Production of collagenase and inhibitor (TIMP) by normal, rheumatoid and osteoarthritic synovium in vitro: effects of hydrocortisone and indomethacin

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Summary

1. The amounts of latent and active collagenase and of collagenase inhibitor (TIMP) produced by two normal, three rheumatoid and two osteoarthritic synovial specimens in culture were compared. Normal synovia produced TIMP, but little latent enzyme. Rheumatoid synovia produced higher levels of total collagenase activity than normal, of which up to 50% in one sample was present in the medium in an active form, whereas no specific inhibitory activity due to TIMP was detectable. The amounts of collagenase and TIMP produced by osteoarthritic synovia were more variable and appeared to reflect the degree of inflammation in the tissue at the time of initiating the cultures.

2. Concentrations of TIMP were usually higher in the culture media of normal, rheumatoid and osteoarthritic synovia when hydrocortisone was present. Correspondingly, amounts of total collagenase were reduced. Production of prostaglandin E (PGE) were inhibited in a dose-dependent manner by hydrocortisone.

3. Indomethacin had no consistent effect on the production of TIMP by rheumatoid and osteoarthritic synovia, although it tended to depress production of collagenase. The production of TIMP by normal synovia was depressed by indomethacin. No PGE was detectable in the media when indomethacin was present.

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4. These results are consistent with those from previous animal studies, and we conclude that the balance between production of collagenase and TIMP may be critical in determining the extent of the destructive processes in arthritis. The ability of hydrocortisone to suppress production of collagenase and to increase free TIMP concentration, as well as to inhibit synthesis of prostaglandin, may explain in part how the drug exerts its therapeutic effects in patients with rheumatoid arthritis.

Key words: collagenase, hydrocortisone, indomethacin, inhibitor (TIMP), osteoarthritis, synovium.

Abbreviations: PG(B2, E, E1, E2), prostaglandin (B2, E, E1, E2); TIMP, tissue inhibitor of metallo-proteinases.

Introduction

Collagenase is the only mammalian enzyme that acts at neutral pH to degrade the native helix of collagen, the major structural component of connective tissue matrices. Consequently, the elucidation of the control of collagenase activity is important in the understanding of destructive pathological events. Collagenase has long been associated with the extracellular degradative processes of rheumatoid arthritis and cultures of rheumatoid synovium can produce large amounts of this proteinase [1, 2]. Dayer et al. [3] showed that a heterogeneous population of cells prepared from rheumatoid synovium produces large
quantities of collagenase and prostaglandin E₂ (PGE₂). Addition of dexamethasone to such cultures depressed production of both collagenase and PGE₂; although treatment with indomethacin also decreased PGE₂ levels, the production of collagenase was increased.

We found that collagenase is often produced in a latent form by many mammalian tissues in culture and demonstrated that under certain conditions an inhibitor is produced [4]. We have purified this inhibitor from rabbit, pig and human sources and named it TIMP (tissue inhibitor of metallo-proteinases [5]), because it blocks the activities of other metallo-proteinases in addition to collagenase. The widespread occurrence and properties of TIMP [6], and changes in its production in response to variations in incubation conditions, led us to propose that TIMP may be of major importance in the regulation of collagenase and other metallo-proteinase activities in vitro [7, 8].

Although production of collagenase by rheumatoid synovial cultures has been well characterized [9], normal human synovium has been little studied, and there is no previous information about production of TIMP by human synovium. In the present paper we show that normal human synovium produces both collagenase and TIMP in culture and that rheumatoid and osteoarthritic synovia have altered patterns of production in vitro. We also report changes in the production of TIMP as well as collagenase and PGE in response to hydrocortisone and indomethacin. The aim of this part of the study was to determine whether the responses of human tissues to disease processes and hydrocortisone treatment were similar to those which have been found with respect to metallo-proteinase and TIMP production by rabbit joint tissues in experimental arthritis [10–12]. Finally, the potential significance of TIMP in determining the activity of collagenase in human pathological tissues is discussed.

Materials and methods

Materials

All materials, other than those specifically mentioned below, have been described [13].

Methods

Culture techniques. Normal synovium was obtained from knee joints after above-knee amputation for either trauma or vascular insufficiency. Rheumatoid synovium was obtained either at synovectomy or during prosthetic knee replacement. Osteoarthritic synovium was obtained after either amputation or knee replacement. The synovium was dissected free from the underlying connective tissue and placed in culture, usually within 2–6 h after operation [although leaving the tissue at 4°C in NaCl solution (150 mmol/l: saline) overnight did not seem to affect tissue viability]. Small pieces (2–3 mm³) of synovium were cultured in 16 mm Linbro tissue culture wells containing Eagle’s minimal essential medium with Earle’s salts (Gibco Bio-Cult), either with 10% (w/v) foetal calf serum or without serum. Although it was necessary to culture the tissue in the absence of serum for inhibitor (TIMP) assays, collagenase and PGE could be assayed in medium either with or without serum. Less PGE and collagenase could be detected in the absence of serum, although relative levels paralleled those measured in the presence of serum. Media contained penicillin and streptomycin (100 units/ml and 100 µg/ml respectively). Cultures were incubated at 37°C in an atmosphere of CO₂ + O₂ (1:19, v/v). Hydrocortisone (sodium hemi-succinate) and indomethacin (Sigma) were added at the stated concentrations at the initiation of the cultures, and were prepared freshly in medium at each medium change. Culture media were collected during 9 days of culture and stored at −20°C until assayed. At the end of the culture period explants were blotted and weighed: each tissue culture well contained 20–50 mg of tissue and 0.5–0.75 ml of medium.

Histological assessment of the dissected synovial tissue agreed with the macroscopical observations and the clinical diagnosis. No inflammatory cells were seen in sections of normal synovium stained with haemotoxylin–eosin, whereas dense aggregates of chronic inflammatory cells (lymphocytes and plasma cells) were observed in the sections of rheumatoid and inflamed osteoarthritic synovia. Selected fragments of synovium were examined histologically after culture and compared with fragments from the same synovium fixed at the time of initiating the cultures: no loss of viability was observed. The DNA [14] and protein [15] contents were determined in homogenates of representative tissue specimens and were found to correlate with the wet weight of the tissue. Therefore the results were expressed in terms of g wet weight of tissue.

One specimen of osteoarthritic cartilage was obtained after knee replacement and sliced into 1 mm x 3 mm fragments. Three fragments (15.1 ± 0.6 mg) were placed in each well with 0.75 ml of medium and cultured in the same way as the
synovial fragment cultures, without and with hydrocortisone, except that the cultures were only maintained for 6 days.

Assays. Concentrations of prostaglandin were determined in unextracted culture media by radioimmunoassay, utilizing dextran-coated charcoal to separate bound from free [3H]PGE2 tracer. Antiserum to PGE was generously provided by Dr Lawrence Levine, Brandeis University, and had similar specificities for PGE2 and prostaglandins E1 and B2 (PGE1 and PGB2). It had been shown previously that concentrations of PGB2 produced under these conditions of culture are low relative to PGE2 [17]. Results were therefore calculated as equivalents of PGE2.

Collagenase and collagenase inhibitor in culture media were assayed with 14C-acetylated collagen fibrils [18]; 1 unit of collagenase hydrolyses 1 pg of collagen/min at 35°C; 1 unit of collagenase inhibitor blocks the activity of 2 units of collagenase by 50%. Total collagenase activity was assayed in the presence of 4-aminophenylmercuric acetate (0.3 mmol/l) as activator of latent enzyme. This compound was found to activate human synovial collagenase as efficiently as optimal levels of trypsin [19], as described for the rabbit tissue enzyme [4]. Inhibitor assays were routinely performed with partially purified rabbit skin collagenase (0.06 unit), but activity against human collagenase was found to be comparable [19].

Statistical analysis. All results are expressed as mean values ± SEM (standard error of the mean) and comparisons were analysed by Student's t-test for significance.

Results

The accumulative production of collagenase and TIMP by synovial tissues, for which we have complete data for all the parameters evaluated, are summarized in Tables 1 and 2. The next paper [19] characterizes collagenase and TIMP as well as other metallo-proteinases from synovium and cartilage. To condense a large body of data we show total amounts produced during the arbitrary time period of 9 days, and illustrate certain points within this time span in Fig. 1, which contains data for specimens of normal (no. 1) and rheumatoid (no. 1) tissue.

Total collagenase production by normal synovium over 9 days was always very low (Fig. 1c) and all of the activity was latent. Over the same period, rheumatoid synovium released larger amounts of collagenase activity (Fig. 1d), of which part was in the active form (not shown). The first rheumatoid sample produced more collagenase than the second (Table 1). Of interest was a sample of rheumatoid synovium which produced very low levels of collagenase; this sample was from a patient who had been treated with prednisolone (Table 1, rheumatoid no. 3).

In contrast to the production of collagenase, the inhibitory activity seemed to be lower than normal in the rheumatoid samples (Table 2). The inhibitory activity detectable on day 1 in the latter cases had a high molecular weight by gel-filtration

<table>
<thead>
<tr>
<th>Synovial sample no.</th>
<th>No addition</th>
<th>Hydrocortisone</th>
<th>Indomethacin (14 x 10^-5 mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 x 10^-6 mol/l</td>
<td>2 x 10^-7 mol/l</td>
<td>2 x 10^-4 mol/l</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>33.9 ± 3.5</td>
<td>13.5 ± 2.4</td>
<td>5.6 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>7.4 ± 2.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rheumatoid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1186 ± 164</td>
<td>863 ± 114</td>
<td>404 ± 93</td>
</tr>
<tr>
<td></td>
<td>(414 ± 13)</td>
<td>(376 ± 46)</td>
<td>(86 ± 29)</td>
</tr>
<tr>
<td>2</td>
<td>75.6 ± 9.0</td>
<td>75.9 ± 5.9</td>
<td>66.3 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>(24.7 ± 6.0)</td>
<td>(15.9 ± 5.9)</td>
<td>(2.5 ± 2.0)</td>
</tr>
<tr>
<td>3*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoarthritic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11.6 ± 6.7</td>
<td>26.4 ± 1.8</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>(17.8 ± 2.5)</td>
<td>(21.4 ± 1.6)</td>
<td>(1.2 ± 0.6)</td>
</tr>
<tr>
<td>2</td>
<td>31.7 ± 6.3</td>
<td>36.7 ± 3.2</td>
<td>6.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>(17.8 ± 2.5)</td>
<td>(21.4 ± 1.6)</td>
<td>(1.2 ± 0.6)</td>
</tr>
</tbody>
</table>

* Synovium from patient treated with corticosteroids.
The culture technique is described in the Materials and methods section and the results are given as the mean cumulative totals ± SEM for four cultures.

<table>
<thead>
<tr>
<th>Synovial sample no.</th>
<th>No addition</th>
<th>Hydrocortisone</th>
<th>Indomethacin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.8 x 10^{-4} mol/l</td>
<td>2.8 x 10^{-2} mol/l</td>
<td>14 x 10^{-5} mol/l</td>
</tr>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>38.0 ± 3.2</td>
<td>52.1 ± 5.6</td>
<td>65.0 ± 3.2</td>
</tr>
<tr>
<td>2</td>
<td>19.8 ± 3.5</td>
<td>20.9 ± 2.4</td>
<td>25.7 ± 1.8</td>
</tr>
<tr>
<td>Rheumatoid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>23.6 ± 0.5†</td>
<td>24.3 ± 0.5</td>
<td>89.0 ± 7.0</td>
</tr>
<tr>
<td>2</td>
<td>12.0 ± 4.3†</td>
<td>8.6 ± 1.6</td>
<td>27.0 ± 3.5</td>
</tr>
<tr>
<td>3*</td>
<td>27.0 ± 3.7</td>
<td>26.5 ± 3.8</td>
<td>21.4 ± 3.0</td>
</tr>
<tr>
<td>Osteoarthritic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>52.1 ± 6.7</td>
<td>62.5 ± 15.5</td>
<td>55.2 ± 5.6</td>
</tr>
<tr>
<td>2</td>
<td>8.6 ± 1.2†</td>
<td>37.6 ± 7.5</td>
<td>21.8 ± 3.9</td>
</tr>
</tbody>
</table>

* Synovium from patient treated with corticosteroids.
† Inhibitory activity remained unchanged after day 1 and was shown to be due to α2-macroglobulin remaining in the tissue after dissection.

The addition of hydrocortisone to the culture media at doses between 28 nmol/l and 2.8 μmol/l had a rapid effect in increasing concentrations of free inhibitor in the media of normal synovium (Fig. 1a). In rheumatoid culture media no TIMP was normally present, but higher doses of steroid stimulated production of this specific metalloproteinase inhibitor by day 6 or day 9 of culture, production after 9 days becoming comparable with that of the normal synovium exposed to similar concentrations of hydrocortisone. Production of collagenase by either normal or rheumatoid tissue was markedly reduced in the presence of hydrocortisone and became undetectable at a concentration of 2.8 μmol/l (Fig. 1c, d). When free inhibitor became detectable in rheumatoid culture media (Fig. 1b) there was no measurable active enzyme in the culture media. In contrast to the consistent effects of hydrocortisone, no such pattern was seen with the non-steroidal anti-inflammatory drug indomethacin, although production of collagenase and TIMP were depressed in some instances.

Prostaglandin production by tissues in culture was examined in detail and Table 3 shows the results obtained with cultures of synovium from a normal (no. 1) and a rheumatoid joint (no. 1). Hydrocortisone rapidly reduced the production of PGE by rheumatoid synovium and at 280 nmol/l completely inhibited production of prostaglandins by day 6. Indomethacin also prevented the appearance of PGE in the culture media.

Osteoarthritic synovium produced amounts of collagenase that were similar to normal, but the relative amounts of enzyme and TIMP were more variable than those found in normal and rheumatoid synovial cultures. Histological examination revealed that the first osteoarthritic specimen (ostearthritic no. 1, Tables 1, 2) was relatively normal with no evidence of inflammation, whereas the second sample was grossly inflamed. Hydrocortisone increased the production of TIMP in the media of osteoarthritic synovial cultures and inhibited the production of collagenase in a manner similar to that observed for normal and rheumatoid synovia.

One specimen of osteoarthritic cartilage was examined. TIMP was synthesized steadily over 6 days of culture and production was susceptible to a dose-dependent stimulatory effect of hydrocortisone. Production of collagenase was low and could be inhibited by hydrocortisone (data not shown).

**Discussion**

The overall aim of this study was to determine whether cultures of human tissue responded to anti-inflammatory agents in ways analogous to cultures of animal tissues. Taking into account the inability to control the quality of the human specimens and the difficulty of obtaining the materials, the results were reasonably consistent and agreed with those of our previous animal studies. Normal synovium, which is turning over connective tissue components in a controlled fashion, produces an excess of collagenase inhibitor (TIMP) under our culture conditions. If this occurred in vivo, then collagenase activity...
Fig. 1. Production of collagenase and inhibitor (TIMP) by normal and rheumatoid synovium in culture and the effects of hydrocortisone and indomethacin. Synovium was cultured for 9 days as described in the Materials and methods section: ●, alone; ▲, with indomethacin (14 μmol/l); ○, with hydrocortisone (28 nmol/l); □, with hydrocortisone (280 nmol/l); △, with hydrocortisone (2.8 μmol/l). Media were harvested at the times indicated and assayed for inhibitory activity against collagenase (a, normal; b, rheumatoid tissue) and for total collagenase in the presence of 4-aminophenylmercuric acetate (c, normal; d, rheumatoid tissue). The results are expressed as cumulative totals, means ± SEM from four treated and eight untreated cultures in each case.

would be suppressed [7, 8]. Compared with normal tissue the production of TIMP by rheumatoid synovium seemed to be insufficient to counteract the increased amount of enzyme being synthesized and in one sample large amounts of active collagenase were detectable. Such an occurrence in vivo might be expected to lead to excessive degradation of collagen in joint tissues. The results of the present study suggest that the production of TIMP may be important in
TABLE 3. PGE production by normal and rheumatoid synovium in culture and the effects of hydrocortisone and indomethacin

Synovium (normal no. 1 and rheumatoid no. 1; Table I) was cultured as described in the Materials and methods section. PGE levels in the media (ng/mg wet wt. of tissue) are expressed as the means of the cumulative totals ± SEM, with eight untreated cultures and four each of cultures treated with varying concentrations of either hydrocortisone or with indomethacin.

<table>
<thead>
<tr>
<th>Day no.</th>
<th>Normal</th>
<th>Rheumatoid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prostaglandin E (ng/mg of tissue)</td>
<td>Prostaglandin E (ng/mg of tissue)</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>Rheumatoid</td>
</tr>
<tr>
<td></td>
<td>No addition</td>
<td>Hydrocortisone (280 nmol/l)</td>
</tr>
<tr>
<td>3 6 9</td>
<td>47.4±6 61.8±6 105±12</td>
<td>91.6±17 511±61 1057±90 1204±68</td>
</tr>
</tbody>
</table>

Determining the amounts of active enzyme present in human synovial tissue, as animal studies have suggested [10, 11].

Rheumatoid synovium consists of a heterogeneous population of cells, including fibroblasts, synovial lining cells, lymphocytes, monocytes and endothelial cells [12, 20, 21]. Mixed populations of such cells [22] produce very large amounts of both collagenase and prostaglandins early on in culture. Dayer et al. [3] have also shown that mononuclear cells produce activating factors which stimulate further the production of collagenase and prostaglandins. Furthermore, we have shown that normal synovium produces much less PGE than rheumatoid, and that normal synovial cells can also be stimulated to produce high levels of PGE in response to mononuclear cell factors [23].

Since the rheumatoid synovium in the cultures described in this paper contain chronic inflammatory cells, including mononuclear cells, it seems likely that the synovial lining cells will have already been stimulated by endogenous activators to produce the observed large amounts of collagenase and PGE. Normal synovial cells are also able to produce low levels of collagenase, but activators are presumably not present because there is no mononuclear cell infiltrate. The difference in the amounts of collagenase produced by rheumatoid synovia probably reflects either a difference in disease activity or were due to treatment with drugs, as in the rheumatoid specimen no. 3, before operation.

Our observations on the response of synovium in culture to hydrocortisone are similar to those described by Dayer et al. [22] for synovial cells, in that production of collagenase is markedly reduced. Production of PGE by rheumatoid synovium is also reduced in the presence of hydrocortisone. Robinson et al. [24] have suggested that the reduction is due to the inhibition of synthesis, rather than to inhibition of the generation of the precursor of arachidonic acid. The effect of hydrocortisone on collagenase inhibitor levels gives additional insight into the mechanism of action and efficiency of this drug, in that the raising of TIMP levels will result in the increase in the amount of inactive collagenase present. At present we cannot be sure that the increased concentrations of TIMP when hydrocortisone is present are solely a result of increased synthesis; depressed enzyme levels could be the minor determining factor.

Inhibitors of general protein synthesis, such as cycloheximide, are known to prevent production of TIMP [18]. Although it is not known whether latent collagenase is either normally activated in vivo or is a form of the enzyme destined for removal, it is possible that the presence of large amounts of proteinases in pathological conditions could result in the activation of latent collagenase [25]. The production of large amounts of TIMP in such situations would reduce the effect of such activating proteinases by allowing collagenase–inhibitor complexes to form.

The marked reduction of production of PGE by rheumatoid synovium cultured in the presence of indomethacin was not accompanied by significant effects on the production of either collagenase or TIMP. Indeed, Dayer et al. [22] showed a slight stimulation of the production of collagenase by rheumatoid synovial cells in the presence of indomethacin. Our present results and those of Dayer et al. [22] suggest that production of prostaglandins and collagenase are not necessarily under the same control, or that they are produced by the same cells, since the
stimulation of their production in response to mononuclear cell factors can be dissociated from each other by indomethacin. This is further supported by the observation that production of prostaglandins fell off much more quickly than that of either collagenase or TIMP in the present tissue culture system, as well as that of Dayer et al. [21], who used cell preparations.

Dayer & Krane [26] showed that production of collagenase is dependent on the presence of small amounts of PGE₂ and suggested that prostaglandins may modulate production of collagenase by synovial cells. Such data could help to explain the variability in the effects of indomethacin on collagenase production. Our data also suggest that inhibitor production may also involve a prostaglandin-modulated control mechanism.

The use of osteoarthritic synovium provides additional support for the role of TIMP, since the absence of inhibitor appeared to be associated with the inflamed, and presumably more degenerative, state. The variability in the osteoarthritic culture media probably reflects the fact that osteoarthritis is a disease which is defined by cartilage abnormalities, the synovial changes being secondary. We suggest that the absence of TIMP production appears to be diagnostic for the pathological pathogenic state of synovium, and could imply that in rheumatoid arthritis collagenase may be free to cause breakdown in localized areas, for example at the cartilage–pannus junction. We have observed similar patterns of enzyme and TIMP production with a proliferative arthritis in rabbits [10–12] and all our studies to date encourage us to believe that more sensitive methods could be developed to measure changes in joint-tissue metabolism.

Acknowledgments

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References


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