LYMPHOKINE-MEDIATED BONE RESORPTION REQUIRES ENDOGENOUS PROSTAGLANDIN SYNTHESIS*

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Localized bone loss can be associated with certain inflammatory, hypermetabolic, and neoplastic diseases. The pathogenesis of osteolytic lesions in most of the latter conditions has not been elucidated. Lymphocytes have been shown to release factor(s) with osteolytic activity (1–3). One lymphokine, osteoclast activating factor (OAF), a product of T or B lymphocytes, has in part been chemically characterized (4–7). Bioassays have not been able to distinguish osteoclast-mediated bone resorption initiated by parathyroid hormone (PTH) from that caused by OAF. Both agents cause the release of calcium from bone with similar dose-dependency curves (8), both cause osteoclast activation as measured by increased cell number and size (9), and both agents activate adenylate cyclase in bone cells (10, 11). We therefore decided to look more closely at the sequence of biochemical events that occurs during lymphokine-mediated bone resorption. In particular, we examined endogenous bone synthesis and release of prostaglandins after exposure to a lymphokine preparation that probably contained OAF activity.

Materials and Methods

Prostaglandin Synthesis by Explanted Fetal Rat Bone Culture. The radii and ulnae from 20-d-old fetal rats were placed into culture as previously described (8). The classes of prostaglandins synthesized by the explanted bones were followed by measuring the conversion of tritiated arachidonic acid ([5,6,8,9,11,12,14,15]H)arachidonic acid, Amersham Corp., Arlington Heights, Ill.) and by radioimmunoassay, as previously described for monocytes and macrophages (12, 13).

Bone Calcium Release. To measure calcium release from bone, the fetal rat bones were labeled in utero by injecting the mother with 0.2–0.4 mCi of 45CaCl2 on the 18th d of gestation (8). Calcium release was determined by counting the supernate media in a liquid scintillation counter; the data are expressed as the ratio of calcium release in counts per minute (cpm) of the experimental bone per cpm released by the paired control bone (E:C).

Preparation of Lymphokine. Lymphokine-containing media was prepared from human peripheral blood mononuclear cells that had been isolated by differential centrifugation (12, 13). McCoy’s 5A media supplemented with 10% heat-inactivated fetal calf serum was conditioned by 5 million cells/ml in the presence of 2.5 μg/ml phytohemagglutinin (PHA; Wellcome Research Laboratories, Kent, Great Britain) for 24–48 h at 37°C in a humidified 5% CO2 atmosphere. The cell-free media was harvested and frozen at ~80°C until tested in the bone cultures. The bone resorbing activity of the lymphokine-containing media was retained at neutral pH by a dialysis membrane having a 6,000–8,000 mol wt cutoff (Spectrapor TM, Spectrum Medical Industries, Inc., Los Angeles, Calif.), which is similar to previous findings with OAF (7). Measurement of prostaglandin (PG)E levels in the lymphokine-containing media showed it to have <10% of the levels released by fetal rat bones (8). Therefore, the aliquots of lymphokine added to the bone cultures could not have contributed significantly to the PGE levels measured in the explanted bone culture supernates.

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Reagents. Purified PTH was prepared by Sephadex column chromatography from urea-HCl-cysteine, trichloroacetic-precipitated bovine parathyroid gland extracts (14).

Results and Discussion

Bones that had been radiolabeled with arachidonic acid released several classes of tritiated prostaglandins (Table I). Lymphokine-stimulated bones showed a marked and selective increase in [3H]PGE2 release (Table I). Indomethacin and flurbiprofen, structurally unrelated cyclooxygenase inhibitors, markedly reduced the amount of [3H]PGE2 released by lymphokine-stimulated bones. Greater than 85% of the [3H]arachidonic acid could be recovered from the bones by chloroform-methanol extraction at the end of the 96-h culture. A similar release pattern and quantitative increase in [3H]PGE2 was seen if a bacterial endotoxin was substituted for lymphokine (data not shown). PGE levels measured in bone by radioimmunoassay showed a highly significant correlation, \( P < 0.001 \) with [3H]PGE2 release (\( r = 0.91, n = 19 \)). Such correlation would suggest that tritiated arachidonic acid conversion to [3H]-PGE2 adequately reflects PGE-synthetase activity. It is not known whether such a correlation exists for the other prostaglandins because their absolute levels were not measured.

A comparison of the dose-dependent release of PGE and 45Ca by the explanted bones that had been exposed to the test agents was made. Lymphokine-induced PGE levels were significantly greater than control or PTH-treated bones (\( P < 0.001 \)) at all concentrations tested (Fig. 1A and B). PHA alone had no significant effect on PGE or 45Ca release.

When PGE synthesis was followed as a function of time in culture, a characteristic sequence of biochemical events was seen. Similar levels of PGE were released from bones equilibrating in control media; (Fig. 2; mean PGE release ± SEM from 36 bones after the first 18 h in culture was 1.13 ± 0.07 ng/bone). At the end of a 24-h exposure to lymphokine, bone PGE synthesis showed a 20-fold increase compared with untreated bones (20.55 ± 1.05 vs. 1.05 ± 0.1 ng/bone) for the experiment shown. Four separate experiments gave similar results, and, in every case, the addition of structurally unrelated prostaglandin-synthetase inhibitors caused a significant de-

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Number of plates*</th>
<th>Mean cpm [3H]prostaglandin released ± SEM/plate</th>
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<tbody>
<tr>
<td></td>
<td>PGE2a</td>
<td>6K-PGF2α</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>113 ± 40</td>
</tr>
<tr>
<td>PTH</td>
<td>2</td>
<td>31</td>
</tr>
<tr>
<td>Lymphokine</td>
<td>3</td>
<td>313 ± 48‡</td>
</tr>
<tr>
<td>Lymphokine + indomethacin</td>
<td>1</td>
<td>117</td>
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* Each plate represents the pooled, extracted supernatant media from four cultured bones.
‡ Significantly greater than control (\( P < 0.02 \)) as determined by the \( t \) test for two means. After an 18-h equilibration period, two pairs of ulnae and radii per culture were incubated with 10 μCi of [3H]-arachidonic acid for 4-6 h. The nonincorporated label was removed by washing with fresh media. The bones were then incubated for 24 h with fresh media (control) or with media that contained bovine PTH (2.0 μM final concentration) or 0.3 ml of lymphokine. At the 48th h of culture (representing 24 h of continuous exposure to the test substances), the media was removed from the bone cultures and analyzed for the tritiated prostaglandin conversion products. The tritiated prostaglandins were separated by two-dimensional thin-layer chromatography on silica gel plates (12, 13). Abbreviations used: PGE2a, 6K-PGF2α, PGE2, and TXB2 for prostaglandins F2α, 6-keto F2α, E2, and thromboxane B2, respectively.
Fig. 1. Dose-dependent release of PGE (A) and $^{45}$Ca (B) from explanted fetal rat bones treated in culture with PTH (●) or lymphokine (○). PTH was tested from 0.01 to 10 μM final concentration; aliquots of a lymphokine-containing media conditioned by 5 million mononuclear cells/ml in the presence of PHA. Data given as the mean (SE = 4) ± SEM of the ratio counts per minute (cpm) $^{45}$Ca released from experimental bones/cpm $^{45}$Ca released from paired control bones (E:C). Mean PGE release by 32 control bones ± SD = 0.9 ± 0.3 ng/bone; mean E:C for 24 control bones ± SEM = 1.0 ± 0.03. PHA alone had no effect on PGE or $^{45}$Ca release from bones. * Significantly different from controls ($P < 0.001$) as determined by t test of two means.
FIG. 2. The effect of PTH and OAF on PGE and 45Ca release from fetal rat bones. (A) PGE levels were measured by radioimmunoassay in the media conditioned by fetal rat bones after an initial equilibration period (0-18 h), after 30 h in the presence of test substances (18-48 h), and finally, 48 h after the removal of the test substances (48-96 h). Each bar graph represents the mean ± SEM of at least six samples; each sample represents one culture dish containing one bone. All data were derived from a single representative experiment. (B) Release of 45Ca from the explanted bone cultures was followed as a function of time. The fetal rat forelimb bones were derived from littermates and the data are expressed as the ratio of counts per minute released by the experimental bones divided by the mean of the six control bones in that particular test group (E/C). * Significantly greater than control, P < 0.005. † Significantly greater than control, P < 0.001.

crease in the levels of PGE released, suggesting that de novo synthesis of prostaglandins was being measured. For the experiment shown in Fig. 2, flurbiprofen (10 μM added 20 min before the test substance) caused a significant (P < 0.001) reduction in lymphokine-induced PGE release measured at 48 h (21.4 ± 1.1 to 11.5 ± 1.2 ng PGE released/bone, n = 8). Higher levels of flurbiprofen and indomethacin reduced PGE release to control levels. No significant lowering of PGE levels was noted in the control or in PTH-treated bones.

Radiolabeled calcium release measured at the end of culture (48 h after the removal of the test substances) of both the PGE and lymphokine-treated bones showed markedly enhanced calcium release as compared with control bones (E/C = 1.64 ± 0.1 and 1.45 ± 0.12, respectively, compared with 1.0 ± 0.05, P < 0.005; Fig. 2). In every case studied (n = 5), 45Ca release occurred with a delayed time-course compared with that noted for PGE release and occurred after the removal of the stimulus from the culture media. The concomitant administration of indomethacin or flurbiprofen with PTH had no effect on the calcium release induced by PTH (E/C = 1.64 ± 0.1
vs. $1.58 \pm 0.11$, $n = 4$ bones/experimental point). In contrast, the addition of prostaglandin-synthetase inhibitors with lymphokine significantly abrogated its ability to induce calcium release ($E:C = 1.45 \pm 0.12$ reduced to $1.11 \pm 0.08$, $P < 0.05$). The data strongly suggest that the early burst of PGE synthesis caused by lymphokine was required for the subsequent calcium release.

The induction of bone prostaglandin synthesis by lymphokine is not a response that is unique for that agent. Endotoxin as well as antigen-antibody plus complement have been reported to cause increased calcium as well as PGE release from bone (15, 16). In another model of localized bone loss, indomethacin could be shown to block resorption caused by the application of gingival fragments to bone but not that induced by PTH (17).

Bone resorption caused by inflammatory stimuli tends to be highly localized and dependent on the secretion of locally produced factors. Prostaglandins are likely mediators of inflammatory cell-induced bone resorption because they are locally produced, have a short half-life in the circulation, and because continuous stimulation is required to maintain locally elevated levels. Furthermore, prostaglandins are known to amplify or even initiate several cell activities associated with bone resorption. They can, for example, augment adenylate-cyclase activity in bone, which is a necessary concomitant step in the factor-mediated bone resorption (10, 11, 18). Alternatively, prostaglandins can act by accelerating lysosomal enzyme release, which is necessary for collagen matrix digestion and mineral dissolution to occur (19, 20). Prostaglandin synthesis and release after macrophage exposure to endotoxin has been shown to be essential for neutral protease release from lysosomes (21). Finally, prostaglandin secretion by monocytes has been shown to be necessary for OAF release from lymphocytes (22). Prostaglandins may thus be acting through a positive feedback loop to maintain elevated OAF levels.

To our knowledge, initiation of prostaglandin synthesis is the first biochemical response that clearly distinguishes lymphokine and PTH-stimulated bones. The biochemical response of enhanced PGE synthesis could have several different roles in facilitating inflammatory-factor-initiated bone resorption. Our data from explanted fetal rat bones provide evidence that endogenous prostaglandin synthesis within bone is necessary for lymphokine-induced bone resorption; the subsequent mechanisms by which prostaglandins mediate bone mineral dissolution remain to be elucidated.

Summary

Enhanced synthesis of prostaglandin (PGE) by explanted fetal rat bones was initiated by lymphocyte-conditioned media but not by physiological levels of parathyroid hormone. Rapid release of PGE from bone occurred only when the lymphokine was present. Synthesis of PGE preceded and was necessary for the bone resorption caused by the lymphokine preparation. Local production of prostaglandins in response to inflammatory cell or tumor-derived factors may in part be responsible for the localized bone loss that occurs in certain pathological states.

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References


