LOCALIZATION OF CHONDROMUCOPROTEIN IN CARTILAGE

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The histochemical localization of acid polysaccharides involves difficulties of fixation and usually reactions of uncertain specificity. Thus, the aqueous phase used in most fixatives is liable to elute at least part of the material, while most staining reactions lack specificity. The latter problem has lately been successfully attacked by the use of the salt-controlled alcian blue technique (Scott and Dorling, 1965). The present paper describes the application of an immuno-histochemical technique to this problem. Chondromucoprotein (CMP) in cartilage was detected by the application of antibody directed against this substance. The immunological characteristics of the reaction and nature of antigen and antibody have been fully described elsewhere (Loewi and Muir, 1965; Muir, 1958). Antiserum to CMP is predominantly directed against the protein part; for this reason parallel studies have been carried out with the alcian blue technique to demonstrate the acid polysaccharide part of the CMP complex.

Material and Methods

Cartilage was taken from fully-grown pigs immediately after death in the slaughter-house, including specimens of costal cartilage, of tracheal cartilage, and of articular cartilage from the ankle joint. Part of each specimen was snap-frozen in a mixture of solid CO₂ and ethanol, and another part was fixed in 95 per cent. ethanol at 4° C. Some specimens were fixed in a mixture of 1 part of 40 per cent. formaldehyde and 9 parts of absolute ethanol at -20° C. Fixed material was subsequently embedded in paraffin wax and sections were cut at 5 μ thickness. The method followed with cold-ethanol fixed tissue was that of Sainte-Marie (1962). Unfixed, frozen material was cut in a cryostat cabinet and then rapidly dried before further treatment.

Sections of Pig Aorta were cut from cold-ethanol fixed tissue.

Embryo Pig Tissue was obtained from a litter *in utero*.

Fluorescent Staining was done by the sandwich technique. Antibody to pig laryngeal cartilage CMP was obtained in rabbits (Loewi and Muir, 1965). This antiserum, at a dilution of 1:5 in Coons's buffered saline was

applied to sections for 30 min., followed by washing for 30 min. After this, fluorescein-conjugated goat-anti-rabbit γ -globulin was layered on the sections for 30 min., followed by another wash of at least 30 min. Controls treated with an unrelated rabbit antiserum, were included in every experiment.

Alcian Blue Method for acid polysaccharides was used on sections of fixed or unfixed tissue. Varying molarities of MgCl₂ were used, the critical point being different for the several acid mucopolysaccharides (Scott and Dorling, 1965).

Results

Costal Cartilage.—Sections treated with antibody showed specific staining of the rims of lacunae surrounding chondrocytes (Fig. 1, opposite). This region was unstained in control sections, whereas some staining of chondrocytes was frequently seen in control (Fig. 2, opposite) as well as in antibody-treated sections. The zone surrounding chondrocyte clusters, beyond the stained rim, was often, but not always, seen to be unstained as in Fig. 1. This contrasts strongly with the alcian blue stained section (Fig. 3, opposite) of costal cartilage taken from the same small block of tissue and cut and prepared in the same way as the fluorescent section. Here the circum-lacunar space is strongly stained, in contrast to the next zone, known as the interterritorial region. This showed stippled staining with antibody, whilst being virtually unstained by alcian blue. This contrasted staining was, however, not so well marked in other types of cartilage or even in all parts of sections of costal cartilage examined. The effect of pre-treatment of sections with hyaluronidase (Seravac Laboratories, Ltd.) also showed different results when examined by antibody and alcian blue-staining. Treatment for 2 hrs caused marked generalized increase of fluorescent staining, while a regional staining distribution could still be recognized. Some alcian blue staining was retained in the lacunar regions. After 24 hrs, however, alcian blue was not taken up, while fluorescence was very bright and generalized. These results are illustrated in Figs 4 and 5 (opposite) and Figs 6 and 7 (overleaf).

Fig. 1.—Fresh-frozen section of pig costal cartilage, treated with rabbit antibody to pig CMP, followed by fluorescein-conjugated goat anti-rabbit globulin. $\times 160$.

Fig. 2.—As Fig. 1, but treated with rabbit antibody to dextran. $\times 160$.

Fig. 3.—As Fig. 1, stained with alcian blue, 0.5 M MgCl_2 . $\times 84$.

Fig. 4.—Fresh-frozen section of pig costal cartilage, treated with hyaluronidase for 2 hrs followed by CMP antibody and conjugate. $\times 200$ approx.

Fig. 5.—As Fig. 4, stained with alcian blue, 0.5 M MgCl_2 . $\times 100$ approx.

For control purposes, some sections were incubated in saline for 24 hrs; this did not affect alcian blue staining and only slightly increased the intensity of fluorescent staining. Hyaluronidase treatment did not cause any non-specific uptake of fluorescent

conjugate in control sections treated with rabbit anti-dextran or other rabbit antisera.

Articular Cartilage.—Articular cartilage showed specific lacunar rim staining by antibody, as did

Fig. 6.—Fresh-frozen section of pig costal cartilage, treated with hyaluronidase for 24 hrs followed by CMP antibody and conjugate. $\times 200$ approx.

Fig. 7.—As Fig. 6, stained with alcian blue, 0.5 M $MgCl_2$. $\times 100$ approx.

costal cartilage (Fig. 8). This was particularly strongly demonstrated in the deeper layers adjoining bone (Fig. 9). The distinction between the circum-lacunar spaces and the interterritorial regions was not marked, although it could be made out here and

there in sections. A line of staining on the surface of the cartilage was usually seen (Fig. 8). Such a line could occasionally be seen in control sections (Fig. 10), though there it was less strong.

Alcian blue (Fig. 11, opposite) showed a lacunar

Fig. 8.—Fresh-frozen section of pig articular cartilage from ankle joint, treated with CMP antibody and conjugate. $\times 160$.

Fig. 9.—Same section as Fig. 8, another field, bordering underlying bone. $\times 160$.

Fig. 10.—As Fig. 8, but treated with rabbit anti-dextran serum. $\times 160$.

Fig. 11.—Fresh-frozen section of pig articular cartilage, stained with alcian blue, 0.5 M MgCl_2 .
 $\times 125$.

rim accentuation, but there was, except in the deeper layers, considerable interterritorial staining also. In the deeper layers, interterritorial regions were only slightly stained. Hyaluronidase treatment led to great generalized increase in fluorescent staining as with costal cartilage, while abolishing alcian blue uptake (Figs 12 and 13).

Fig. 12.—Fresh-frozen section of pig articular cartilage, treated with hyaluronidase for 24 hrs, followed by CMP antiserum and conjugate.
 $\times 240$.

Fig. 13.—As Fig. 12, stained with alcian blue, 0.5 M MgCl_2 .
 $\times 125$.

Tracheal Cartilage.—In addition to lacunar rim staining, there was widespread stippled fluorescence of cartilage matrix with only indistinct territorial distribution (Fig. 14). A control section is shown in Fig. 15. Alcian blue showed fairly uniform staining of matrix (Fig. 16).

Embryonic Cartilage.—Sections taken from a femur showed bright fluorescence of lacunar rims as well as fairly uniform stippled staining of surrounding matrix (Fig. 17). Alcian blue showed fairly heavy uptake by the cartilage matrix without much regional accentuation (Fig. 18).

Fig. 14.—Pig tracheal cartilage, treated with CMP antiserum and conjugate. $\times 160$.

Fig. 15.—As Fig. 14, control section, treated with antiserum to dextran. $\times 160$.

Fig. 16.—As Fig. 14, stained with alcian blue, 0.5 M $MgCl_2$. $\times 84$.

Fig. 17.—Cold-ethanol fixed section of pig embryo cartilage, treated with CMP antiserum and conjugate. $\times 240$.

Fig. 18.—As Fig. 17, stained with alcian blue, 0.5 M $MgCl_2$. $\times 125$.

Other Tissues.—Amongst other tissues in which CMP could be demonstrated by the fluorescent antibody method was aorta. Interfibrillar material of the aortic media, as well as the intima, showed fluorescent staining (Fig. 19). A control section is shown in Fig. 20. Chemical analysis of aorta has shown the presence of considerable amounts of acid mucopolysaccharides, some or all of which are probably in protein combination (Meyer, 1964).

Controls.—Apart from sections treated with unrelated rabbit antisera, which served as controls on every occasion on which fluorescent staining was carried out, experiments were performed in which the antibody had been absorbed. Such absorption with CMP led to complete absence of fluorescent staining. Absorption of anti-serum with pig serum or with collagen, however, did not affect the fluorescent staining of cartilage or other tissues. Fluorescent conjugates other than those directed against the antibody in the sandwich also failed to stain. The specificity of antibodies to CMP is further described in an earlier paper (Loewi and Muir, 1965).

Discussion

The results show that tissues rich in CMP are capable of being stained by antibody to CMP, using the fluorescent method. The antibody used in the present work was made against CMP from pig trachea, the acid polysaccharide constituent of which was predominantly chondroitin sulphate (Muir, 1958). We have previously presented evidence (Loewi and Muir, 1965) showing that this antibody is directed against the protein constituent of CMP. This is presumably also the site of reaction in tissue sections. We have, however, at present no evidence as to the possible reaction of antibody with proteins bound to other acid polysaccharides, such as keratosulphate. The alcian blue technique, as described by Scott and Dorling (1965), allows the differentiation of acid polysaccharides from other negatively charged materials and the distinction between various acid polysaccharides by the use of different salt concentrations. In the experiments reported here, 0.5 M $MgCl_2$ has been used. At this molarity, only chondroitin sulphate,

Fig. 19.—Cold ethanol-fixed section of pig aorta, treated with CMP antiserum and conjugate. $\times 240$.

Fig. 20.—Control to Fig. 19, treated with anti-dextran and conjugate. $\times 240$.

keratosulphate, and heparin are stained. Thus, combining the fluorescent and alcian blue techniques, it seems possible that both the protein and polysaccharide of CMP would be demonstrable. As shown in the accompanying illustrations, in many sections of cartilage, there was some degree of inverse relationship between the results of staining by the two methods. The interterritorial region showed fluorescent staining, but little alcian blue uptake while there was often a peri-lacunar zone which showed the reverse relationship. The effect of hyaluronidase was to cause widespread increase of fluorescent staining while alcian blue uptake was abolished. The reactivity of antibody with CMP is greatly increased by prior hyaluronidase treatment of CMP; this is presumably due to degradation and removal of polysaccharide and freeing of reactive sites on the protein moiety of CMP (Loewi and Muir, 1965). This explanation can also account for increased staining of sections by fluorescence, while polysaccharide loss has led to diminution or lack of alcian blue uptake. The different results with the two methods in the peri-lacunar and interterritorial regions could be explained on a similar basis: a relatively polysaccharide-rich and protein-poor complex, or at least one with polysaccharide groups covering protein combining sites might be present in the peri-lacunar region, while a reverse relationship might apply in the interterritorial region. On the other hand, it must be remembered that polysaccharides have excluded volume (Laurent and Ogston, 1963) which would lead to failure to penetrate, especially by a molecule of the size of γ -globulin. Thus, a region such as the peri-lacunar one which is stained heavily by alcian blue might be relatively unstained by the fluorescent technique because the antibody might fail to penetrate (Scott, 1965). It was thought that treatment of cartilage, especially fixation, would have an effect on staining, and nearly all the work reported here was therefore carried out both on alcohol-fixed and fresh-frozen material. In general, there was found to be no difference in the results obtained on the two types of sections by either staining method, with the exception that the effect of hyaluronidase was more marked on fresh-frozen than on fixed tissue. It was also noted that cold formaldehyde-ethanol-fixed material showed a rather more even distribution and deeper staining by alcian blue; such fixation, however, was unsuitable for the fluorescent antibody technique.

Summary

Antibody to porcine cartilage chondromucoprotein has been used to stain sections of costal,

articular, and tracheal cartilage by the fluorescent antibody technique. The results have been compared with alcian blue uptake under controlled conditions.

The presence of chondromucoprotein was shown in the lacunar rim. In the surrounding matrix, fluorescent staining was less marked, but was particularly associated with the interterritorial regions. Alcian blue staining gave, in some instances, the reverse of the fluorescent staining pattern, in that there was strong staining of a fairly wide peri-lacunar zone, but very little uptake in interterritorial regions.

Treatment of sections with hyaluronidase greatly increased fluorescent antibody staining both in intensity and extent. At the same time alcian blue uptake was abolished. The action of hyaluronidase was most marked on unfixed sections.

The hyaluronidase effect may be attributed to degradation and removal of polysaccharide providing for increased access of antibody to the protein moiety of chondromucoprotein. Regional staining differences could be due to different composition of the chondromucoprotein polymer in the various territories, but could also be accounted for by the property of excluded volume.

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Localisation de la chondromucoprotéine dans le cartilage

RÉSUMÉ

On s'est servi de l'anticorps contre la chondromucoprotéine du cartilage du porc pour colorer des coupes du cartilage costal, articulaire et trachéal par le procédé de fluorescence. On a comparé les résultats avec ceux obtenus avec l'absorption du bleu d'alcian dans des conditions déterminées.

On a démontré la présence de la chondromucoprotéine dans le rebord lacunaire. Dans la matrice environnante la coloration fluorescente était moins marquée, mais elle se trouvait associée tout particulièrement avec des régions interterritoriales. Le bleu d'alcian donnait

quelquefois une image opposée à celle produite par la méthode de fluorescence: une coloration forte et assez étendue de la zone périlacunaire, mais très faible dans les régions interterritoriales.

Lorsqu'on traitait les coupes par l'hyaluronidase, la coloration fluorescente de l'anticorps devenait plus intense et plus étendue, tandis que l'absorption du bleu d'alcan cessait. L'action de l'hyaluronidase était plus accentuée sur des coupes non fixées.

L'effet de l'hyaluronidase peut être attribué à la dégradation et l'enlèvement du polysaccharide, ce qui facilite l'accès de l'anticorps à la portion protéique de la chondromucoprotéine. Les différences régionales de coloration pourraient être dues à une composition différente du polymère chondromucoprotéique en territoires différents ou bien à la propriété du volume exclu.

Localización de la condromucoproteína en el cartílago

SUMARIO

El anticuerpo contra la condromucoproteína del cartílago porcino fué empleado para colorar cortes del

cartílago costal, articular y traqueal por el procedimiento de fluorescencia. Se compararon los resultados con los obtenidos con la absorción del azul alciano en condiciones determinadas.

Se demostró la presencia de la condromucoproteína en el borde lacunar. En la cercana matriz la coloración fluorescente fué menos marcada, pero se vió asociada muy particularmente con regiones interterritoriales. El azul alciano daba a veces un cuadro opuesto al producido por el método de fluorescencia: una coloración fuerte y bastante extensa, de la zona perilacunar pero muy débil en las regiones inter-territoriales.

Al tratar los cortes con hialuronidasa, la coloración fluorescente del anticuerpo se volvía más intensa y más amplia, mientras que la absorción del azul alciano cesaba. La acción de la hialuronidasa fué más marcada en cortes sin fijar.

El efecto de la hialuronidasa puede atribuirse a la degradación y a la eliminación del polisaccharido, facilitando así el acceso del anticuerpo a la porción proteínica de la condromucoproteína. Las diferencias regionales de coloración pudieran deberse a una composición diferente del polímero condromucoprotéico en varios territorios o, quizás, a la propiedad del volumen excluido.