CELL ADHESION AND PROTEOGLYCANS.

I. THE EFFECT OF EXOGENOUS PROTEOGLYCANS ON THE ATTACHMENT OF CHICK EMBRYO FIBROBLASTS TO TISSUE CULTURE PLASTIC AND COLLAGEN

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SUMMARY

Proteoglycan was isolated from cartilage and freed from contaminating glycoproteins and hyaluronic acid. The macromolecule consists of a protein core covalently linked to a number of glycosaminoglycan side chains, namely chondroitin sulphate and keratan sulphate. This proteoglycan retards the attachment of a variety of cell types to tissue culture plastic and to collagen. Glycosaminoglycans alone, have no significant effect on rates of attachment. Similarly, trypsinized proteoglycan is without effect. It is concluded that the structural integrity of the proteoglycan macromolecule is essential for its effect on cell adhesion.

INTRODUCTION

When cells grow attached to a solid surface such as glass or tissue culture plastic they secrete onto this substratum a microexudate (Rosenberg, 1960; Yaoi & Kanaseki, 1972). The secretion is an active process; it varies quantitatively between cell types (Poste et al. 1973), and changes through the cell cycle (Weiss, Poste, Mackearnin & Willett, 1975). Cells can be detached from the cell substratum without removing the microexudate and the latter can then be analysed (Culp & Buniel, 1976). Serum protein, a necessary component of growth medium, also binds to the substratum and the combination of serum protein and microexudate is referred to as substrate attached material or SAM (Culp & Buniel, 1976). In addition to the serum glycoprotein in SAM, the cell-derived components include fibronectin (Culp, 1976) (otherwise known as LETS or cell surface protein), collagen (Schwartz, Hellerqvist & Cunningham, 1978) and proteoglycans (Culp & Buniel, 1976; Mapstone & Culp 1976).

High concentrations of SAM appear to be present beneath morphological protrusions which make close contact with the substratum (Rosen & Culp, 1977). Since SAM lies between cell and substratum it is likely to play a major role in adhesive interactions. There is now extensive evidence that fibronectin is a mediator of cell adhesion; fibronectin isolated from the surface of cells in culture, as well as the cold-insoluble...
globulin from plasma, increases the adhesion of virally transformed cells deficient in this glycoprotein (Yamada, Yamada & Pastan, 1976; Ali, Mautner, Lanza & Hynes, 1977). In fact the presence of fibronectin is an absolute requirement for the attachment of cells to collagen (Klebe, 1974).

Proteoglycan has also been postulated by Culp & Buniel (1976) to have a role in the adhesion between cell and substratum. Proteoglycans are a covalent combination of glycosaminoglycan and a protein core. More specifically the production of hyaluronate proteoglycan has been correlated with cell attachment. Moscatelli & Rubin (1977) have shown an increase in the rate of hyaluronic acid synthesis accompanying growth stimulation of cells by serum or elevated pH; an elevated level of the enzyme hyaluronate synthase is responsible. Barnhart, Cox & Kraemer (1979) have measured hyaluronic acid synthesis in a number of CHO variants; they find a correlation between increased synthesis and a more rounded morphology.

Rollins & Culp (1979) have used radioactive precursors of the various glycosaminoglycans to show that specific classes of glycosaminoglycans may be associated with both attachment and detachment of cells. Similarly, Pratt et al. (1979) have found that the antibiotic tunicamycin selectively prevents the synthesis of sulphated proteoglycan whereas the production of hyaluronic acid is unaffected; in addition tunicamycin causes cells to round up, i.e. lose adhesion. Rollins & Culp (1979) have suggested that glycosaminoglycans are able to act as a bridge between the cell surface fibronectin and the cold-insoluble globulin absorbed on to the substratum. However, there is now general agreement that glycosaminoglycans such as chondroitin sulphate, keratan sulphate and heparan sulphate do not exist in a free state; rather they are part of proteoglycan molecules. Even in the case of hyaluronic acid, the existence of free polysaccharides is doubtful and evidence has been presented that hyaluronate is also a proteoglycan (Scher & Hamerman, 1972).

In this study we have prepared proteoglycan monomer from bovine nasal cartilage and report on the effect of this material on the attachment of chick embryo fibroblasts to tissue culture plastic.

MATERIALS AND METHODS

Proteoglycan extraction

Nasal cartilage from a 15-month-old steer was freed of adhering non-cartilaginous tissue and perichondrium and frozen in liquid nitrogen within 20 min of death. After thawing on ice the cartilage was cut into approximately 0.5-mm slices and added immediately to the extraction medium which contained: 4 M guanidinium hydrochloride, 0.1 M Tris, 0.1 M aminohexanoic acid, 0.01 M sodium-EDTA, 0.005 M benzamidine hydrochloride, 2 mM phenylmethylsulphonylfluoride, 5 mM iodoacetate, 1 mM dithiothreitol, 5 µg/ml pepstatin, adjusted to pH 7.5 with HCl. Benzamidine hydrochloride and phenylmethylsulphonylfluoride were added immediately prior to use.

After extraction for 24 h at 4 °C the solution was clarified by centrifugation at 40000 g, for 15 min at 4 °C. The extract was diluted with extraction medium to give a uronate concentration of 1 mg/ml, adjusted to a density of 1.50 g/ml by the addition of calcium chloride and centrifuged in a Beckman Ti-50 rotor (12 × 13.5 ml) at 40000 rev/min (100000 g) for 48 h at 20 °C. Tubes were frozen in liquid nitrogen and cut into fractions. Fractions with a density greater than 1.63 g/ml were isolated, pooled, exhaustively dialysed against water and lyophilized.
**Analytical ultracentrifugation**

For molecular weight analysis, solutions of proteoglycan were dialysed against 0.005 M sodium phosphate - 1 M sodium chloride, pH 7.4. Equilibrium sedimentation, using the meniscus depletion method of Yphantis (1964), was performed at 25 °C using a Beckman Model E analytical ultracentrifuge equipped with interference optics. Fringe displacements were measured using a microcomparator. The calculation of point average $M_a$ and $M$, molecular weights across the cell allowed an estimate to be made of sample polydispersity.

The partial specific volume for both proteoglycan and trypsinized proteoglycan was taken to be 0.55 ml/g (Hascall & Sajdera, 1970).

**Analysis of proteoglycan**

For hexosamine determination, hydrolysis was carried out in sealed tubes in vacuo, with 4 M HCl at 110 °C for 8 h. After hydrolysis, excess acid was neutralized and differential determination of glucosamine and galactosamine performed by ion-exchange chromatography using a Locarte analyser. Amino acids were determined using a Locarte amino acid analyser after hydrolysis of samples for 24 h at 110 °C. No corrections were made for hydrolytic losses. Dry weight was determined on samples exhaustively dialysed against water and dried at 78 °C in vacuo for 10 h over phosphorus pentoxide.

**Preparation of glycosaminoglycans**

Keratan sulphate was prepared according to the method of Matthews & Cifonelli (1965). Chondroitin sulphate from bovine trachea was purchased from British Drug Houses and hyaluronic acid from human umbilical cord was obtained from Sigma (Grade I).

**Trypsin digestion of proteoglycan**

Three hundred milligrams of proteoglycan were dissolved in 60 ml of 0.05 M sodium phosphate, pH 7.6. After the addition of 1.5 mg of trypsin (Type XI, Sigma), the solution was maintained at 24 °C for 24 h under toluene; after dialysis against water at 4 °C, the material was lyophilized.

**Chick embryo fibroblasts (CEF)**

Skin was dissected from the back of 11-day chick embryos and after washing in PBS (0.2 g/l. KCl; 0.2 g/l. KH$_2$PO$_4$; 8.9 g/l. NaCl; 1.15 g/l. Na$_2$HPO$_4$; pH 7.4), each skin was finely minced with sterile scissors and incubated for 15 min at 37 °C with 10 ml PBS containing 0.1 % trypsin (Flow Laboratories). After removing most of the supernatant liquid, 10 ml of growth medium (Ham's F10 (Flow Laboratories) supplemented with 10 % foetal calf serum (Flow Laboratories) was added and the tissue dispersed by suction with a pipette. Cell suspensions were pooled and non-dispersed tissue removed by passage through sterile gauze. Cell density was measured with a Coulter counter (Model ZB with Channelizer) and adjusted to 10⁶/ml. The suspension was plated out in 9-cm Petri dishes (Sterilin) and incubated in a 37 °C humidified incubator gassed with 5 % CO$_2$/95 % air. After 24 h cultures were washed and refed to remove unattached cells.

**Attachment assay with CEF**

After reaching confluence, primary cultures of CEF were passaged at a density of 5 x 10⁴/ml. After 2 days these cultures were pulsed for 48 h with 10 μCi/ml [6-³H]thymidine (30 Ci/mmol, Radiochemical Centre, Amersham). After 5 washes with PBS, each dish was treated with 1.5 ml 0.05 % trypsin/0.02 % EDTA (combined solution purchased from Flow Laboratories) at 37 °C for 1 min by which time cells begin to 'round-up'. Cells were removed with a Pasteur pipette and resuspended in growth medium. Cell density was adjusted to approximately 5 x 10⁴/ml and the suspension divided into aliquots. After adding proteoglycan or glycosaminoglycan solutions, cells were plated out in 3-cm Petri dishes (Sterilin). At specific time
intervals, non-adherent cells were removed by decanting and washing with 2 ml PBS; adherent cells were removed by the addition of 2 aliquots of 2 ml 1% Triton X-100. The suspensions of adherent and non-adherent cells were added to 10 ml ice-cold 10% trichloroacetic acid solution (TCA) and the resulting precipitate collected on 2.1-cm GFC niters (Whatman). Filters were used directly for scintillation counting and after background subtraction and quench correction attachment was calculated from the equation:

\[
\text{% cell attachment} = \frac{\text{cpm (adherent)}}{\text{cpm (adherent)} + \text{cpm (non-adherent)}} \times 100.
\]

The range of variation between duplicate Petri dishes was 0 - 14%.

**Ehrlich ascites cells**

Ehrlich ascites cells (donated by Dr M. J. Clemens) were grown continuously in spinner vessels using Eagle's minimum essential medium modified for suspension culture (Flow Laboratories) supplemented with 10% donor calf serum (Flow Laboratories). These cells divide every 14 h; every 2 days suspensions were centrifuged at 1000 g/min and resuspended at 5 x 10⁴/ml and excess to requirement was discarded.

**Attachment assays with Ehrlich cells**

After resuspension at 5 x 10⁴ cells/ml, cultures were pulsed for 48 h with 10 μCi/ml [6-³H]-thymidine; the suspension was then centrifuged (1000 g/min) and washed 3 times with PBS (20 ml/5 x 10⁴ cells). After the last wash cells were resuspended at a density of 5 x 10⁴/ml in Ham's F₁₀ supplemented with 10% donor calf serum. Attachment assays were then carried out as for CEF cells.

**Collagen**

Rat tail collagen was prepared and used to coat Petri dishes according to the method of Klebe (1974).

**RESULTS**

Table 1 shows a typical composition of the proteoglycan used in these studies. As prepared by caesium chloride density gradient centrifugation in a dissociative solvent, proteoglycan is recognized to be both polydisperse and heterogeneous. It should however, be free of contaminating glycoprotein and hyaluronic acid (Hascall & Sajdera, 1969). The possibility of contamination was reduced further by isolating proteoglycan only from fractions with a density greater than 1·53 g/ml rather than at a density of 1·53 as described by Hascall & Sajdera (1969). Most of the glycosaminoglycan is chondroitin sulphate with a much smaller proportion of keratan sulphate. Before use in cell attachment assays, proteoglycan subunit was exhaustively dialysed in order to remove traces of proteolytic inhibitors used during preparation. Following lyophilization, the material was stored at -70 °C.

Chick embryo fibroblasts cells were used for most of the studies and attachment assays involve the readhesion of freshly dispersed monolayers. The attachment assay makes use of cells that have been prelabelled with tritiated thymidine. Duplicate plates are taken at specified time intervals and unattached cells are removed by decantation and attached cells are removed from the substratum with detergent. TCA precipitates of both attached and unattached cells are collected on glass-fibre filters.
Table 1. Molecular weight range and chemical composition of proteoglycan

<table>
<thead>
<tr>
<th>Mol. wt range</th>
<th>Proteoglycan</th>
<th>Trypsinized proteoglycan</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 x 10^6 - 2 x 10^6</td>
<td>5 x 10^3 - 60 x 10^6</td>
</tr>
<tr>
<td>% dry wt of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chondroitin sulphate*</td>
<td>77.0</td>
<td>76.0</td>
</tr>
<tr>
<td>Keratan sulphate†</td>
<td>4.4</td>
<td>5.1</td>
</tr>
<tr>
<td>Protein§</td>
<td>8.3</td>
<td>7.5</td>
</tr>
<tr>
<td>Galactosamine/glucosamine molar ratio</td>
<td>16:1</td>
<td>13:1</td>
</tr>
</tbody>
</table>

* Calculated from galactosamine content.
† Calculated from glucosamine content.
§ Calculated by summation of amino acids.

Fig. 1. The effect of proteoglycan on cell attachment to tissue culture plastic. Attachment assays were carried out with chick embryo fibroblasts in the presence of proteoglycan, µg/ml: □, 100; ○, 300; ●, control.

which can be used directly for scintillation counting. The percentage of cells attached can then be calculated directly.

Fig. 1 shows the effect of exogenous proteoglycan on the rate of attachment of CEF cells. Proteoglycan retards attachment in a dose-dependent fashion. When plates of cells are examined with an inverted microscope then the following phenomenon is observed: in the presence of proteoglycan subunit, attachment is delayed and unattached cells float to the middle of the Petri dish. This results in a high concentration of cells in the middle of the dish and after a small proportion of the cells have become attached there is clearly no space for further cell attachment. Thus the values for attachment after 6 h probably do not indicate the true number of cells that could have attached in this time. This phenomenon has no effect on the initial rate of attachment and so this is the most reliable parameter.
Fig. 2. The effect of individual glycosaminoglycans and trypsin-digested proteoglycan on cell attachment to tissue culture plastic. Attachment assays were carried out with chick embryo fibroblasts in the presence of glycosaminoglycans or trypsinized proteoglycan. ●, Control; ○, 300 µg/ml proteoglycan; △, 300 µg/ml trypsin-digested proteoglycan; □, 300 µg/ml keratan sulphate; 300 µg/ml hyaluronic acid; ▲, 300 µg/ml chondroitin sulphate.

Different batches of cells give significantly different rates of attachment but the proteoglycan effect is entirely consistent. Proteoglycan is predominantly glycosaminoglycan, with only a small proportion of protein, and so the effect of individual glycosaminoglycans was examined. Chondroitin sulphate and keratan sulphate were prepared and added to CEF cells during attachment assays. Fig. 2 shows that the glycosaminoglycans have no significant effect on rates of attachment. The concentration of glycosaminoglycan is 0.3 mg/ml and this is clearly greater than the glycosaminoglycan present in levels of proteoglycan that produce inhibition of attachment. We have also found that combinations of keratan sulphate and chondroitin sulphate are without effect. Fig. 2 also shows that hyaluronic acid is without effect in this attachment assay.

Trypsinization of the proteoglycan macromolecule results in the formation of a large number of polydisperse fragments. Table 1 shows that the overall chemical composition of these fragments is not significantly different from that of intact proteoglycan. The average molecular weight of the preparation is, however, reduced by an order of magnitude. Fig. 2 shows that these fragments are without effect on attachment. This again indicates that the structural integrity of the macromolecule is necessary for inhibition of attachment.

When cell monolayers are dispersed with trypsin, cell surface components are likely to be proteolytically cleaved; fibronectin, for example, is very sensitive to trypsin. Thus it is necessary to distinguish between effects on the recovery from trypsinization and effects on attachment per se; to determine the effect of proteoglycan on the attachment of cells that have not been subjected to trypsinization, Ehrlich ascites cells were used. These cells were grown in spinner cultures prior to use and after centrifugation,
Fig. 3. Pretreatment of cells or substratum with proteoglycan. ●, attachment of control chick embryo fibroblasts. □, Petri dishes were treated with a 1 mg/ml proteoglycan solution in PBS; after 3 washes the dishes were used for attachment assays with untreated chick embryo fibroblasts. ○, before carrying out attachment assays, suspended chick embryo fibroblasts were treated for 30 min with 1 mg/ml proteoglycan in growth medium. After centrifuging and washing once, cells were resuspended in growth medium and used in attachment assays to untreated Petri dishes.

Fig. 4. The effect of proteoglycan on the attachment of cells to collagen. Attachment of chick embryo fibroblasts to collagen-coated Petri dishes was carried out in the presence of proteoglycan, μg/ml: □, 100; ○, 300; ●, control.

cells were simply resuspended at the required density and used immediately for attachment assays. The percentage of cells attaching in control cultures was always lower than CEF cells and also varies between experiments, but there was always a dose-dependent inhibition by proteoglycan.

The attachment of BHK cells and human skin fibroblasts is also inhibited by the proteoglycan preparation (unpublished data). BHK cells seem to be less sensitive than CEF cells while human skin fibroblasts behave in a similar fashion to CEF.
To determine whether the effect of proteoglycan is on the cell or on the substrate, cells or substratum were treated with 1 mg/ml proteoglycan solution. After thorough washing, attachment assays were carried out and Fig. 3 shows the results of such an experiment. While there is no effect when the substratum is treated with proteoglycan, when cells are treated, then after washing there is a significant effect on the rate of attachment although this is substantially less than that produced if the proteoglycan is present during the attachment assay (Fig. 1).

Chick embryo fibroblasts will readily attach to collagen in the presence of serum. Such substrata are clearly more physiological than tissue culture plastic. Fig. 4 shows the effect of proteoglycan on the attachment of chick embryo fibroblasts to collagen. Again proteoglycan produces a dose-dependent lowering of the rate of attachment. In fact there is little difference in the effects of proteoglycan on attachment to collagen or attachment to tissue culture plastic.

**DISCUSSION**

To our knowledge, no studies have been made on the effect of exogenously added proteoglycan to cells in culture. The availability of cartilage proteoglycan in a pure undegraded and well characterized state makes it possible to relate structural features of the proteoglycan to any effect which may be produced in such experiments. In intercellular matrices, proteoglycans exist as aggregate structures which can be readily extracted using dissociative solvents such as 4 M guanidinium hydrochloride. Monomeric proteoglycan, also known as proteoglycan subunit (PGS) (Sajdera & Hascall, 1969), can subsequently be isolated by centrifugation of the extract in a dissociative cesium chloride gradient (Hascall & Sajdera, 1969). The proposed model for PGS has the following features (Hascall & Sajdera, 1970; Pasternack, Veis & Breen, 1974; Luscombe & Phelps, 1967; Hascall & Rio, 1972): PGS is polydisperse with an average molecular weight of \(2.5 \times 10^6\) and a range of \(1.0 \times 10^6\) to \(4.0 \times 10^6\). It contains about 100 chondroitin sulphate chains, each with an average mol. wt of about \(2 \times 10^4\), and 25–50 keratan sulphate chains, each with an average mol. wt of about \(5 \times 10^3\). Polysaccharide chains are covalently linked to a protein core, which has a weight average mol. wt of \(1.8–2 \times 10^6\) (Hascall, Rio, Hayward & Reynolds, 1972). One end of the protein core interacts with hyaluronic acid and this end is relatively free of polysaccharide chains (Hardingham & Muir, 1972, 1974; Hascall & Heinegard, 1974; Rosenberg, Hellmann & Kleinschmidt, 1975).

The results described in this communication show that exogenous proteoglycan reversibly inhibits the attachment of CEF cells to collagen or to tissue culture plastic. Earlier reports invoked proteoglycans as effectors of adhesion (Culp & Buniel, 1976) rather than as inhibitors although no direct effect was demonstrated. Since the microexudate also contains fibronectin, which is known to have a role in adhesion, this might explain the correlation between timing of attachment and microexudate secretion. Another approach has been to radiolabel cells with glycosaminoglycan precursors. However, there is now general agreement that glycosaminoglycans such as chondroitin sulphate, keratan sulphate and heparan sulphate exist as covalently
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linked parts of the proteoglycan molecule; hyaluronic acid, if not covalently linked, can certainly be associated with proteoglycan.

The results of glycosaminoglycan labelling studies present a complex picture. Barnhart et al. (1979), have implicated hyaluronic acid in reducing the adhesion of cells while on the other hand Rollins & Culp (1979) have suggested that specific glycosaminoglycans may promote adhesion by acting as a bridge between cell surface fibronectin and plasma cold-insoluble globulin which becomes bound to the substratum. There may however be no contradiction, since the latter authors postulate that different classes of glycosaminoglycans may have operationally distinct functions. Indeed they show that initial adhesion is accompanied by a predominant synthesis of heparan sulphate but that in older cultures the synthesis of undersulphated chondroitin and dermatan sulphates predominate.

Thus specific glycosaminoglycans seem to be associated with either attachment or detachment. If the functional molecule is proteoglycan rather than glycosaminoglycan, then there may well be proteoglycan species at the cell surface operating in both positive and negative fashions with respect to adhesion. Clearly the species we have studied should be referred to as inhibitory proteoglycan.

Certainly if the results of Barnhart et al. (1979) reflect a correlation between decreased cell adhesion and higher levels of proteoglycan rather than higher levels of hyaluronic acid then this would be perfectly compatible with our results, namely that exogenous proteoglycan decreases cell adhesion. Individual glycosaminoglycans were found to be without effect on our attachment assay; hyaluronic acid, keratan sulphate and chondroitin sulphate are totally without effect, even at high concentration (0.3 mg/ml). Thus the native configuration of the proteoglycan macromolecule is likely to be important for the inhibitory effect. This has been confirmed by the observation that mild trypsinization of proteoglycan, a procedure which does not affect glycosaminoglycans, completely removes the inhibitory effects.

One mechanism by which proteoglycan might affect cell attachment is by competing with cold-insoluble globulin or fibronectin for binding sites on the substratum. Attachment of cells to collagen requires the presence of cold-insoluble globulin (Klebe, 1974) and this glycoprotein binds avidly to collagen (Engvall & Ruoslahti, 1977). Proteoglycan also binds to collagen and so may thus prevent or slow down adsorption of cold-insoluble globulin.

Although an attractive hypothesis it is more likely that the effect is on the cell surface, since pretreatment of cells with proteoglycan causes significant retardation of attachment. In addition we have found that pretreatment of collagen-coated coverslips with proteoglycan does not retard attachment (unpublished observations).

Proteoglycan subunits in solution have large excluded volumes and hence limited mobility. This suggests that only a small percentage of the proteoglycan will make contact with the cell surface and further suggests that proteoglycans are rather more potent than the concentrations used in this study would suggest.

Morphological studies have revealed that the cell is not uniformly attached to its substratum (Izzard & Lochner, 1976). Rather, there are specific areas where the cell surface comes into close contact with the substratum; these areas are known as focal
contacts. There are several ways in which the cell might regulate the amount of its surface that is in close apposition to the substratum; for instance, cell surface receptors may cluster. Another possibility is that receptors are evenly distributed, but areas become masked and hence areas of 'non-contact' are produced. Proteoglycans have been detected in both cell surface coat and surface attached material, and since proteoglycan inhibits attachment, one possibility is that this component of surface coat, by inhibiting attachment in localized areas, controls the amount of 'close contact'. This may be especially significant in cells which are highly motile.

Focal contact points turn over and are formed de novo at the lamellipodium or leading edge of the cell (Lochner & Izzard, 1973); the trailing edge of the cell has few focal contacts and this may be a necessary feature of motile cells. It would seem likely that if the trailing edge becomes firmly attached to the substratum then cell motility would be seriously retarded.

Of course one serious criticism is that the proteoglycan used in these studies is not representative of the type of proteoglycan found at the surface of cells in culture, although this possibility is not ruled out by the labelling studies of Culp and collaborators (Culp, Terry & Buniel, 1975; Culp & Buniel, 1976; Rollins & Culp, 1979). More information is needed on the precise nature of proteoglycans at the cell surface and on such parameters as half-lives.

Perhaps the most significant finding to emerge from these studies is that the effects of proteoglycan on attachment are only exhibited by the intact molecule. Certainly the glycosaminoglycans, once they have been removed from the peptide core, are without effect. This suggests that much of the data referring to glycosaminoglycans in fact relate to proteoglycan macromolecules and that it will be necessary to investigate more fully the effects of proteoglycan rather than that of individual glycosaminoglycans.

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