Technical Note

Characterization of Hyaluronic Acid on Tissue Sections with Hyaluronectin¹,²

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An affinity immunological procedure for hyaluronic acid detection on tissue sections is described. This new, sensitive, and specific technique is based on the high affinity of hyaluronectin for hyaluronic acid, utilizing anti-hyaluronectin–hyaluronectin immune complexes. Elimination of binding when the reagent was supplemented with hyaluronic acid or when Streptomyces hyaluronidase-digested tissues were used emphasizes the specificity of the assay. This technique made possible accurate HA localization in embryonic mesenchyme, in neural tissue, in kidney medulla, and in tumors.

KEY WORDS: Hyaluronic acid; Hyaluronectin; Affinity; Immuno-histochemistry.

Introduction

Glycosaminoglycans (GAGs) are an essential component of the extracellular substance in several normal, embryonic, or tumor tissues. Available techniques to detect hyaluronic acid (HA) and other GAGs usually require dissociative extraction of tissues (1,13). For histochemistry, Alcian blue staining is generally used, in combination with critical electrolyte conditions at a definite pH (6,8,11,15). A recent paper suggests that addition of cetylpyridinium chloride to the fixative enhances the staining with Alcian blue or colloidal iron (10). Although all these methods produce a relatively specific staining of all GAGs (6,15), they do not distinguish HA from the other GAGs when they are juxtaposed. The purpose of our study was to develop a histochemical procedure specific for HA, using a hyaluronic acid-binding protein, hyaluronectin (HN). The high specific affinity of this glycoprotein for HA (3,4) makes it an excellent choice for histochemical determinations.

Materials and Methods

Tissues were fixed either in glacial acetic acid–absolute ethanol (2:98, v/v) or in formalin before embedding in paraffin. Tissue samples were also frozen in liquid nitrogen and stored at −76°C. Sections 5 μm thick were used.

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Hyaluronectin (HN) was extracted from human brain and purified on a column of HA coupled to AH Sepharose (Pharmacia Fine Chemical, Uppsala) with carbodiimide (2,16). Anti-HN antibodies were raised in rabbits and purified by immunoadsorption on HN coupled to AH Sepharose by glutaraldehyde (4). The concentration was estimated by the absorbance at 280 nm.

Human umbilical cord HA (grade I) was purchased from Sigma Chemicals (St. Louis, MO). It was dissolved in water and heated at 100°C for 40 min in order to destroy residual proteins, especially HN bound to HA.

Soluble immune complexes were obtained by mixing anti-HN antibodies with purified HN in an adequate ratio, determined by enzymimunological titration. The assay was performed in 96-well plates, each well being coated with an HA solution (4 μg/ml in 0.1 M bicarbonate, pH 8.5) overnight at 37°C. Antibodies (20 μg/ml) were absorbed by adding an equal volume of increasing amounts of HN from 1 μg/ml to 40 μg/ml in PBS (pH 7.4, 0.01 M phosphate-buffered saline with 8 g/liter sodium chloride) containing 5% bovine albumin. These reagents were incubated in tubes overnight at 37°C. Plates were then washed with Tween 20 (0.05% in PBS) and the pre-incubated anti-HN–HN complexes were distributed into the wells for 8 hr at 37°C in order to measure the residual antibody activity. After washing, peroxidase-labeled anti-rabbit immunoglobulin antibodies (Inst. Pasteur, Paris) were added overnight at 37°C. The peroxidase activity was then detected using azin-diethylbenzthiazoline sulphonate (ABTS, Boehringer–Mannheim, France) as the chromogen in the presence of 1/5000 H2O2, and the optical density was read at 405 nm. A titration curve was employed to determine the equivalence point. The concentration of HN used in further experiments was slightly greater than the equivalence point to obtain soluble complexes absolutely devoid of antibody activity and providing an efficient reagent for HA detection.

Hyaluronic acid was characterized on tissue sections using the following double-step affinity-immunofluorescence assay: the first layer reagent was soluble rabbit anti-HN–HN immune complexes (IC), the second layer was fluorescent anti-rabbit immunoglobulin antiserum. IC containing 40 μg anti-HN antibodies and 60 μg HN per ml were incubated on tissue sec-

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Residual anti-HN activity after incubation with increasing amounts of HN. Equivalence point (arrow) was obtained at 10 pg/ml antibodies and 10 pg/ml antigen (final concentrations). At concentrations of HN below this point, free antibodies were still present. Soluble immune complexes were prepared in the ratio of 10 μg anti-HN:15 μg HN for HA detection.

Results

The titration curve (Figure 1) showed that the anti-HN activity decreased with the amount of HN added to the antibodies. Complete absorption was obtained from a ratio of 10 μg anti-HN:10 μg HN. This point corresponded to the equivalence zone. When HN concentration was increased beyond this ratio, soluble immune complexes which could bind to tissue HA appeared. From these data, we concluded that the most efficient reagent for HA detection would be soluble immune complexes prepared in slight excess of antigen, in the ratio of 10 μg anti-HN:15 μg HN. For histological assay concentrations, 40 μg/ml anti-HN and 60 μg/ml HN gave the best results.

Such IC were used for HA detection on various normal tissues. Results on rat cerebellum sections were previously described (7). A dense granular pattern was observed on the white matter and the granular layer. The interneuronal substance in the grey nuclei...
was also labeled, and the perineuronal staining seemed like thick coronas. On peripheral nerve sections, fine connective tissue between the fibers was stained. In kidney, HA was detected in the connective tissue surrounding interstitial cells in the medulla (Figure 2A).

This procedure was also performed on tumor sections. In skin basocellular carcinoma, HA was detected in normal dermis, in carcinomatous nodules, and small deposits were located in the intercellular substance of the stratum spinosum. In a typical epithelial mesothelioma, HA was associated with the connective stroma reaction. It was also detected along the mesothelial borders and inside the epithelial clusters (Figure 2B).

Experiments performed in rat embryo confirmed HA abundance in the whole undifferentiated mesenchymal tissue, with significant staining between migrating sclerotomal cells.

In all cases, incubations with anti-HN–HN–HA abolished the staining, whereas complexes with other GAGs did not block the fixation, thus demonstrating the HA specificity of IC binding. In addition, digestion of tissue with hyaluronidase eliminated fluorescence on tissue sections.

Discussion

The new immunohistochemical technique described here is based on the high specific affinity of HN for HA. Its principle makes it similar to a technique published very recently, using cartilage proteoglycan core protein as a probe for HA (9). Our purpose was to detect only HA with anti-HN–HN immune complexes and to have a reagent devoid of antibody activity so that tissue HN could not be detected. This condition was realized with IC prepared in slight excess of antigen. A too large proportion of antigen would hinder the assay, because free HN would link to tissue HA and would not be detected by FITC conjugate. Negative reaction with IC supplemented with HA both verified that there were no residual free anti-HN antibodies in the reagent and demonstrated the specificity of the IC affinity for HA. The blocking reaction with HA also enables us to assert that HN bound strictly to HA and not to other carbohydrate residues. Specificity for HA is confirmed by two other results: 1) other GAGs did not modify IC fixation; and 2) elimination of staining when tissue sections were incubated with Streptomyces hyaluronidase, whose activity is known to be restricted to HA and to have no chondroitinase activity. Although the combination of Streptomyces hyaluronidase digestion and Alcian blue staining also provides specific HA detection, it may be inaccurate when other GAGs are juxtaposed. The technique described in this paper gives positive staining, providing clear-cut indication of the presence of HA.

Our results are in agreement with the findings of others: HA is widely distributed in the interstitial spaces of most organs. It is localized mainly in mesenchyme from early embryonic stages (12), in adult loose connective tissue (14) and ground substance of brain (5), and also in tumors, particularly in pleural mesotheliomas (17).

In conclusion, the affinity–immunohistochemical technique described here introduces a specific tool for histological localization of HA. Its sensitivity provides an improvement with regard to other classical stainings. It should prove useful in studies of cancer invasiveness and of embryonic processes, since HA is involved in cell migration and tissue differentiation.

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Literature Cited