Interference with Carcinoembryonic Antigen Radioimmunoassays by Glycosaminoglycans, and Their Removal

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Using the reagents from the CEA-Roche kit, we found that solutions containing only glycosaminoglycans (GAG) yielded false concentrations of carcinoembryonic antigen (CEA), and mixtures of CEA and GAG produced falsely high values. On the other hand, solutions of GAG yielded no additional CEA concentration when reagents from the Abbott CEA kit were used; rather, the CEA result was decreased with the Abbott kit for a CEA solution also containing GAG. These effects of GAG were not ascribable to contamination, because neither gel filtration nor ion-exchange column chromatography separated the false Roche CEA content related to GAG from their peaks of uronic acid or from their anticoagulant activity. In addition, an enzyme specific for GAG eliminated these GAG-related false concentrations. Both the positive and negative effects are correlated with concentrations of GAG. We found that the concentration of GAG could be decreased in a solution containing plasma proteins by either heating (70°C, 15 min) or treating with perchloric acid (0.6 mol/L). The former is superior because essentially all GAG added to a solution containing plasma proteins were removed by heat, whereas as much as 25% to 80% of the GAG still remained after acid treatment. The effect of GAG was also completely eliminated by treating the specimens with chondroitin ABC lyase (EC 4.2.2.4).

Radioimmunoassays for carcinoembryonic antigen (CEA) are used as an adjunctive diagnostic test for certain malignant diseases, especially carcinoma of the human digestive system (1, 2); however, the test is nonspecific (2). One way to improve its specificity is to identify the interfering substances in the blood and then study the nature of their inhibitory effect. Earlier, we found that heparin inhibits CEA and anti-CEA antibody binding and in this way interferes with CEA assay (3). Conceivably, other glycosaminoglycans (GAG) such as the chondroitin sulfates and hyaluronic acid may behave similarly. Although heparin is only rarely detectable in blood, other GAG are found in the plasma and physiological fluids of normal persons in much higher concentrations (4–6), and in increased concentrations in the plasma of patients with various mucopolysaccharidoses, rheumatoid arthritis, and mental disorders (7, 8). Therefore, an understanding of the nature of GAG interference and of the methods for their elimination would be helpful in improving the specificity of CEA assays. Such an understanding would also help us understand why CEA results so often differ when the same specimen is measured by two different commercial assays kits for CEA (9–11). In this communication we evaluate GAG interference with CEA as measured with two CEA kits and describe how GAG can effectively be removed from plasma samples.

Materials and Methods

Materials

Hyaluronic acid, grade III, from human umbilical cord; chondroitin sulfate, grade III, from whale cartilage; chondroitin ABC lyase (chondroitinase, EC 4.2.2.4); and the kit used for the measurement of anticoagulant activity (12) were all purchased from Sigma Chemical Co., St. Louis, MO 63178. Noncommercial dermatan sulfate and heparan sulfate were obtained from Dr. A. Linker, Department of Pathology, University of Utah School of Medicine. Dextran sulfate, Sepharose 6B, and concanavalin A–Sepharose 4B were from Pharmacia Co., Piscataway, NJ 08854. Celer D and Biogel A-5M were purchased from BioRad Laboratories, Richmond, CA 94804. To measure the concentration of CEA, we used two commercial kits: Abbott CEA-RIA (Abbott Laboratories, North Chicago, IL 60064) and CEA-Roche Kit (Roche Diagnostics, Nutley, NJ 07110). All chemicals used in this study were of analytical grade.

Procedures

Chromatography on diethylaminoethylcellulose. The procedure described by Hallen (13) was adopted with a slight modification. A 1.5 × 20 cm Pharmacia column packed with Cellex D was used. Elution was at room temperature with a linear gradient of lithium chloride (100 mL of 0.2 mol/L LiCl and 100 mL of 3 mol/L LiCl) in a 50 mmol/L sodium acetate buffer solution, pH 4. Samples were dialyzed against the sodium acetate buffer before being applied to the column. The eluate was dialyzed against water before being used for other determinations.

Methods for gel filtration chromatography and chromatography on Sepharose 4B have been described (3). Uronic acid was measured by the m-hydroxydiphenyl method of Blumenkrantz and Asboe-Hansen (14). Anticoagulant activity was evaluated with a Sigma “Heparin in Plasma” kit (12).

Enzyme treatment. To test whether chondroitin ABC lyase is also capable of removing chondroitin sulfates from solutions containing plasma proteins, we used the following conditions. The incubation mixture, total volume 1 mL, contained 0.5 mL of normal human plasma that had been dialyzed against water, 100 µg (20 µL) of chondroitin sulfate, 20 ng (160 µL) of Roche CEA standard, 2 units (40 µL) of chondroitin ABC lyase, and 10 µL of Tris · HCl buffer (pH 7.8), the last added to adjust the final volume. For the various controls, any substance not included was replaced by buffer. The same buffer was also used to prepare enzyme and chondroitin sulfate solutions. After a 2-h incubation at 37°C, 0.1-mL aliquots were withdrawn and mixed with 0.1 mL of 0.2 mol/L sodium acetate buffer, pH 5, before analysis by the Abbott CEA assay. Other 0.25-mL aliquots, after dialysis against water, were used for the Roche CEA assay (15).

Heat and acid treatments. To find out how much GAG could be removed by either perchloric acid (final concentration 0.6 mol/L) or heat (70°C, 15 min) treatment, we used
the following procedure. The solutions contained 1 mL of normal human plasma that had been dialyzed against water, and various amounts of GAG in 10 mmol/L phosphate buffer, pH 7.4; the final volume was 2 mL. Aliquots were mixed with an equal volume of 0.1 mol/L sodium acetate buffer, pH 5, before heat treatment, or with an equal volume of 1.2 mol/L perchloric acid for acid treatment.

Results

CEA Measurement in Solutions Containing GAG Only

As in our previous findings with heparin (3), all GAG produced false CEA measurements with the Roche kit, whereas no CEA could be detected with the Abbott kit (Table 1). Before the Roche CEA measurements, all GAG solutions were dialyzed against water, so that the false CEA results shown in Table 1 were truly derived from interference by GAG and not by the presence of any salt in the solution (15). Dextran sulfate, a synthetic compound with properties similar to GAG, also exhibits strong positive interference. The false concentration of CEA produced by dextran sulfate presumably is related to its degree of sulfation, which varies with different preparations.

Figure 1 illustrates the relationship between the false presence of CEA and the different amounts of the various GAG. The false CEA is proportional to the amount of GAG, but not linearly. Straight lines were produced only for a semilogarithmic plot. Figure 1 also provides some idea of the effectiveness of various GAG in inhibiting the binding between CEA and anti-CEA. Dextran sulfate appears to be the most inhibitory, heparan sulfate the least.

Positive and Negative Effects of GAG in the Presence of CEA

The effect of GAG on CEA measurements in the presence of CEA is also similar to that found earlier with heparin (3). As illustrated in Figure 2B, the Abbott CEA result for a sample containing 8.5 ng of CEA per milliliter gradually decreases with increasing concentrations of chondroitin sulfate or hyaluronic acid. This negative effect is apparently concentration dependent. Figure 2A illustrates the negative effect on results with the Abbott kit and the positive effect with the Roche kit. When the Abbott kit was used, 100 µg of chondroitin sulfate decreased the apparent CEA concentration from 9 to 4.5 ng/mL, although 100 µg of chondroitin sulfate by itself produced almost no apparent CEA with the Abbott kit. On the other hand, with the Roche kit, the same amount of chondroitin sulfate increased the apparent CEA concentration from 24 to 43 ng/mL.

The Possibility of Contaminants in GAG Preparations

Chromatography of the various GAG on columns containing either Sepharose 6B or diethylaminoethylcellulose ruled out the possibility that the false CEA concentration ob-

Table 1. Effect of GAG on Apparent CEA as Measured with the Roche and Abbott Kits

<table>
<thead>
<tr>
<th>Type of GAG</th>
<th>GAG, µg/assay</th>
<th>Measured CEA, ng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin sulfate</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>100</td>
<td>0.1</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>500</td>
<td>0.15</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>10</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Fig. 1. Correlation of false CEA with GAG (Roche kit)

Aliquots of different GAG solutions (1 mg/mL) were mixed with 2 mL of water and dialyzed overnight against water. After transferring the dialysate to a test tube, 0.5 mL of 0.1 mol/L ammonium acetate buffer, pH 6.8, and additional water were added to each tube to adjust the volume to 5 mL, before the addition of anti-CEA. The CEA standard curve was also prepared in 10 mmol/L ammonium acetate buffer, pH 6.8. GAG solutions used include heparan sulfate (●), chondroitin sulfate ABC from Sigma (○), hyaluronic acid (△), and dextran sulfate (△). The amount of GAG used per individual assay is plotted against the nanograms of apparent CEA measured.

Fig. 2. Positive and negative effects of GAG on Roche and Abbott CEA assays

A: Bars indicate the amount of Roche or Abbott CEA before and after additions of chondroitin sulfates (CS) to CEA, and before and after enzyme treatments (E, chondroitinase ABC). The composition of the individual incubation mixtures is listed above each bar. B: 9 ng of Roche CEA standard was mixed with various amounts of chondroitin sulfates (CS, ○) or hyaluronic acid (HA, ●) in a final volume of 1 mL. Sodium acetate buffer, 0.1 mol/L, pH 5, was used to adjust the volume and to dissolve CS and HA.
served with the Roche kit was caused by contaminants (Figures 3 and 4). As in our findings with heparin, none of the peaks of false Roche kit CEA ascribable to GAG could be clearly separated from the peaks of anticoagulant activity or from the uronic acid peak characteristic of these GAG preparations. The chromatographic procedure used in Figure 4 is a method favored for separating and identifying GAG (13); most other biological molecules are not retained by the column at pH 4. Therefore, the association of the false Roche kit concentration of CEA with both the anticoagulant activity and the uronic acid concentration during GAG chromatography strongly supports the idea that these properties are derived from the same molecule.

In addition, as Figure 5 shows, both the CEA standard of the Roche kit and CEA isolated from colon tumor appear at the void volume of the diethylaminoethylcellulose column, well separated from the false CEA peaks associated with the GAG. On the other hand, all the GAG tested in Figure 4 appeared very close to the position of $^{125}$I-labeled CEA in the elution profile, even though these molecules are much smaller than the CEA molecule.

**Removal of GAG from Plasma**

*By heat and acid treatments.* Before CEA is determined in plasma, most of the plasma proteins, which interfere, must be removed. The methods used in the Abbott and Roche procedures are heat (70 °C for 15 min) and perchloric acid (0.6 mol/L) treatments, respectively. We found that, in solutions containing GAG only, concentrations of uronic acid were unaffected by either treatment. However, when plasma proteins were present, both heat and perchloric acid could decrease the concentrations of the GAG.

In our initial experiments, we monitored the concentrations of GAG by use of a modified carbazole reaction (16), but the high background color produced by this reaction in plasma samples prevents accurate assessment of the uronic concentrations either before or after treatment. For this reason, we used the more specific m-hydroxydiphenyl method (14). CEA in the solution after either heat or acid treatment was determined by the Roche procedure. The amount of GAG remaining after each treatment was indicated both by the concentration of uronic acid and by the Roche kit apparent CEA concentration (the false CEA content could, of course, serve only as a qualitative indicator). Very little GAG remained after the heat treatment (Figure 6A), indicating that the GAG was effectively precip-
chondroitin sulfates, to treat the solution containing the plasma proteins, the Roche CEA standard, and 100 μg of chondroitin sulfates. After incubation with this enzyme and without further treatment by either heat or acid, aliquots were drawn for use in both the Abbott and the Roche CEA assays. Because the normal human plasma selected contained very little CEA (the plasma had also been dialyzed against water to remove all salts before the experiment), the amount of normal human plasma contained in the aliquot yielded a negligible background of CEA in both assays (Figure 2A). The results clearly indicate that, under the conditions we used, the chondroitin sulfates in the solution were effectively removed by chondroitin ABC lyase. The results also confirm our earlier observations concerning the negative and positive effects of GAG in the presence of CEA when monitored by either the Abbott or Roche assays. As Figure 2 shows, when the Abbott kit was used, the apparent concentration of the CEA standard decreased when the chondroitin sulfate was added. The same amount of chondroitin sulfate alone or in a mixture with the enzyme showed no appreciable apparent CEA content. Most interesting was the full analytical recovery of CEA concentration, devoid of any negative effect of chondroitin sulfates, in the mixture of CEA and chondroitin sulfate after the enzyme treatment. On the other hand, both the chondroitin sulfate solution and the chondroitin sulfates added to plasma solution produced falsely high CEA values with the Roche kit. Both of these false CEA results could be eliminated by enzyme treatment. Moreover, because there was no loss in CEA concentration (with use of the Roche CEA standard) in either the Roche or the Abbott assay after enzyme treatment, we conclude that it is the GAG that produce the observed positive and negative effects on CEA assays.

**Discussion**

GAG are widely distributed in human physiological fluids and tissues (4–6, 17). Normal plasma contains as much as 5 to 10 μg of GAG per milliliter (5–8, 18, 19), a concentration that may contribute from 1 to 2 ng of false CEA per milliliter with the Roche procedure, depending on the type of GAG involved. Urine from normal adults contains nearly 10 mg of GAG per 24-h urine (4), with greater amounts in urine from younger persons. Moreover, GAG concentrations increase in physiological fluids and tissues in several diseases (6, 8, 18, 19).
The false CEA concentration produced by GAG probably is related to their acidic properties. For example, dextran sulfate, a synthetic anticoagulant, produces the greatest false CEA activity (Roche kit) per unit weight and also requires the greatest salt concentration to elute it from the column (Figure 5). At pH 4, very few major plasma proteins carry a negative charge and are retained by the diethyl aminooethylcellulose column. The association of a low isoelectric point for a molecule with its false Roche CEA activity raises the question that perhaps other, non-GAG molecules in physiological fluids, because of their acidic nature, may also interfere with the binding between CEA and anti-CEA. Such molecules have not yet been detected, but various mucous substances and proteoglycans are probably good candidates (20).

The only two commercially available kits for CEA assay are well known not to produce similar results for the same specimens (9–11). Although some of these differences are related to variations in the preparations of CEA and anti-CEA, to the ionic strength and pH of their assay conditions, and to sample preparation, they may also be related to the effects of the GAG on CEA determination (Figure 2). The much higher concentration of CEA measured by the Roche direct procedure (21) may also be caused, at least in part, by GAG interference. Until a simpler and more economical way is found to remove GAG specifically from test specimens before CEA assay, we recommend the heat treatment used in the Abbott method. Such treatment should result in a substantially improved correlation between the two methods.

Supported by the Development Fund of the Department of Pathology, University of Utah School of Medicine.

References