

DETECTION OF CARTILAGE PROTEOGLYCAN-RELATED ANTIGENS ON THE
SURFACE OF 3T3 CELLS

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The presence of glycosaminoglycans (GAGs), particularly of heparan sulphate, chondroitin sulphate, hyaluronic acid and dermatan sulphate on the surface of a variety of cultured cells has been well documented (Kraemer 1971; Malmström et al 1975; Vogel and Kelley 1977). In some instances, the corresponding cell associated proteoglycans i.e. proteoheparan sulphate and dermatan sulphate have been isolated and characterized (Oldber et 1979; Malmström et al 1975).

The most, so far, well characterized proteoglycan molecules are those from hyaline cartilage. The basic proteoglycan subunit (PGS) consists of a core protein onto which are covalently bound several chains of chondroitin sulphate, Keratan sulphate (Tsiganos and Muir 1967) and additional oligosaccharides containing mannose (Lohmander et al 1980).

Three regions with distinct chemical, structural and functional features can be recognised along the proteoglycan macromolecule. The carboxyl end part which is devoid of polysaccharide chains, has a rather globular structure and binds reversely onto hyluronate; the intermediate region containing most of the keratan sulphate and the other end containing most of chondroitin sulphate and its protein has simple structure (Hardingham 1981).

It has been demonstrated by several workers that cartilage proteoglycans are antigenic and that the determinants reside on the protein part of the molecule (Loewi and Muir

1965; Tsiganos and Muir 1969). At least two antigenic determinants are present; one being species common and the other species specific (Loewi and Muir 1965). The species common is located on the chondroitin sulphate rich part of the molecule and the species specific on the hyaluronate binding region (Wieslander and Heinegard 1979). Recent studies however, with a proteoglycan from mammalian cornea which contains only keratan sulphate, have shown that in addition to the protein moiety the carbohydrate chains may also react with antisera raised against the whole molecule (Conrad et al 1981).

On the basis of the presence of a species common protein antigen on the proteoglycans we undertook the present study and we report the detection of components on the external surface of 3T3 cells that cross-react with specific antisera directed against proteoglycan subunit from mammalian hyaline cartilage.

METHODS

Cell cultures

Balb/c 3T3 cells were cultured in antibiotics free Dulbecco's modified medium (Flow Laboratories, U.K.), supplemented with 10% foetal bovine serum (Flow Labs). Cells were harvested after reaching confluent densities by trypsinisation.

Isolation of proteoglycans

Proteoglycans from sheep nasal or pig laryngeal cartilage were isolated by extraction of the tissue with 4M-Guanidinium chloride pH 5.8 and subsequent centrifugation in a linear density gradient first in 0.5M i.e. aggregated form and then in 4M Guanidinium chloride i.e. disaggregated form or basic subunit (PGS) as described by Sajdera and Hascall (1969). The PGS fraction was further purified by gel chromatography on Sepharose-4B in the presence of 4M-Guanidinium chloride. The fraction that is eluted in the void volume of the column was used for immunization.

Production of antisera

New Zealand white rabbits weighing 2-2,5 Kg were immunized with 4mg of PGS (about 400µg of protein) in Freund's complete adjuvant. Each animal received one ml of emulsion subcutaneously in multiple injection sites on the back of the neck. The injection was repeated one week later and three weeks thereafter (Loewi, G. and Muir, H., 1965; Tsiganos, C.P.

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and Muir, H., 1969). The animals were bled a week after the last injection and the sera were separated, heated at 56°C to inactivate the complement and were stored at -20°C until required.

Immunofluorescence method

The indirect immunofluorescence method was used to detect proteoglycans on the surface of 3T3 cells. About 3×10^5 cells were incubated with antisera against PGS at 1:20 dilution, followed by fluorescein isothiocyanate conjugated sheep antirabbit serum (Burroughs Wellcome). After three washes the cells were resuspended in 50% glycerol, mounted on slides and examined under a Zeiss microscope equipped with epi-illumination for fluorescence. For cytoplasmic staining cytocentrifuged smears were fixed in 5% (vol/vol) glacial acetic acid in ethanol and washed in PBS. The slides were then stained by the indirect method described above in a moist chamber.

Enzyme treatment of the cells

Cells (3×10^5) were washed three times with MEM without FCS and were resuspended in 0.5ml of phosphate buffer pH 7.4 in the case of trypsin digestion, or in 0.5ml 0.1M acetate 0.15M NaCl buffer pH 5.0 for hyaluronidase digestion. Treatment with enzymes was performed at 37°C for 30 min with intermittent shaking. DNAase was included in the incubating mixture at a final concentration of 0.1mg/ml during treatment with trypsin. After incubation with the enzymes the cells were washed three times with MEM supplemented with 10% FCS and the indirect immunofluorescence method was performed.

Reagents

Colchicine, trypsin and testicular hyaluronidase (1300 U/mg) were obtained from Sigma.

The cytochalasin B (Aldrich Chemical Company) was dissolved in dimethyl sulfoxide (DMSO) (B.D.H., Poole) at a concentration of 5mg/ml, and DMSO controls were included each time cytochalasin B was used.

RESULTS

Immunofluorescence staining

The antisera against pig and sheep PGS were tested by the Ouchterlony double diffusion technique after hyaluronidase digestion and produced several precipitin lines one of

which is species common as it has been observed by Loewi and Muir (1965); Tsiganos and Muir (1969).

Viable 3T3 cells produced a patchy pattern of membrane spots when stained 22°C with rabbit antisera to either sheep or pig proteoglycans (Fig.1a). This patchy staining was seen on all cells examined under the microscope. When staining

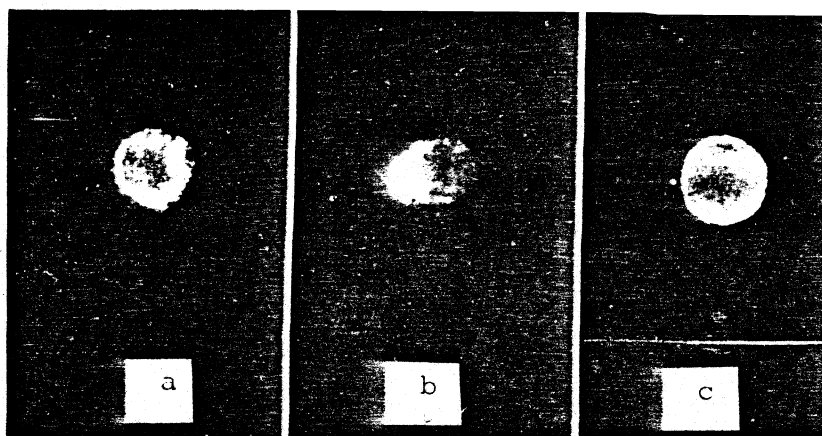


Fig. 1. a, b, c . Membrane immunofluorescence of 3T3 cells with rabbit anti-proteoglycan serum; reaction at 22°C, 37°C and at 4°C respectively.

with anti-proteoglycans was carried out at 37°C cap formation was seen in few cells, about 2%, (Fig.1b). Preimmune normal rabbit sera did not stain the cells. Absorption of antiproteoglycan sera with their respective proteoglycan antigens abolished completely the staining of the cells. In contrast, preincubation of these antisera with culture medium containing 10% or 20% foetal calf serum (FCS) for 30min and 4 hr did not change the intensity of the staining reaction. This indicates that the culture medium does not contain proteoglycan like antigen(s) possibly derived from FCS and which could have been passively absorbed on the surface of 3T3 cells. No effect on the staining pattern was observed when the antisera were first absorbed with purified cartilage hyaluronic acid or protein free chondroitin sulphate chains. Thus providing further evidence that the antigenic determinants are not on the chains. When the staining reaction was performed at 4°C the pattern of staining was homogeneous in all cells, (Fig.1c). The transition from 4°C to 37°C (that is transition

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of staining patterns from homogeneous to patch) involved the appearance of discrete membrane spots (Fig.1b). This transition did not occur in the presence of both colchicine (10^{-4} M) and cytochalasin B (5-10 μ g/ml), but it was observed when these reagents were used alone. When cytocentrifuged smears were stained intracellular staining was not observed but only a patchy pattern of fluorescence was seen corresponding to the cell membrane.

Enzyme treatment

Cells treated with bovine testicular hyaluronidase for 30min at 37°C under optimal conditions for the enzyme still gave the usual staining pattern with anti-proteoglycan sera. The enzyme was active upon chondroitin sulphate chains when a sample was incubated under the same conditions and at the end it was precipitated with 4 vol. of ethanol. In contrast, when cells were treated with trypsin at 0.1 and 0.5mg/ml at 37°C for 30 min the staining pattern was completely abolished (Table I).

Table I: Effect of trypsin and hyaluronidase on surface antigen(s) of 3T3 cells.

	Trypsin		Hyaluronidase
	0.1mg/ml	0.5mg/ml	1500U/ml
Normal rabbit serum	-	-	-
Anti-proteoglycan antiserum	-	-	++

To show further that the proteoglycan like antigen(s) were not absorbed from the medium but synthesized by the cells, the trypsin treated and negative to staining cells were incubated at 37°C in tissue culture condition for four hours.

Table II: Effect on surface antigen(s) of short term culture of trypsin treated 3T3 cells.

	Intensity of staining at		
	0h	30min	4h
Normal rabbit serum	-	-	-
Anti-proteoglycan serum	-	-	++

Cell samples were taken after 30 min and four hours washed and stained. The capacity to react with antisera was restored after four hours of incubation (Table II). These results together with the absence of any effect on the staining by

absorption of the antisera with FCS indicate that the antigen(s) on the cell surface are actively synthesized by the cells.

DISCUSSION

The proteoglycan antigens used for immunization expected to contain no extraneous proteins, non-covalently bound, since they were isolated after chromatography in the presence of 4-M guanidinium chloride. Furthermore, no proteins were detected when they were electrophoresed before and after reduction with 2-mercaptoethanol in 7% polyacrilamide gels in SDS (Laemmli 1970). It is therefore justifiable to assume that the antisera used are specific to the proteoglycan subunit.

Previous work on Balb/c 3T3 cells have indicated that hyaluronic acid, heparan sulphate and chondroitin sulphate are components of the cell surface and can be removed by digestion with specific enzymes but not by rinsing (Vogel and Kelley, 1977). The work so far done has been based on the incorporation of labelled glycosaminoglycan precursors and release of glycosaminoglycan chains upon trypsinisation of the cell surfaces. On this evidence (Kleinman, Silbert and Silbert, 1975; Vogel and Kelley, 1977) suggested that glycosaminoglycans may be bound to proteins on the cell membrane.

Staining 3T3 cells with specific antisera directed against the protein core of proteoglycans provides evidence for the presence of similar protein antigens on the cell surface. Since no cross reaction of anti-proteoglycan sera with glycoproteins has been reported and since chondroitin sulphate chains had no effect on the staining it may be concluded that some glycosaminoglycans are bound to protein(s) which are in part structurally similar to the regions of cartilage proteoglycans containing the species common determinants. However, it is not known whether the protein core of heparan sulphate reacts with antisera to proteoglycans of chondroitin sulphate. Whether heparan sulphate shares the same protein with chondroitin sulphate or it is bound to a different protein core with the same or different antigenic properties remains to be elucidated. The absence of intracellular staining by the anti-proteoglycan antisera may be due to low turnover rates of their synthesis and to the sensitivity of our method; a local concentration of about 5000 molecules are required before staining can be seen.

The membrane bound antigen is mobile and temperature sensitive; its membrane mobility is inhibited by colchicine and cytochalasine B two drugs known to disrupt microtubules

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and microfilaments respectively (Weisenberg and Timasheff, 1970; Wessells et al 1971). Thus a role for microfilaments and microtubules in the mobility of membrane-bound proteoglycan components can be suggested. From our results it is also concluded that hyaluronic acid and chondroitin sulphate chains may be released from the cell surface by enzymic treatment without the concomitant release of the antigens which are assumed to be protein(s). This might indicate that hyaluronic acid is not a backbone for cell surface glycosaminoglycan complexes. Another possibility is that the protein component is partly embedded into the cell membranes as it has been suggested for heparan from rat liver plasma membranes (Kjellén et al 1981).

Trypsin treatment removed the proteoglycan-related component from the cell surface but it was resynthesized by the cells following tissue culture conditions. After trypsinisation there was at least a culture period of 4 hours before the antigens reappeared on the cell surface (Table II). This and the lack of inhibition by FCS on the staining reaction clearly indicate that the proteoglycan-related component is synthesized by the cells and it is not passively absorbed from the tissue culture medium.

The results of the present study suggest the presence of cartilage proteoglycan-related antigen(s) on the surface of 3T3 cells. The use of specific antisera may be extended in the elucidation of the role of these macromolecules in some biological functions of the cells.

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