PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 161, 99–103 (1979)

Collagenase Activity and Morphological and Chemical Bone Resorption Induced by Prostaglandin E_2 in Tissue Culture (40499)

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Although the mechanisms involved in the degradation of collagen during bone resorption are still unclear, tissue culture studies have provided indirect evidence that a specific tissue collagenase (E.C. 3.4.24.3) is involved (1–3). A serious drawback of these studies (2, 3), however, is the fact that the collagenase activity could only be indirectly demonstrated, since serum containing collagenase inhibitors was present in the tissue culture medium. As a result of a new procedure for the isolation of tissue collagenase from tissue culture medium containing serum (4), it is now possible to isolate and to directly quantitate the total amount of bone tissue collagenase released into tissue culture medium containing serum, under which conditions *living bone is undergoing active cellular resorption.* Thus it is possible to compare the morphological assessment of active bone resorption with chemical indices of bone mineral and bone matrix resorption. Previous study demonstrated that there was an increased amount of active bone collagenase released into the tissue culture media when bone resorption is increased by the addition of parathyroid hormone extract (PTE) (4). The present communication reports the studies correlating the levels of total bone collagenase released into the media (latent plus active) and bone resorption measured morphologically and chemically, when the resorption is induced by prostaglandin E_2 (PGE_2). Particular emphasis is placed on the simultaneous time-course changes of these parameters.

Materials and methods. Tissue culture techniques. Tissue cultures of calvaria of 5-day-old mice of the Swiss Webster strain were prepared as previously described (5). The medium was composed of heated horse serum and Gey's balanced salt solution (6:4). A stock solution of PGE_2 (generously provided by Dr. John Pike of the Upjohn Co.) was prepared in 70% ethanol (1 mg/ml) and diluted with heated horse serum immediately before addition to the medium. The final concentration of ethanol in the culture medium of PGE_2-treated cultures was less than 0.01% by volume and was not toxic to the tissue. The culture medium (2 ml) was renewed at 0.5, 1, 1.5, 2, 3, 4 and 6 days, and maintained for 8 days. The cultures were examined daily under a dissecting microscope to assess and grade the extent of bone resorption (5). The culture medium from each medium change as well as nonincubated medium for zero time controls were collected and aliquots were taken for calcium analysis. The medium samples from individual cultures were then pooled according to the experimental groups and analyzed for collagenase activity and hydroxyproline content as described in appropriate sections below.

Isolation of collagenase from culture medium. Collagenase was isolated from pooled culture medium samples which contained collagenase-inhibitors by affinity chromatography using heparin-Sepharose 4B gel as previously described in detail (4). Briefly, the sample (12 ml) was charged on a column (1.6 × 5 cm) of heparin-Sepharose 4B gel which had been equilibrated with 50 m*M* Tris–HCl buffer, pH 7.6 containing 5 m*M* $CaCl_2$. The sample was washed with 50 ml of the same buffer, eluted by a linear gradient of $Ca(CH_3COO)_2$ and NaCl, and fractions of 5 ml collected. Under these conditions, collagenase emerged between fraction 35 and 51. The combined fractions (85 ml) were concentrated to 1–2 ml on a Diaflo PM-10 membrane (Amicon), dialyzed overnight against 50 m*M* Tris buffer, and centrifuged.

Activation of latent collagenase and collagenase assay. Aliquots of the supernatants were incubated with trypsin (6) (Trypsin-

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TPCK, Sigma) at a concentration of 40 μg/ml for 5 min at ambient temperature (22°C). After incubation, soybean trypsin inhibitor (15× excess, Sigma) was added and incubated for 10 min prior to the collagenase assay. Collagenase activity of activated and nonactivated samples were assayed by measuring the release of [^{14}C]labelled peptides from reconstituted fibrils of radioactively labelled rat skin collagen (7). One collagenase unit was defined as the amount of enzyme activity necessary to digest 1 μg of collagen in 1 min under the stated conditions. In the present communication, collagenase activity in the samples was calculated as units of activity per ml of the culture medium.

Calcium analyses. The calcium content of one-tenth ml aliquot samples of culture medium samples was measured fluorometrically with a Corning Model 940 Calcium Analyzer. In some experiments, the calcium contents of calvarial explants were measured by ashing them at 560°, dissolving the ash in perchloric acid, and analyzing aliquot samples.

Hydroxyproline measurement. Hydroxyproline contents of zero time and incubated explants as well as those isolated from the tissue culture medium samples were measured as previously described (8).

Results and discussion. Almost all of the bone tissue collagenase isolated from the tissue culture medium during active bone resorption was found to be in the form of the latent (inactive) enzyme. In a typical experiment, for example, 0.001 units/ml and 0.042 units/ml of collagenase activity were found without trypsin activation in the media obtained from control and PGE_2-treated calvaria, respectively, whereas 0.02 units/ml and 0.75 units/ml, respectively, were measured in the same samples after trypsin activation. The ratio of active enzyme to latent enzyme was relatively constant and did not change during the period of tissue culture. It seems likely that latent enzyme which had escaped from serum collagenase inhibitors was partially autoactivated during the isolation procedure. This may account for the recovery of small amount of active collagenase from the culture medium of parathyroid extract-treated calvaria in our previous study (ref. 4, wherein collagenase unit is defined as the amount of enzyme activity necessary to digest 1 μg of collagen in 1 hr, therefore, previous one unit is equal to one-sixtieth of the present unit). The values for collagenase activity after trypsin activation were determined in all cases and are presented in the present communication.

Table I demonstrates that there is very little morphological evidence of bone resorption after 8 days of tissue culture in the control group. Chemical evidence for the resorption of bone based on the amount of calcium released is consistent with this morphological data. Although in the calcium determination rather small changes were measured in large numbers, the deviations among eight cultures were surprisingly small as shown in the tables. The total hydroxyproline content (medium plus incubated explant) increased to 141% of the original hydroxyproline content of the explant; thus the relatively large amount of hydroxyproline released into the tissue culture media (54% of the total) does not represent active resorption of structural collagen present in the calvaria prior to explant. The results rather indicate the release and partial degradation of newly synthesized collagen into the tissue culture medium which has either not been incorporated into the tissue fabric or degraded rapidly after its incorporation (9–11). Table II demonstrates that the extent of active cellular bone resorption induced by PGE_2 assessed morphologically, likewise correlates closely with the chemical indices of both bone mineral and bone matrix dissolution. The total hydroxyproline content increased to 108% of the original content and the value was not so high as in the control experiment, which might possibly be due to the inhibition of collagen synthesis by PGE_2 (12, 13) like PTE (14). Thus bone mineral and bone matrix resorption roughly parallel one another in the PGE_2 experiment.

The data presented in Tables I and II also demonstrate that there is a definite correlation between the overall amount of active cell mediated bone resorption observed morphologically, the two chemical parameters of bone resorption [bone mineral (calcium) and bone matrix (hydroxyproline)], and the total amount of bone tissue collagenase released into the tissue culture medium, even when allowances are made for the fact that a significant amount of the hydroxyproline in the

TABLE I. THE TIME-COURSE RELATIONSHIPS OF COLLAGENASE ACTIVITY, THE EXTENT OF MORPHOLOGICAL BONE RESORPTION, AND THE AMOUNTS OF CALCIUM AND HYDROXYPROLINE RELEASED INTO THE MEDIUM FROM BONE EXPLANTS IN CONTROL CULTURES.[a]

Day	Collagenase activity[b] in medium units/ml	Cumulative morphological bone resorption (%)[c]	Calcium concentration (mg% ± SE)	Hydroxyproline[d] concentration (μg/ml)
0	0	—	8.2 ± 0.08	1.37
0.5	0.09	—	8.2 ± 0.09	4.17
1	0.03	2.1	8.6 ± 0.07	2.55
1.5	0.03	—	8.4 ± 0.10	2.35
2	0	2.4	8.2 ± 0.09	2.07
3	0	2.6	8.7 ± 0.09	2.80
4	0	3.1	8.5 ± 0.08	2.30
6	0	3.8	8.7 ± 0.06	2.75
8	0	4.3	8.0 ± 0.07[e]	2.75
	Total: 0.30 units/culture			Total: 21.56 μg/culture

[a] Heparin was added to all culture media at a concentration of 10 units/ml. Average values from eight-bone cultures.

[b] Total collagenase activity (latent plus active enzyme).

[c] The percentage of bone surface area resorbed was calcualted from the scores obtained by morphological observation (see ref. 5).

[d] Hydroxyproline content of zero time medium (1.37 μg/ml) reflects hydroxyproline present in the horse serum lot used. Total hydroxyproline recovered in the culture was 56.58 μg (medium: 21.56 plus incubated explant: 35.02), thus hydroxyproline increased to 141% of the zero time value (average hydroxyproline content of zero time explant: 40 μg) indicating active collagen synthesis during the culture. Total hydroxyproline released into the medium during 8 days culture period (21.56 μg/culture) was derived by summing the differences between individual value and 0 time value. Since medium hydroxyproline like medium collagenase activity was measured for aliquots of pooled culture medium samples no SE was obtained.

[e] The value indicates calcium uptake by explant from medium. It was not clear whether this was due to calcification or nonspecific deposition of calcium on degenerated tissue.

TABLE II. THE TIME-COURSE RELATIONSHIPS OF COLLAGENASE ACTIVITY, THE EXTENT OF MORPHOLOGICAL BONE RESORPTION, AND THE AMOUNTS OF CALCIUM AND HYDROXYPROLINE RELEASED INTO THE MEDIUM FROM BONE EXPLANTS IN PGE_2-TREATED CULTURES.[a]

Day	Collagenase activity[b] in medium units/ml	Cumulative morphological bone resorption (%)[c]	Calcium concentration (mg% ± SE)	Hydroxyproline[d] concentration (μg/ml)
0	0	—	8.4 ± 0.05	1.50
0.5	0.95	—	8.6 ± 0.10	2.52
1	0.70	2.6	9.4 ± 0.09	2.88
1.5	0.16	—	9.6 ± 0.11	2.62
2	0.24	14.0	10.2 ± 0.10	2.88
3	0.60	30.4	11.3 ± 0.09	5.07
4	0.25	55.4	11.1 ± 0.10	4.90
6	0.07	77.1	10.8 ± 0.08	4.30
8	0.04	87.6	9.3 ± 0.09	3.57
	Total: 6.02 units/culture			Total: 33.48 μg/culture

[a] PGE_2 (100 ng/ml) and heparin (10 units/ml) were added to the tissue culture media. Average values from eight-bone cultures.

[b] Total collagenase activity (latent plus active enzyme).

[c] The percentage of bone surface area resorbed was calculated from the scores obtained by morphological observation (see ref. 5).

[d] Total hydroxyproline recovered in these cultures was 43.07 μg (medium: 33.48 plus incubated explant: 9.59), thus hydroxyproline increased to 108% of the zero time value indicating far less collagen synthesis than the control cultures (Table I). For the derivation of total hydroxyproline see footnote *d* of Table I.

control culture medium undoubtedly represents newly synthesized collagen rather than structural collagen present in the bone prior to explant.

Since our preliminary experiments showed that a large amount of collagenase was re-

leased into the medium during the first two days of culture, close attention was paid to this time period in order to assess in greater detail the early changes in the rates of collagenase release and its relationship to bone resorption measured morphologically and chemically. The control group (Table I), in which very little morphological bone resorption occurred, showed a small peak of collagenase activity in the medium during the initial half day of incubation. There was no detectable collagenase activity in the control medium collected after two days of culture. The early peak of collagenase activity after explantation is probably related to the trauma associated with explantation. Table II shows that when extensive morphological bone resorption is induced by PGE_2, it is accompanied by very large increases in the collagenase activity recovered in the tissue culture medium. Although there is no direct correlation between the amount of collagenase released and the amount of bone resorption which occurs either morphologically or chemically for single time periods, such a relationship clearly exists when the data are summed over 2- to 3-day periods. This time lag may, at least in part, be due to a rate limiting step which converts the latent enzyme to the active form. A similar proposal has been suggested by Werb *et al.* (15). The findings in the present study that most of the bone collagenase in the tissue culture medium is in the latent form makes this suggestion particularly pertinent.

Stimulation of bone resorption by PGE_2 has been well documented (16–18). The present report has demonstrated, that like PTE (4), PGE_2 also stimulates the synthesis and/or release of tissue collagenase during the active cellular resorption of living bone, and the extent of bone resorption observed morphologically correlated well with the amount of bone tissue collagenase released into the tissue culture medium. The differences between our results and those of Lenaers-Clays and Vaes (19) who found no significant correlation between bone resorption and the amount of either collagenase or procollagenase which accumulated in the culture medium of PTE stimulated bone is difficult to explain. It may possibly be due to differences in culture technique and/or enzyme assay methods, but it is not possible to discuss these points at this time since the detailed methodology of culture and analyses are not given in their abstract.

Summary. The isolation of collagenase present in serum-containing medium harvested from bone cultures was effected by heparin-Sepharose affinity chromatography. Almost all of the bone tissue collagenase released into the medium was of the latent, inactive type, which could be activated *in vitro* with trypsin. Over the course of the experiment a 20-fold greater amount of collagenase was released into the medium by the resorbing cultures stimulated with prostaglandin E_2 as compared to the control cultures. A significant increase in calcium released into the medium correlated in time with rapid bone resorption as measured microscopically. The increase in collagenase activity did not demonstrate the same precise time course.

The authors wish to acknowledge the generous gift of prostaglandin E_2 from Dr. John Pike of The Upjohn Co., and the skilled technical assistance of Miss Frederica Ward, Mr. Gene Belch and Mr. Daniel Penzer. This investigation was supported by grants from the National Institutes of Health (DE 02849 and AM 15671), The New England Peabody Home for Crippled Children, Inc., and the Research Career Development Award KO4-DE-00048 for S.S.

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Received November 22, 1978. P. S. E. B. M. 1979, Vol. 161.