An Inhibitor of Mammalian Collagenase from Foetal Rabbit Bone in Culture

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There is ample evidence to indicate an important role for specific collagenases in the turnover of connective-tissue collagens both in normal and pathological conditions (Harris & Krane, 1974a,b,c). It has been the aim of many investigators to define the stimuli controlling the synthesis and secretion of collagenase, but it is clear that the extracellular control of the activity of collagenase is also important. Two mechanisms have been proposed for this control. In the first it is suggested that collagenase is released from cells or tissues as a proenzyme which is converted into active collagenase extracellularly by limited proteolytic cleavage (Vaes, 1972; Harper & Gross, 1972; Kruze & Wojtecka, 1972; Hook et al., 1973). The second mechanism proposes that active collagenase is released from cells and tissues and that the extent of its subsequent extracellular action is modified by inhibitors (Bauer et al., 1972, 1975; Nagai, 1973; Woolley et al., 1976). The relative contribution of these two mechanisms to collagenase regulation in vivo is not yet clear. In this communication we report the production, by rabbit bone, of a low-molecular-weight inhibitor of collagenase and speculate on its role in the control of extracellular collagenase activity.

Parietal bone explants from foetal rabbits (22–29 days of gestation) in culture synthesize a specific collagenase as characterized by its action on collagen in solution at 25°C and its sensitivity to inhibitors of the various classes of proteinases. The enzyme, which is a metalloproteinase, exists in both latent and active forms. We call the active enzyme native collagenase to distinguish it from activated collagenase produced by treatment of latent

![Graph](image)

Fig. 1. Relationship between the release of inhibitor and the production of latent and active collagenase by foetal rabbit bone explants in culture

Explants were cultured in a modified form of BGJ medium (Reynolds, 1976). Samples of medium were assayed for inhibitory activity (△) and for latent (●) or active (○) collagenase. Collagenase activity was determined as described by Werb & Reynolds (1974). A unit of collagenase hydrolysed 1 µg of collagen/min at 35°C. One unit of inhibitor blocked by 50% the activity of 0.1 unit of collagenase under the assay conditions. Results are means ± S.E.M. for four cultures.
Table 1. Action of the bone inhibitor on collagenases from various sources

Bone inhibitor was assessed for its ability to block the lysis of 14C-reconstituted collagen fibrils by equivalent activities of collagenases from a variety of tissues and species. Sufficient inhibitor was added to each assay to ensure a 70–80% inhibition of native rabbit bone collagenase. Results are expressed as percentage gel lysis. Collagenase was either trypsin-activated for 1 min at 25°C by using 0.5 mg of bovine pancreatic trypsin/ml of activation mixture or activated by preincubation with 4-aminophenylmercuric acetate (NH₂PhHgAc; 0.5–1.0 mM final concentration) for 4 h at 35°C then chromatographed on Ultrogel AcA 44. n.d., Not determined.

<table>
<thead>
<tr>
<th>Type of enzyme activity</th>
<th>Native</th>
<th>Activated by NH₂PhHgAc</th>
<th>Activated by trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+Inhibitor</td>
<td>Control</td>
</tr>
<tr>
<td>Enzyme source</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rabbit bone</td>
<td>54</td>
<td>10</td>
<td>41</td>
</tr>
<tr>
<td>Mouse bone</td>
<td>n.d.</td>
<td>n.d.</td>
<td>60*</td>
</tr>
<tr>
<td>Rabbit skin</td>
<td>55</td>
<td>32</td>
<td>55*</td>
</tr>
<tr>
<td>Rabbit uterus</td>
<td>n.d.</td>
<td>n.d.</td>
<td>50</td>
</tr>
<tr>
<td>Pig synovium</td>
<td>n.d.</td>
<td>n.d.</td>
<td>45</td>
</tr>
<tr>
<td>Clostridium histolyticum</td>
<td>45</td>
<td>47</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

* 4-Aminophenylmercuric acetate was present in the assay at a concentration of 0.1 mM.

Collagenase with either trypsin, 4-aminophenylmercuric acetate (NH₂PhHgAc) or 4-chloromercuribenzoate (ClHgBzO⁻).

There is a lag of 1–8 days before the detection of collagenase in the culture medium. During this period the bone produces an inhibitor of collagenase (Fig. 1). Typically the concentration of the inhibitor in the culture fluid rises until the onset of collagenase production, at which time the measurable concentration of the inhibitor begins to fall. Initially collagenase is detected in a latent form, which is assayable after treatment of the culture medium with trypsin or NH₂PhHgAc. As increasing amounts of latent collagenase are produced the concentration of inhibitor becomes undetectable. The progress of the culture is further characterized by the appearance of native collagenase in increasing amounts, such that up to 80% of the total enzymic activity may eventually be in this form.

The bone inhibitor of collagenase has apparent mol. wt. 30000 by gel filtration and is synthesized by the bone explant, since tissue cultured in the presence of cycloheximide (0.2 mM) or after having been frozen and thawed three times does not release inhibitor into the surrounding medium. The activity of the inhibitor in culture medium is destroyed by incubation with trypsin or chymotrypsin or by heat treatment, and its action is antagonized by NH₂PhHgAc or ClHgBzO⁻. The inhibitor is neither tissue- nor species-specific and when present in culture fluid it will block the action of native collagenases from rabbit bone and skin and collagenase activated by NH₂PhHgAc from rabbit bone, skin, uterus, mouse bone and pig and human synovium (Table 1). It is largely ineffective against trypsin-activated collagenases from rabbit tissues and against bacterial collagenase from Clostridium histolyticum and collagenase from human granulocytes.

The synthesis of inhibitor by the rabbit bone explants and its apparent low molecular weight qualify it as a potentially important extracellular regulator of collagenase activity. The lack of tissue specificity and species specificity suggests that inhibitors similar to the
bone inhibitor may exist in a variety of tissues. We think it significant that the gradual disappearance of inhibitory activity from the culture media of bone explants is paralleled by the appearance of latent, and then native collagenase.


Collagenase Activity from Cultured Non-Gravid Rabbit Uterus

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A considerable amount of work has been carried out on collagenase production by the rat uterus. Woessner (1969) described extensive breakdown of collagen during post-partum involution; this process was inhibited by prior administration of oestradiol (Woessner, 1969; Ryan & Woessner, 1974) and progesterone (Halme & Woessner, 1975). Other studies using tissue culture (Jeffrey & Gross, 1970; Jeffrey et al., 1971a) showed that cultured post-partum rat uterus released a specific collagenase which was secreted, after an initial lag period, for up to 10 days. Cyclic nucleotides, theophylline and progesterone diminished activity considerably (Koob & Jeffrey, 1974), but oestradiol was without effect (Jeffrey et al., 1971b). No activity was detectable in medium from non-gravid uterus cultures.

During our studies on the control of latent collagenase production by rabbit tissues, we have examined medium from cultures of non-gravid rabbit uterus. The tissue was excised, washed three times in Dulbecco's phosphate-buffer, pH 7.4, and minced finely. The fragments were cultured in serum-free Dulbecco's Modified Eagle's Medium, which was harvested daily. Collagenase activity was detected by the measurement of solubilization of [14C]glycine-labelled peptides from reconstituted rat collagen fibrils (Werb & Burleigh, 1974).

We have shown that, in this system, considerable amounts of collagenase are released into the medium, but only in a latent form, which may explain the previous failure to detect the enzyme in the non-gravid uterus.

The collagenase can be activated by trypsin (0.5 mg/ml for 1 min at 25°C; a fivefold molar excess of soya-bean trypsin inhibitor is used to stop the reaction) or non-enzymically by the thiol-blocking agent 4-aminophenylmercuric acetate (1 mM). Production of collagenase commences after 4-5 days in culture and continues until at least the tenth day (Fig. 1). Included in the Figure are results obtained from cultures of uterus taken 9 days pre partum and 48 h post partum; the non-gravid uterus produces comparable amounts of collagenase. In contrast with previous reports that collagenase is present in

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