglycan macromolecules was perhaps essential for the creation of structures with specific steric relationships between their parameters in the tissues of the body [3, 8].

Associations formed in a mixture of HUA and PCKS had no strictly orderly structure, they were formed by means of noncovalent forces, and the possibility of their appearance has been confirmed by other indirect methods [3]. The nonaggregated fraction of proteoglycans from hyaline cartilage, on interaction with HUA, evidently does not create complexes with a strictly orderly structure, such as are characteristic of aggregates of the aggregating fraction of proteoglycans. By contrast with the latter, PCKS—HUA complexes readily dissociate in solutions of weak ionic strength. The ease of formation and dissociation of these complexes suggests that in the cell and tissue systems of the body they can easily appear and disappear.

The macromolecular formations of PCKS and HUA thus revealed are present in films formed by proteoglycans themselves in preparations without the addition of cytochrome c. It can tentatively be suggested that this structureless electron-transparent film, which does not react with uranyl acetate, is formed from the lowest-molecular-weight fraction of proteoglycans in the presence of acetic acid. Relations between the structured and nonstructured phases of the proteoglycans are unchanged during film formation. No difference as regards the formation of such films is observed between PCKS and HUA.

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