REVIEWS

Proteoglycans and Cells

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Studies of proteoglycans for their capacity to perform the function of steric exclusion of cells are reviewed. The ability of the proteoglycans hyaluronic acid and protein-chondroitin-keratosulfate and their aggregates to effect cell aggregation and steric exclusion is determined by the structure of glycosaminoglycan components and the amount of covalently bound protein in their macromolecules. These actions of proteoglycans, in which physicochemical processes predominate, can be stimulated or suppressed by protein substances and heparin fractions present in the medium. Results of model tests are fully consistent with those of animal experiments.

Key Words: *proteoglycans; cells; aggregation; steric exclusion; adhesion; dispersion*

Among the many biological functions performed by hyaluronic acid (HUA), protein-chondroitin-keratosulfate (PCKS), and their aggregates (proteoglycan aggregates, PA), of special note is the nonspecific steric exclusion of cells, biopolymers, and various low-molecular-weight substances by these proteoglycans from the space they occupy in solution [7,12]. While carrying out this task, the proteoglycans can concurrently perform their other functions [12].

The mechanism by which steric exclusion of cells is effected by HUA, PCKS, and PA has been chiefly studied using tissue or cell cultures [12]. In addition, suspensions of rabbit and other mammalian erythrocytes in salt solutions (0.15 M NaCl, pH 7.2-7.4) have been used as models in these studies. Although somewhat simplistic, such a model makes it possible to estimate, by direct counting, the number of aggregated erythrocytes and thus the volume of the phase displaced from a stable suspension. These estimates are necessary in order to gain insight into the mechanism of steric exclusion and to evaluate the influence of various factors on it [6]. Erythrocytes have the advantage of being kinetically stable in a salt solution and of lacking receptors reactive with proteoglycans, because of which the process of steric exclusion is not complicated by their interaction with the cell surface. The quantitative results of studies with erythrocytes are similar to those of experiments using tissue cultures [19].

Cell adhesion and the exclusion of adherent cells as an isolated phase from the total space are important events in the formation of tissue structures in the animal organism [12]. The use of erythrocyte suspensions as models of isolated cells provided a rationale for better differentiation between adhesion and aggregation, the latter of which is the initial step in the steric exclusion of adherent and free cells accomplished by HUA, PCKS, and PA. Cell adhesion, which is interpreted by some investigators as a cell-cell reaction similar to the antigen-antibody interaction [12], involves specific biochemical processes. The transferases present on the cell surface can combine with another cell whose surface contains glycoproteins with incomplete carbohydrate ends; as a result, cell-cell complexes arise whose number depends on the presence of uridine diphosphate-monosaccharide in

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the medium. Cell adhesion has been shown to depend on the concentration of L-glutamine, a compound essential for the synthesis of glycosaminoglycan components of proteoglycans. A factor regulating cell adhesion may be altered glutamine synthase activity. Cell-cell interactions involve the participation of a number of protein substances (e.g., fibronectin, lipo- and glycoproteins, and certain specific proteins) and of actomyosin, Ca^{2+}, and Mg^{2+}, which control the distribution of electric charges on the cell surface and determine their form. Many cells are capable of binding HUA and PCKS directly. Receptors present on the cell surface may be specific for HUA and nonspecific for PCKS. The maximal binding of these proteoglycans to their receptors occurs at 18°C, i.e., at the temperature of phase transition for plasma membranes. The predominant type of cell adhesion in animals appears to be that involving HUA and PCKS [7,12]. Cell adhesion as a whole, in which both specific and nonspecific factors are implicated, depends, of course, on the metabolism proceeding in the body, and it should be emphasized in this connection that a large proportion of the studies devoted to cell adhesion have used tissue or cell cultures which lack many of the systems controlling metabolic processes *in vivo* [12].

Cells made adherent by various methods are displaced from the total space and concentrated in a limited volume by the action of HUA, PCKS, and PA. The following results from studies on mechanisms of cell aggregation and steric exclusion were all obtained with erythrocyte suspensions in salt solutions using normal Na^+ salts of HUA, PCKS, and PA in concentrations of the order of $mg \times ml^{-1}$.

It has been shown experimentally that the rate at which erythrocytes are sterically excluded from their stable suspension to a separate phase is a linear function of the proteoglycan concentration provided the erythrocyte numbers remain constant and the system does not contain substances influencing the steric exclusion process. After the linear course of phase separation is completed, a slow increase occurs in the density of the isolated phase. The highest phase-separating activity is displayed by PA [11]. When HUA and PCKS are co-present in the erythrocyte suspension, the total effect of steric exclusion is greater than the sum of the effects produced by HUA and PCKS separately (independently of each other) at equal concentrations [2]. If they occur in a given solution together at equal concentrations, HUA and PCKS may associate to form complexes in the absence of a binding protein required for the formation of more stable PA from these proteoglycans [7]. This can explain the phenomenon of more than additive effects mentioned above. If, on the other hand, HUA and PCKS are present in different concentrations, then a proportion of one of them remains free after complex formation, and the magnitude of steric erythrocyte exclusion will be the sum of the effects produced by the HUA-PCKS complex and by the free proteoglycan. Tissues that do not contain PA always contain HUA and PCKS in various proportions [10], a phenomenon which may have implications for the steric exclusion accomplished by these proteoglycans and for their actions as factors essential for the formation of particular cellular structures. The aggregates formed by the action of HUA and PCKS consist of erythrocytes closely adhering to one another. Similar structures arising under the action of PA have a looser packing and free spaces may be present within individual aggregates. The differences mentioned above are undoubtedly associated with individual properties of the proteoglycans under consideration [3].

Biochemical and physiological properties of proteoglycans are determined by the glycosaminoglycan components of their macromolecules. The covalently bound protein components of such molecules also have a role to play in proteoglycan formation [9,12]. To evaluate the contribution of these components to the steric exclusion effected by HUA, PCKS, and PA, high-molecular polymers with unchanged glycosaminoglycans but different amounts of covalently bound protein were obtained from these proteoglycans. The HUA- and PA-derived polymers containing relatively large quantities of protein were found to be much more active in displacing erythrocytes from their suspension than those with less protein [15]. Hence, it may be concluded that the steric exclusion effected by HUA, PCKS, and PA may be enhanced or weakened by their covalently bound protein components depending on how much of these they contain. However, the major role in the steric exclusion process is played by the glycosaminoglycan components of these biopolymers. Glycosaminoglycan components mainly determine the structure of supramolecular formations produced by HUA, PCKS, and PA, whereas the contribution of protein components to their formation is relatively small, and so, accordingly, is their contribution to the steric exclusion of cells. The reason that the protein contents of HUA and PA have equal influences on the steric exclusion effected by these proteoglycans is due to the fact that HUA incorporated into PA retains many of its properties on which the activity of these aggregates depends. Tissues contain several HUA, PCKS, and PA fractions differing in levels of covalently bound protein [8,10], and these differences probably have a direct bearing on the regulation of the steric exclusion process.

The steric exclusion of erythrocytes from their suspension depends on the nature of the cation with which the carboxyl and sulfate groups of proteoglycans

combine. The effects of Ca^{2+}-HUA and Ca^{2+}-PCKS are more dependent on the concentration of these salts than of the Na^+ salts of these proteoglycans. Ca^{2+}-PA are much more active than Na^+-PA. The differences in activity between Na^+ and Ca^{2+} salts of the proteoglycans are due to the higher molecular weights of the latter salts [5]. In the presence of Ca^{2+} salts of proteoglycans, Ca^{2+} may exert a direct action itself.

Formaldehyde-treated erythrocytes remain capable of aggregation and can be displaced to form a separate phase in the presence of HUA, PCKS, and PA. The effects of proteoglycans depend on their concentration only slightly and the cell aggregates that form do not differ much in structure. This indicates that the steric exclusion accomplished by proteoglycans, unlike the specific and nonspecific cell adhesion, cannot be attributed to their chemical interactions with the surfaces of the elements they displace [4].

However, erythrocytes fail to aggregate and are not displaced to a separate phase when HUA, PCKS, and PA concentrations exceed a certain limit. This is also true of tissue cultures [12]. In such cases, the entire space of the solution becomes filled with supramolecular structures of proteoglycans and the formed elements are no longer able to move freely. This may be how barriers performing protective functions are created in tissues.

Albumins, $(\alpha+\beta)$-globulins, or γ-globulin isolated from rabbit plasma, which by themselves neither aggregate erythrocytes nor cause their release into an isolated phase from their suspension, greatly reduce the ability of HUA to do so, and γ-globulin is more active in this respect than the other two protein fractions. However, when albumins, $(\alpha+\beta)$-globulins, and γ-globulin proteins are present in an erythrocyte suspension together, HUA remains as active as in their absence, i.e., the proteins neutralize each other's inhibitory actions on this proteoglycan. The ability of PCKS to aggregate and displace erythrocytes is also inhibited by each of these protein fractions (the globulins being more inhibitory than the albumins) but is enhanced by mixtures of albumins and $(\alpha+\beta)$-globulins and, even more so, by mixtures of albumins and γ-globulin [9]. These differences between the effects of proteins on HUA and PCKS are due to the formation of more complex three-dimensional structures from PCKS macromolecules as compared to similar HUA molecules.

Heparin, which is also incapable of aggregating erythrocytes and displacing them from their suspension, alters the ability of both HUA and PCKS to produce these effects. The use of two fractions of this anticoagulant, one of which contains three (HP-3) and the other four (HP-4) sulfuric acid residues per disaccharide repeating unit of the molecule, showed that HUA's ability to aggregate and displace erythrocytes was inhibited by both fractions (in concentrations of the order of $mg \times ml^{-1}$. The PCKS-effected phase separation of erythrocytes was activated by HP-4 in low concentrations and inhibited in relatively high concentrations, while a mixture of HP-3 and HP-4 was totally ineffective [1]. The equilibrium volumes of the phase of displaced erythrocytes established following HUA and PCKS activation by HP-3 and HP-4 were considerably smaller than the volumes observed when they exerted inhibitory effects on the proteoglycans. It follows that the HP-3 and HP-4 fractions act on the supramolecular proteoglycan structures [1]. Heparin, which is contained in many tissues, may therefore regulate the steric exclusion effected by HUA and PCKS.

The complex influences of protein fractions, HP-3, and HP-4 on steric exclusion may be explained by marked differences among HUA, PCKS, and PA in negative electric charges, which are much weaker in HUA than in the other two proteoglycans. The protein fractions differ much less in this regard and, moreover, are ampholytes. Electric charges are particularly high in HP-3 and HP-4, the latter fraction having a higher charge. When HUA, PCKS, and PA are co-present with protein fractions or with HP-3 and HP-4, electrostatic factors are bound to influence the structure of supramolecular entities formed by these proteoglycans in solutions, and this influence is reflected in their altered ability to accomplish steric exclusion of cells. Physicochemical interactions apparently predominate in this process.

Thus, the ability of HUA, PCKS, and PA to nonspecifically aggregate and effect steric exclusion of cells is directly linked with their molecular weights, hydrodynamic volumes, and electric charges, and with their property to produce supramolecular structures in solutions [7]. These proteoglycans also prevent the dispersion of cellular complexes and, by bringing cells closer together, facilitate their adhesion. As a result, systems of morphologically and physiologically homogeneous cells arise. While promoting the creation of such systems, HUA, PCKS, and PA at the same time mediate water and ion transport and other types of cell communication with the internal milieu of the body. The dependence of the steric exclusion effected by proteoglycans on protein substances and heparin and the reversibility of the exclusion process (cellular dispersion is restored after the removal of proteoglycans from the medium) warrant the conclusion that steric exclusion is a general biological phenomenon.

The results of *in vitro* tests of HUA, PCKS, and PA as factors of steric exclusion agree with those of *in vivo* experiments on animals. In blood plasma, where complete dispersion of formed elements is a physi-

ological necessity, no PA are found, while HUA and PCKS occur in extremely low concentrations. HUA and PCKS added to rabbit blood were reported to cause erythrocyte aggregation that continued until these proteoglycans were completely sorbed from the circulating blood [13,16-18]. Proteoglycans aggregating erythrocytes in a concentration-dependent manner were detected in the blood of rabbits with an experimentally produced disease such as streptococcal sepsis or gas gangrene. In animals that survived infections, erythrocyte aggregation declined more rapidly than did the plasma concentration of proteoglycans, indicating that the regulatory mechanisms mentioned above were operating [14].

HUA, PCKS, and PA are used as therapeutic agents in several areas of medicine [20]. The beneficial effects of these proteoglycans are probably due, directly or indirectly, to their activity in effecting steric exclusion of cells. In this context, the quantitative method of estimating this activity (see above) appears to be quite suitable for determining the optimal doses of proteoglycans.

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