DIRECT EXTRACTION AND CHARACTERIZATION OF ANIMAL COLLAGENASE FROM HUMAN RADICULAR CYSTS

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ABSTRACT

High animal collagenase (EC 3.4.24.7) activity was detected together with gelatinolytic activity directly in human radicular cyst fluids and in 4 M urea extracts of cyst wall tissues. Both cyst wall and cyst fluid collagenases were essentially in latent form and were most effectively activated by p-aminophenylmercuric acetate (APMA). Both APMA-activated collagenases broke down type I collagen preferentially to type III collagen resembling these of polymorphonuclear leukocyte origin.

We now show that a high level of animal collagenase activity can be extracted directly from both radicular cyst walls and cyst fluids and that these collagenases break down type I collagen preferentially to type III.

MATERIALS AND METHODS

Chemicals

[U-¹⁴C]Glycine (10 mCi/mmol) was purchased from Radiochemical Centre, Amersham (Bucks, England). Pepsin was supplied by Worthington Biochemical (Freehold, NJ, U.S.A.); p-aminophenylmercuric acetate (APMA) was from Tokyo Kasei Kogyo (Tokyo, Japan). Ultrogel AcA 44 and 54 were from LKB-Produkter AB (Bromma, Sweden); trypsin (EC 3.4.21.4), from Boehringer-Mannheim GmbH (Mannheim, F.R.G.); soybean trypsin inhibitor from Sigma Chemical (St. Louis, MO, U.S.A.); sodium iodide from Yoneyama Yakuhin Kogyo (Osaka, Japan); and X-ray film (X-Omat AR), fixer, and developer, from Kodak (Rochester, NY, U.S.A.).

Specimens

Specimens of human radicular cysts were obtained during surgical operation and were
stored in a freezer (−20°C) until use. We eliminated all questionable cyst samples such as those containing turbid fluid, which is a good indication of the recurrence of subacute or acute inflammation.

**Direct Extraction of Enzymes**

Collagenase and gelatinolytic activity was extracted from the radicular cyst walls directly with 15 vol of 4 M urea in 30 mM Tris-HCl buffer, pH 7.8, containing 0.2 M NaCl and 5 mM CaCl₂ (buffer A), as reported previously (10). The urea extracts were dialyzed overnight against buffer A and then centrifuged at 10,000 g for 30 min to remove insoluble protein.

**Partial Purification of Collagenase**

Collagenase was purified by gel filtration from pooled radicular cyst fluid and the urea extract of cyst walls. Ten ml (86 mg protein) of the urea extract of cyst walls and 4 ml (117 mg protein) of the cyst fluid were applied to an Ultrogel AcA 44 column (1.6×9.3 cm) and Ultrogel AcA 54 column (1.6×90 cm), respectively, equilibrated with buffer A. In the case of cyst fluid, all fractions containing collagenase activity were combined, concentrated with Sephadex powder, and reapplied to the same column.

**Preparation of Type I and Type III Collagens**

Dental pulps from unerupted bovine teeth were solubilized by limited pepsin digestion, and then collagens were purified according to the procedure of Epstein (2).

**Assay Procedures**

Collagenase activity was determined by the method of Terato et al. (16), using [¹⁴C]glycine-labelled collagen (2,130 cpm/0.15 ml/tube) as a substrate in a 20-h incubation at 35°C. One unit (U) of collagenase was defined as the activity which hydrolyzed 1 μg of native soluble collagen per min.

Gelatinolytic activity was measured at 35°C by the method of Harris et al. (4), using [¹⁴C]glycine-labelled gelatin (2,130 cpm/0.15 ml/tube) as a substrate. [¹⁴C]Collagen was heated at 60°C for 30 min to form [¹⁴C]gelatin. One unit (U) of gelatinolytic activity was defined as the activity which degraded 1 μg of denatured collagen per min.

Activation of latent-type enzymes was achieved by APMA (12), NaI (14), or trypsin (19).

**Electrophoresis and Fluorography**

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn (21) or Laemmli (11), using 6% acrylamide gel after reduction of samples with 10% β-mercaptoethanol.

Interrupted gel electrophoresis was carried out essentially by the method of Sykes et al. (15) with some modifications (6).

Fluorography of SDS-polyacrylamide gel electrophoretograms was carried out by the method of Chamberlain (1). [¹⁴C]Collagen (31.6 μg, 570 cpm) was incubated at 20°C for 6 days with APMA-activated, partially purified collagenase (27 μU of cyst wall and 60 μU of cyst fluid enzyme) in buffer A containing 0.3 M glucose, and the digest was then electrophoresed on an SDS-polyacrylamide slab gel. X-Omat X-ray film was exposed to the dried gel for 7 days at −70°C.

**Preparation of Cyst Wall Sections**

Part of every cyst wall was fixed in 95% ethanol for 2 days, dehydrated in a graded series of ethanol, embedded in paraffin, and then the sections were stained with haematoxylin-eosin for light-microscopic observation.

Protein was determined by the method of Hartree (5), using bovine serum albumin as standard.

**RESULTS**

Table 1 summarizes all the cases we examined in this study. With a total of 21 patients, 15 cyst wall and 6 cyst fluid samples were analyzed for both collagenase and gelatinolytic activities with or without APMA activation. For 4 patients (case Nos. 4, 5, 7 and 9), both cyst wall and fluid were subjected to these analyses. Collagenase activities in both the urea extract of cyst walls and cyst fluids were essentially of the latent type. Gelatinolytic activities were also essentially latent with two exceptions (case Nos. 5 and 10).

Both cyst wall and cyst fluid collagenases were partially purified using Ultrogel AcA 44 and 54 columns, respectively. Chromatograph
Table 1  Collagenase and Gelatinolytic Activities in Human Radicular Cysts and Cyst Fluids

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Collagenase U/g tissue</td>
</tr>
<tr>
<td>Cyst wall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>F</td>
<td>71.20 (29.64)</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>M</td>
<td>64.54 (33.32)</td>
</tr>
<tr>
<td>3</td>
<td>33</td>
<td>F</td>
<td>18.97 (0.53)</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>M</td>
<td>27.57 (4.81)</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>M</td>
<td>17.07 (1.74)</td>
</tr>
<tr>
<td>6</td>
<td>41</td>
<td>M</td>
<td>56.61 (10.05)</td>
</tr>
<tr>
<td>7</td>
<td>42</td>
<td>M</td>
<td>59.59 (15.23)</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>M</td>
<td>12.92 (2.60)</td>
</tr>
<tr>
<td>9</td>
<td>47</td>
<td>M</td>
<td>101.67 (44.87)</td>
</tr>
<tr>
<td>10</td>
<td>59</td>
<td>F</td>
<td>9.93 (2.72)</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>F</td>
<td>46.88 (17.33)</td>
</tr>
<tr>
<td>12</td>
<td>61</td>
<td>F</td>
<td>10.78 (0)</td>
</tr>
<tr>
<td>13</td>
<td>61</td>
<td>F</td>
<td>83.66 (25.59)</td>
</tr>
<tr>
<td>14</td>
<td>65</td>
<td>F</td>
<td>27.97 (2.89)</td>
</tr>
<tr>
<td>15</td>
<td>75</td>
<td>M</td>
<td>73.36 (28.50)</td>
</tr>
<tr>
<td>Cyst fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>27</td>
<td>F</td>
<td>6.53 (0)</td>
</tr>
<tr>
<td>4</td>
<td>34</td>
<td>M</td>
<td>7.65 (0.24)</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>M</td>
<td>6.26 (1.55)</td>
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<tr>
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<td>37</td>
<td>M</td>
<td>6.54 (0.31)</td>
</tr>
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<td>7</td>
<td>42</td>
<td>M</td>
<td>8.57 (0.08)</td>
</tr>
<tr>
<td>9</td>
<td>47</td>
<td>M</td>
<td>6.39 (5.64)</td>
</tr>
</tbody>
</table>

One unit (U) of enzyme activity is defined as the activity which hydrolyzes 1 μg of native soluble collagen for collagenase and denatured collagen for gelatinolytic enzyme per min. Enzyme activities without APMA activation are given in parentheses.

Profiles of these samples are shown in Fig. 1. Both activities were essentially of the latent type, and cyst wall collagenase showed a molecular weight of 64 K. Cyst fluid collagenase, however, showed a relatively broad peak with a provisional molecular weight of 66 K at its peak position.

A typical electrophoretogram of reaction products of [14C]collagen with APMA-activated cyst wall and cyst fluid collagenases is shown in Fig. 2. Two characteristic products, i.e., the N-terminal three-quarter fragment (α³) and the C-terminal one-quarter fragment (α¹) are seen. These are known to be produced by typical mammalian collagenases attacking the collagen molecule at one specific site.

The latent collagenases from both cyst wall and cyst fluid were most effectively activated by APMA, and less effectively by trypsin and iodide. Partially purified and APMA-activated collagenases from both cyst wall and cyst fluid were inhibited by 10 mM EDTA (100% and 89.7%, respectively), 5 mM o-phenanthroline (91.7% and 81.6%), 5 mM dithiothreitol (91.7% and 88.6%), and 10% (v/v) human serum (98.6% and 72.9%).

To study the substrate specificity, isolated type I and type III collagens were incubated with partially purified cyst wall and cyst fluid collagenases. As shown in Fig. 3, both types of collagenase preferentially attacked type I collagen. Type III collagen was less affected. To confirm the preferential degradation, the molar ratio of undegraded chain (%) = (area of a chain) × 100/(area of a chain + 4/3 area of α² chain) (7) was calculated from the densitometric patterns (data not shown) of type I and III collagens treated with collagenases; these were 28.3% and 100%, respectively, for cyst...
Fig. 1 Chromatograph profile of cyst wall urea extract (A) on an Ultrogel AcA 44 column and re-chromatograph profile of cyst fluid (B) on an Ultrogel AcA 54 column. A 50 µl aliquot of each fraction was assayed for collagenase activity with (○) or without (●) APMA activation, and fractions 86-92 and 72-74 were respectively combined and used for further analyses. Vo, void volume; BSA, bovine serum albumin; OA, ovalbumin; Mb, myoglobin. For details, see Materials and Methods.

Wall collagenase and 18.8% and 67.3%, respectively, for cyst fluid collagenase.

In five of the 15 cysts, histological sections showed typical chronic inflammatory cells, lymphocytes, monocytes (macrophages) and plasma cells to be dominant (Fig. 4), but the others were almost free from these inflammatory cells. Few polymorphonuclear (PMN) leukocytes were observed, however, in any of the specimens.

DISCUSSION
We reconfirmed the presence of collagenase activity in human radicular cyst walls, that was initially reported by Uitto and Ylipaavalniemi (18), and showed that the activity was effectively extracted with 4 M urea solution.
Fig. 2  Fluorography of SDS-polyacrylamide gel electrophoretogram. [14C]Collagen was incubated alone (1), with cyst fluid collagenase (2), or with cyst wall collagenase (3). Details of the reaction mixtures are given in Materials and Methods.

Also we demonstrated animal collagenase activity for the first time in radicular cyst fluids. These collagenases were typical animal collagenases which cleave native collagen in solution into three-quarter (α1) and one-quarter (α2) length cleavage fragments.

Analysis of the collagenase from radicular cyst walls and cyst fluids showed that the enzyme is relatively specific for type I collagen, suggesting the presence of PMN leukocyte collagenase at least in part (8). Histological study, however, showed that the cyst walls were essentially free from PMN leukocytes. This suggests that the PMN leukocyte collagenase was not derived directly from PMN leukocytes but came from cyst-wall matrix in which collagenase might have been released from PMN leukocytes accumulated during the acute inflammatory phase. Two other possible origins of the cyst wall collagenase are the macrophages and fibroblasts of cyst wall connective tissues. It has been demonstrated that endotoxin-activated guinea-pig macrophages (20) and thioglycollate-stimulated mouse macrophages (22) produce a typical animal collagenase. At certain sites in the granulation tissue of human radicular cyst walls, where the tissue was edematous and metachromatic, both intracellular and extracellular degradation of collagen bundles has been observed electron-microscopically. Collagenolytic activity has also been demonstrated in
Fig. 3 SDS-Polyacrylamide gel electrophoresis of isolated types I and III collagens and their mixture incubated with or without partially purified cyst wall (A) or cyst fluid (B) collagenases. In A, collagen (6 μg) was incubated at 20°C for 20 h with or without 59 μU of APMA-activated cyst wall collagenase in 43 μl of buffer A containing 0.3 M glucose, and then subjected to SDS-polyacrylamide gel electrophoresis after reduction with β-mercaptoethanol. Mixtures of type I and type III collagen (5 μg each) were incubated at 20°C for 20 h with or without 90 μU of APMA-activated cyst wall collagenase in 60 μl of buffer A containing 0.3 M glucose, and then subjected to interrupted gel electrophoresis. In B, collagen (10 μg) was incubated at 20°C for 74 h with or without 22.5 μU of APMA-activated cyst fluid collagenase in 30 μl of buffer A containing 0.3 M glucose, and then subjected to SDS-polyacrylamide gel electrophoresis after reduction with β-mercaptoethanol. Mixtures of type I and type III collagens (6 μg each) were incubated at 20°C for 74 h with or without 22.5 μU of APMA-activated cyst fluid collagenase in 30 μl buffer A containing 0.3 M glucose and then subjected to interrupted gel electrophoresis. 1, type I collagen alone; 2, type I collagen + collagenase; 3, type III collagen alone; 4, type III collagen + collagenase; 5, collagenase alone; 6, type I and type III collagen mixture alone; 7, type I and type III collagen mixture + collagenase. α, β and γ indicate collagen components. α1, α2 and β1 indicate the bands corresponding to the reduction products of the collagen and collagenase mixture. E indicates the enzyme.
Fig. 4. Histology of human radicular cyst wall. A: Section through membrane showing stratified squamous epithelium overlying fibrous connective tissue which is infiltrated by a typical chronic inflammatory cells, i.e. lymphocytes, monocytes (macrophages), and plasma cells. B and C are higher magnification of granulation and connective tissue regions in A, respectively.
the cyst walls by a collagen gel lysis method, suggesting that radicular cyst walls can themselves produce and secrete a collagenase (9).

We have at present no data to suggest whether the cyst wall and cyst fluid collagenases in vivo are in a latent form ready to be activated, or whether the enzyme is inactivated by some serum inhibitor(s) which prevent any residual inappropriate collagenase activity (13).

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REFERENCES