Title

Two Squamous Cell Carcinomas not Associated with Humoral Hypercalcemia Produce a Potent Bone Resorption-Stimulating Factor which is Interleukin-1α*

Journal

Endocrinology, 125(2)

ISSN

0888-8809

Authors

FRIED, RM

VOELKEL, EF

RICE, RH

et al.

Publication Date

1989-08-01

DOI

10.1210/endo-125-2-742

Peer reviewed

0013-7227/89/1252-0742$02.00/0
Endocrinology

Vol. 125, No. 2
Printed in U.S.A.

Two Squamous Cell Carcinomas not Associated with Humoral Hypercalcemia Produce a Potent Bone Resorption-Stimulating Factor which is Interleukin-1α*

R. M. FRIED, E. F. VOELKEL, R. H. RICE, L. LEVINE†, E. V. GAFFNEY, AND A. H. TASHJIAN, JR.

Charles A. Dana Laboratory of Toxicology, Harvard School of Public Health, and the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115; the Graduate Department of Biochemistry, Brandeis University (L.L.), Waltham. Massachusetts 02254; and the Department of Molecular and Cell Biology, Pennsylvania State University (E.V.G.), University Park, Pennsylvania 16802

ABSTRACT. Conditioned medium (CM) from two squamous cell carcinoma cell lines, SCC-9 and SCC-13, stimulated bone resorption in neonatal mouse calvariae in organ culture. Enhanced bone resorption induced by CM was associated with an increased production of prostaglandin-E_2 (PGE_2) by the calvariae. Complete inhibition of stimulated PGE_2 synthesis by indomethacin only partially inhibited bone resorption-stimulating activity (BRSA) in the CM. Neither SCC-9 nor SCC-13 CM stimulated cAMP production in rat osteosarcoma cells (ROS 17/2.8). The BRSA in CM was completely inhibited by an antibody to interleukin-1α (IL-1α). Fractionation of SCC-9 CM by gel filtration and HPLC ion exchange chromatography revealed a single peak of BRSA and PGE_2 synthesis-stimulating activity at 17–20K (termed SCM_{II}). In mouse calvariae, SCM_{II} increased medium Ca^{2+} and PGE_2 in a dose-dependent manner at concentrations from 20 ng protein/ml to a maximum of 500 ng protein/ml. Preincubation of SCM_{II} with antibody to IL-1α completely inhibited SCM_{II}-induced bone resorption. SCM_{II} also enhanced thymocyte proliferation with activity that was equivalent to 353 U/ml IL-1. Antibodies to IL-1β and tumor necrosis factor had no effect on SCM_{II}-induced bone resorption. Using specific enzyme-linked immunosorbent assays for IL-1α and IL-1β, IL-1α was measured in high concentrations in both crude and partially purified fractions of SCC-9 and SCC-13 CM. In contrast, IL-1β was either undetectable or present in amounts below those that stimulate bone resorption. In addition, SCM_{II} did not enhance cAMP production in bone cells. We conclude that the BRSA produced by the two squamous cell carcinoma cell lines SCC-9 and SCC-13 is IL-1α. (*Endocrinology* **125:** 742–751, 1989)

ALTHOUGH several squamous cell carcinomas associated with the syndrome of humoral hypercalcemia of malignancy (HHM) have been shown to produce bone resorption-stimulating factors (1–9), little is known about whether squamous cell carcinomas not associated with this clinical syndrome also produce such factors. Both PTH-related protein (1–7) and interleukin-1α (IL-1α) have been shown to be produced by squamous cell carcinomas associated with HHM (10). We have studied two squamous cell carcinoma cell lines derived from patients without HHM. Conditioned media (CM) from these cell lines, SCC-9 and SCC-13, were shown to contain potent bone resorption-stimulating activity (BRSA), which was purified on the basis of this biological activity. The active fractions were analyzed for adenylate cyclase-stimulating activity, which detects human PTH-related protein (hPTHrP) and for IL-1 using a thymocyte-stimulating assay, antibody neutralization, and newly developed enzyme-linked immunosorbent assays (ELISA) for IL-1α and IL-1β. Our results demonstrate that all of the BRSA was accounted for by IL-1α; thus, the BRSA produced by all squamous cell carcinomas cannot be attributed solely to PTH-related protein.

Received February 20, 1989.
Address all correspondence and requests for reprints to: Dr. Armen H. Tashjian, Jr., Laboratory of Toxicology, Harvard School of Public Health, 665 Huntington Avenue, Boston, Massachusetts 02115.
* This investigation was supported in part by research grants from the NIH (DK-10206, AR-27130, CA-44677, and CA-45154).
† American Cancer Society Research Professor of Biochemistry (Award PRP-21).

Materials and Methods

Cell culture

Human squamous carcinomas of the tongue (SCC-9) and facial epidermis (SCC-13) were cultured with lethally irradiated 3T3 feeder layers (11, 12). The cells were grown in Dulbecco-Vogt Eagle's Medium-F-12 (3:1) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), T_3 (20 pM), adenine (0.1 mM), and fetal bovine serum (5%) or in the absence of serum (for serum-free experiments). Four-day-old medium was collected from cells that were near or at confluence. This CM was pooled

and frozen at −20 C for later experimentation.

The rat osteosarcoma cell line ROS 17/2.8, obtained from Dr. Gideon A. Rodan (Merck, Sharp, and Dohme, West Point, PA), was grown as previously described in F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (13).

Organ culture of bone

Neonatal mouse calvariae were placed in organ culture as free-floating bones, as previously described (14, 15). The medium was Dulbecco's Modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated (60 C for 1 h) horse serum.

Incubation of whole or half-calvariae (by sagittal sectioning into two symmetrical pieces) from 4- to 5-day-old neonatal mice was performed at 37 C in a roller drum apparatus under an atmosphere of 50% O_2, 5% CO_2, and 45% N_2. Calvariae were preincubated for 24 h before experimental treatment. The medium was changed at this time, and fresh control medium or medium containing specific treatments was then added. Bone resorption was determined by measuring release of calcium ($^{40}Ca^{2+}$) from the calvariae into the culture medium 48 or 72 h after addition of the test agents.

Immunoneutralization

For immunoneutralization experiments, rabbit antiserum (polyclonal), mouse monoclonal antibody, or control serum was added to the test agents in a total volume of 500 μl serum-free DMEM, and incubation was performed for 90 min at 37 C. The samples were then diluted to their appropriate final concentrations (as reported in *Results*) and added to calvariae after the 24-h preincubation period. Incubation was continued for an additional 48 h. The antibodies alone or preimmune rabbit serum had no effect on bone resorption.

Measurement of calcium

The concentration of total calcium in the bone culture medium was measured in 400-μl samples by a calcium-selective electrode using a NOVA 7+7 automatic calcium analyzer (NOVA Biomedical, Waltham, MA).

Measurement of arachidonic acid metabolites

The concentrations in the culture medium of prostaglandin-E_2 (PGE_2) and the hydrolytic product of PGI_2, 6 keto-$PGF_{1\alpha}$, were measured by RIA (16–19).

Measurement of cAMP

Calvariae were prepared and cultured as described above. After preincubation for 24 h, the medium was removed, and calvariae were washed in serum-free DMEM. Serum-free control medium or medium containing specific test agents was added, and incubations were continued for 1 h (37 C; 50% O_2, 5% CO_2, and 45% N_2). Serum-free medium used for the 1-h incubation contained 3 μM isobutylmethylxanthine (IBMX). After the 1-h incubation, medium samples were collected, placed in a boiling water bath for 5 min, and then stored at −20 C. The cAMP concentrations were measured by RIA (20).

ROS 17/2.8 cells were grown to confluence in 35-mm culture wells. The medium was changed 24 h before experimentation. At the beginning of an experiment, the medium was aspirated, and the cells were washed twice with serum-free F-10. F-10 with 100 μM IBMX in the absence or presence of various test agents was then added to the cultures. The cells were incubated for 60 min (37 C; 95% air and 5% CO_2). After incubation, the medium was collected, boiled for 5 min, and stored at −20 C until measurement of cAMP by RIA (20).

Gel filtration chromatography

Serum-free CM from SCC-9 cells was concentrated 10- to 15-fold by lyophilization and then chromatographed at 4 C on a Bio-Gel P-60 (2.5 × 110 cm) column. The column was equilibrated with 0.15 M NaCl containing 0.02 M HEPES, pH 7.4. The eluate was collected in 5-ml fractions, and the absorbance at 280 nm was determined for each fraction. The fractions were stored at −20 C until assay. Mol wt markers were albumin (68K), myoglobin (17K), and cytochrome-*c* (12K).

HPLC

Biologically active fractions from the Bio-Gel P-60 column (a single broad peak) were pooled, concentrated by lyophilization, and then dialyzed (mol wt cutoff, 3500) extensively against 0.02 M HEPES, pH 7.4. The sample was chromatographed on a Mono-Q HPLC anion exchange column at 20 C using a linear NaCl gradient with a 1.0 ml/min flow rate. One-milliliter fractions were collected, and the absorbance at 280 nm was determined. The fractions were stored at −20 C until assay.

Measurement of protein

The protein concentration in all chromatographic samples was determined using a bicinchoninic acid (Pierce Chemical Co., Rockford, IL) assay method (21).

Thymocyte assay for detection of IL-1

Cell suspensions from the thymus glands of 5- to 8-week-old C_3H/HeJ mice were inoculated at 1.5×10^6 cells/well into 96-well plates in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2.5×10^{-5} M 2-mercaptoethanol, and 1 μg/ml phytohemagglutinin (22, 23). Test samples were added to wells and incubated for 72 h. [^{3}H]Thymidine (0.5 μCi; 2 mCi/mmol) was added to each well for the final 8 h of the incubation period, and cells were harvested on glass fiber filters with an automatic cell harvester. Radioactivity was determined by liquid scintillation counting. A unit of IL-1 activity is defined as that amount which resulted in half-maximal [^{3}H]thymidine incorporation into thymocytes (22, 23).

ELISA for human IL-1α and IL-1β

Double antibody sandwich ELISA methods were used to quantitate human IL-1α and IL-1β (22, 23). Ninety-six-well microtiter plates were coated with 100 μl of a dilution of mouse

ascites fluids containing monoclonal antibody to either recombinant (r) IL-1α or rIL-1β and allowed to incubate overnight at 4 C. Wells were washed three times with a solution of 50 mM Tris and 200 mM NaCl at pH 7.4 (TBS), and then blocked for 1 h with 250 μl TBS containing 1% normal mouse serum. The wells were washed and incubated for 2 h at 37 C with 100-μl samples of test materials or IL-1 standards. Wells were washed and incubated for 2 h at 37 C with 100 μl rabbit anti-rIL-1α or -β diluted in TBS, followed by washing and incubation for 1 h with 100 μl alkaline phosphatase-conjugated goat antirabbit immunoglobulin diluted in TBS-BSA. *p*-Nitrophenyl phosphate was added to washed wells, and absorbance was measured at 405 nm after 30 min.

Statistical analysis

Results of each experiment were subjected to an analysis of variance, and the SE values were calculated from the residual error term of that analysis. Comparisons between groups were made using Duncan's multiple range test or Student's *t* test.

Materials

Swiss albino mice (CD-1 strain) were obtained from the Harvard Animal Resource Center. Media and sera were obtained from Gibco (Grand Island, NY) with the exception of DMEM which was from M. A. Bioproducts (Walkersville, MD). Indomethacin and IBMX were obtained from Sigma Chemical Co. (St. Louis, MO). Radioactive tracer for cAMP, adenosine 3′,5′-phosphoric acid [^{125}I]2′,*O*-succinyl (>150 Ci/mmol), was purchased from ICN (Irvine, CA). Anti-cAMP was obtained from Miles (Elkhart, IN). Human PTHrP-(1–34) was obtained from Peninsula Laboratory, Inc. (Belmont, CA). Monoclonal antibody to tumor necrosis factor (TNF) was a gift from Dr. Anthony Cerami (Laboratory of Medical Biochemistry, Rockefeller University) and the Chiron Corp. (Emeryville, CA). Recombinant human IL-1α, IL-1β, and rabbit antihuman IL-1α were obtained from Genzyme (Boston, MA).

Results

Actions of squamous cell carcinoma CM on bone in organ culture

CM from two human squamous cell carcinoma cell lines (SCC-9 and SCC-13) caused a significant increase in bone resorption in neonatal mouse calvariae (Fig. 1). Fresh unconditioned medium or medium conditioned by irradiated 3T3 feeder cells failed to stimulate bone resorption. Although decreases in medium calcium and PGE_2 concentrations were noted with 3T3 CM, they did not reach statistical significance, and this was not a consistent finding in several additional experiments. Both SCC-9 and SCC-13 CM caused a significant increase in PGE_2 production (Fig. 1). SCC-9 CM was chosen for further characterization of the biological activity.

To determine to what extent the bone resorption induced by SCC-9 CM was mediated by PGE_2, the following experiments were performed. Indomethacin, an inhibitor of PGE_2 synthesis in mouse calvariae, only partially inhibited the BRSA of SCC-9 CM (Fig. 2A), although it completely inhibited the SCC-9 CM-induced increase in medium PGE_2. Thus, SCC-9 CM-induced bone resorption appears to be mediated by both PG-dependent and -independent pathways.

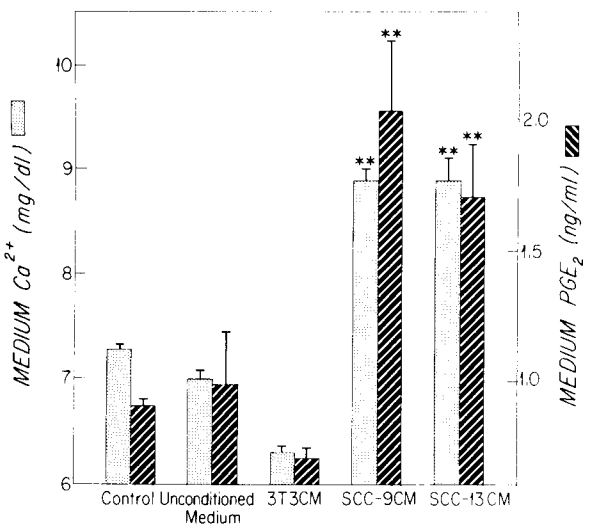

FIG. 1. CM from two squamous cell carcinoma cell lines caused enhanced bone resorption and PGE_2 production in mouse calvariae. Neonatal calvariae were incubated with serum-containing SCC-9 and SCC-13 CM at a 1:5 dilution. SCC-9 and SCC-13 CM caused a significant increase in both medium Ca^{2+} and medium PGE_2. No increase in either medium Ca^{2+} or PGE_2 was obtained with fresh unconditioned medium or medium conditioned by 3T3 feeder cells. Each *bar* gives the mean value, and the *brackets* give the SE for five bones per group. **, Significantly different from control ($P < 0.01$).

Serum-free SCC-9 CM caused a significant increase in bone resorption (Fig. 2B), approximately equal to that observed in serum-containing CM (Fig. 2A). Serum-free SCC-9 CM also caused a large increase in medium PGE_2. Because serum-containing and serum-free SCC-9 CM demonstrated similar responses in mouse calvariae, further purification and experimentation were performed using only serum-free SCC-9 CM.

Gel filtration chromatography of CM

Serum-free CM from SCC-9 cells was concentrated 10-fold by lyophilization and fractioned by gel filtration using Bio-Gel P-60. Fractions were collected and pooled, and protein concentrations were determined; they were then assayed for both BRSA and PGE_2 production at a standardized protein concentration. The BRSA eluted after the bulk of the protein as a single broad peak corresponding to an approximate mol wt of 17–20K (Fig. 3). PGE_2 synthesis-stimulating activity also eluted as a single broad peak coincident with that of the BRSA (Fig. 3A). Neither biological activity eluted with the majority of the protein contained in SCC-9 CM. Data from an

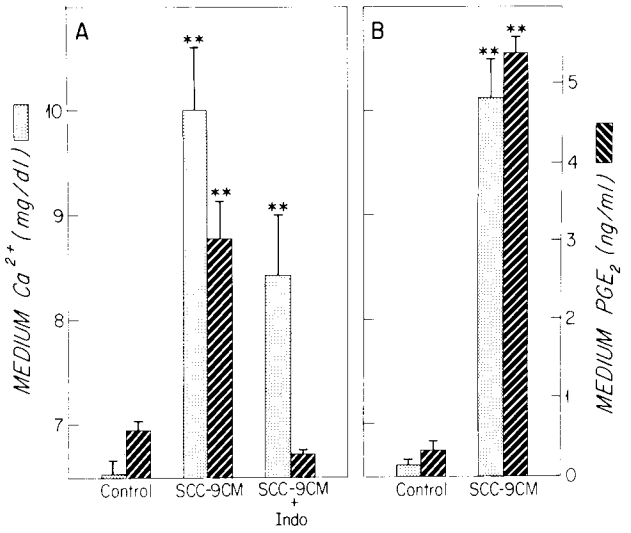

FIG. 2. A, Effects of inhibition of PG synthesis on SCC-9 CM-induced bone resorption. Serum-containing SCC-9 CM was used at a 1:5 dilution. Addition of 200 ng/ml indomethacin (Indo) only partially inhibited the SCC-9 CM-induced increase in medium Ca^{2+}, but it completely inhibited the SCC-9 CM-induced increase in medium PGE_2. The concentrations of PGE_2 in medium of bones treated with Indo alone and Indo plus SCC-9 CM were not different (data not shown). B, Serum-free SCC-9 CM contained BRSA and PG synthesis-stimulating activity. Serum-free SCC-9 CM at a 1:5 dilution caused a quantitatively similar increase in medium Ca^{2+}, as did serum-containing SCC-9 CM (A). Both serum-containing and serum-free CM were collected from SCC-9 cells at the same density. Each *bar* gives the mean value, and the *brackets* give the SE for four bones per group. **, Significantly different from control ($P < 0.01$). For medium Ca^{2+}, SCC-9 CM plus Indo was significantly ($P < 0.05$) lower than SCC-9 CM alone.

independent experiment (Fig. 3B) show that PG synthesis-stimulating activity, measured as 6-keto-$PGF_{1\alpha}$, which is a stable hydrolytic product of PGI_2, also cochromatographed with the BRSA and PGE_2 synthesis-stimulating activities. These results demonstrate the consistency of the chromatography of different batches of SCC-9 CM.

The active fractions from the Bio-Gel P-60 gel column (termed SCM_I) were pooled, and their biological properties were investigated. SCM_I caused a significant increase in bone resorption at a concentration as low as 0.10 μg protein/ml (Table 1); however, at the 0.10 μg protein/ml level, SCM_I-induced bone resorption was not associated with an increase in medium PGE_2. When the concentration of SCM_I was increased to 1.0 μg protein/ml there was an increase in medium PGE_2. SCM_I alone did not contain measurable amounts of PGE_2 (data not shown). When PG synthesis was inhibited by indomethacin, the SCM_I-induced bone resorption was inhibited only partially, although PGE_2 production was completely inhibited. Thus, it appears that SCM_I caused bone resorption through both PG-dependent and PG-independent mechanisms. In addition, both the BRSA and PG synthesis-stimulating activities of SCM_I were greatly reduced or abolished by heating at 100 C for 15 min (Table 1).

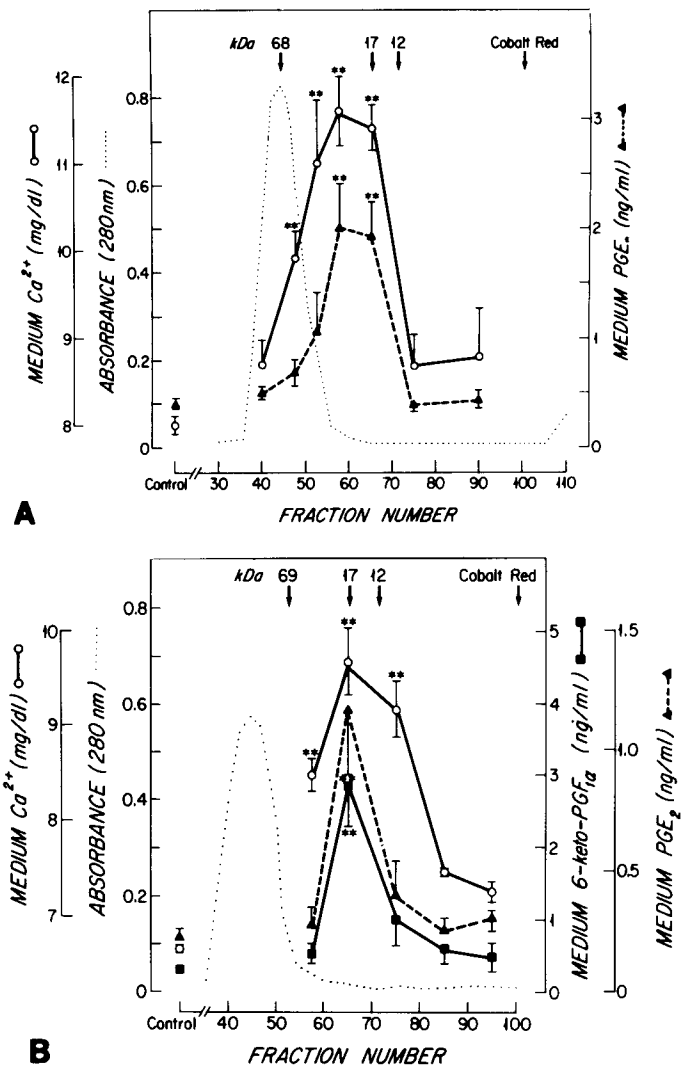

FIG. 3. Gel filtration chromatography of serum-free SCC-9 CM revealed a single broad peak of BRSA and PG synthesis-stimulating activity. SCC-9 CM was concentrated by lyophilization and chromatographed on Bio-Gel P-60 at 4 C. Five-milliliter fractions were collected, and absorbance (A_{280}) was measured (· · ·). Results from two separate pools of SCC-9 CM are shown in A and B. The following fractions were pooled: A, 38–44, 45–48, 49–54, 55–60, 61–70, 71–80, and 81–100; and B, 55–60, 61–70, 71–80, 81–90, and 91–100. Each pool was assayed for BRSA (○—○) and PGE_2 synthesis-stimulating activity (▲– –▲) or PGI_2 synthesis-stimulating activity (■—■). The values obtained were plotted at the midpoint of the fraction number representing each pool. Pooled fractions were assayed at 2.0 μg protein/ml (A) or 2.5 μg protein/ml (B). Single broad comigrating peaks of BRSA and PG synthesis-stimulating activities were noted at 17–20K. Active fractions were pooled and termed SCM_I. Each *point* gives the mean value, and the *brackets* give the SE for four bones per group. **, Significantly different from control ($P < 0.01$).

HPLC purification of SCM_I

SCM_I was fractioned by HPLC anion exchange chromatography. Aliquots (3 μl) were taken from the 1-ml fractions, diluted 10-fold (~50 ng protein/ml), and assayed for BRSA. The biological activities eluted in a distinct peak at about 0.4 M NaCl (Fig. 4). The BRSA

TABLE 1. Characterization of SCM_I

Treatment	Dose (μg protein/ml)	Medium Ca^{2+} (mg/dl)	Medium PGE_2 (ng/ml)
None		6.7 ± 0.09	0.14 ± 0.01
SCM_I	1.0	10.7 ± 0.53[a]	1.6 ± 0.67[a]
SCM_I	0.10	8.5 ± 0.64[a]	0.13 ± 0.02
SCM_I	0.01	7.2 ± 0.14	0.10 ± 0.01
SCM_I + Indo	1.0	8.4 ± 0.41[b]	0.11 ± 0.02
SCM_I (100 C; 15 min)	1.0	7.6 ± 0.06	0.14 ± 0.03

Values are the mean ± SE (four bones per group; 72-h treatment). Neonatal mouse calvariae were incubated with various concentrations of SCM_I. Indomethacin (Indo) was used at a concentration of 200 ng/ml.

[a] Significantly greater than no treatment control ($P < 0.01$).

[b] Significantly greater than no treatment control ($P < 0.05$). Significantly less than SCM_I (1 μg/ml) alone ($P < 0.05$).

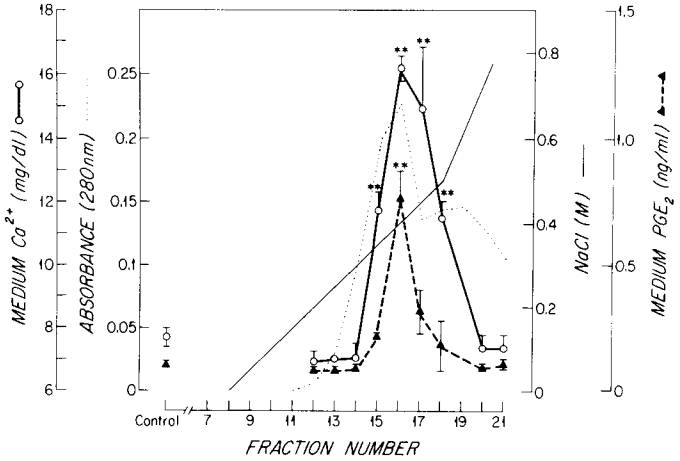

FIG. 4. HPLC ion exchange chromatography of SCM_I. Active fractions from gel filtration chromatography were pooled and dialyzed against 0.02 M HEPES, pH 7.4. A 20-ml sample was chromatographed on a Mono-Q anion exchange HPLC column. One-milliliter fractions were eluted by increasing NaCl concentrations (——). Absorbance was measured at 280 nm (· · ·). Each fraction was assayed for BRSA (O—O) and PG synthesis-stimulating activity (▲– –▲) at dilutions of 0.3 μl/ml bone culture medium. The protein concentrations of the active fractions were 47, 52, 27, and 33 ng/ml for fractions 15, 16, 17, and 18, respectively. BRSA and PG synthesis-stimulating activities coeluted at about 0.4 M NaCl. Fractions 15–18 were pooled and called SCM_{II}. Each *point* gives the mean value, and the *brackets* give the SE for four bones per group. **, Significantly different from control ($P < 0.01$).

again appeared to cochromatograph with the PG synthesis-stimulating activity. The active fractions (tubes 15–18) were pooled and dialyzed for further testing. The pooled active fraction, SCM_{II}, accounted for 22% of the total protein placed on the column.

Dose response of SCM_{II}

SCM_{II} caused a significant increase in bone resorption at 20 ng protein/ml and gave a maximum response at 0.5 μg protein/ml (Fig. 5). There was a parallel dose-dependent increase in BRSA and PGE_2 production caused by

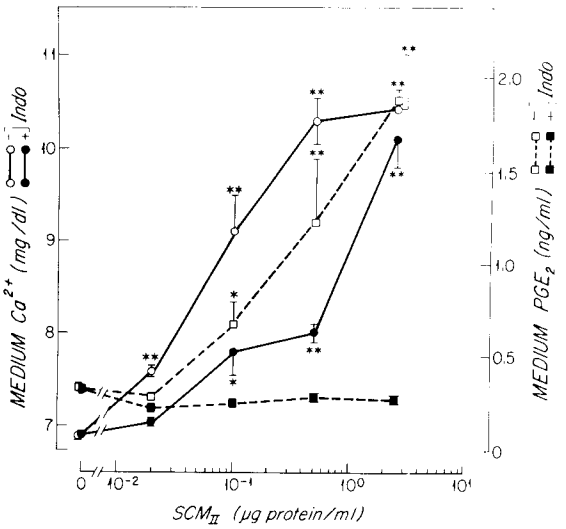

FIG. 5. Dose response for SCM_{II} on bone resorption and PGE_2 production. A significant increase in medium Ca^{2+} (O–O) was obtained at 20 ng protein/ml. SCM_{II} also increased medium PGE_2 (□– –□) in a dose-dependent manner. Addition of 200 ng/ml indomethacin (Indo; ●—●) partially inhibited the SCM_{II}-induced increase in medium Ca^{2+} at submaximal concentrations, but at supramaximal concentrations of SCM_{II}, Indo failed to inhibit SCM_{II}-induced bone resorption. Indo completely inhibited the SCM_{II}-induced increase in PGE_2 (■– –■). Each *point* gives the mean value, and the *brackets* give the SE for five bones per group. *, Significantly different from control ($P < 0.05$). **, Significantly different from control ($P < 0.001$).

SCM_{II} at concentrations of 100 ng protein/ml and above. However, when indomethacin was used to inhibit PGE_2 synthesis completely, SCM_{II}-induced bone resorption was inhibited only partially, and the dose-response curve was shifted to the right (Fig. 5). In addition, at high concentrations of SCM_{II}, indomethacin failed to inhibit SCM_{II}-induced bone resorption. These results again indicate that SCM_{II} stimulates bone resorption via PG-dependent and PG-independent mechanisms.

Immunoneutralization studies with SCM_{II}

Antibody neutralization studies were performed to characterize the biologically active bone resorption-stimulating factor(s) in SCM_{II}. Because IL-1α and -β both stimulate bone resorption (24–26), enhance PGE_2 production (25–27), and have mol wts similar to that of SCM_{II} (28), the effects of neutralizing antibodies to IL-1α and -β on SCM_{II}-induced BRSA were investigated.

Rabbit antihuman IL-1β completely neutralized recominbant human (rh) IL-1β-induced bone resorption (Fig. 6A), but did not inhibit the action of rhIL-1α (Fig. 6B). Anti-IL-1β failed to inhibit SCM_{II}-stimulated bone resorption (Fig. 6C). Rabbit antihuman IL-1α completely neutralized rhIL-1α-induced bone resorption, while it failed to inhibit rhIL-1β-induced BRSA (Fig. 7A). These data demonstrate that in the mouse calvarial bioassay, anti-IL-1α and anti-IL-1β are specific for their homolo-

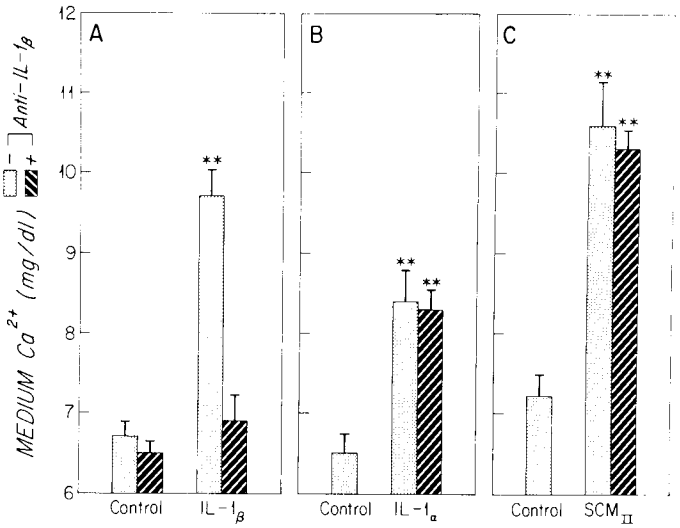

FIG. 6. Antibody to human IL-1β completely neutralized IL-1β-induced bone resorption, but had no effect on SCM_{II}-induced bone resorption. Both rhIL-1β at 5 U/ml (A) and rhIL-1α at 1 U/ml (B) caused significant increases in medium Ca^{2+}. When antihuman IL-1β antibody (1:250 dilution) was preincubated with rhIL-1β at 5 U/ml for 90 min at 37 C, there was no subsequent increase in medium Ca^{2+} (A). Incubation with IL-1β antibody had no effect on rhIL-1α-stimulated bone resorption (B). SCM_{II} (500 ng protein/ml) caused a significant increase in medium Ca^{2+} that was unaffected by preincubation with IL-1β antibody (C). Each *bar* gives the mean value, and the *brackets* give the SE for five bones per group. **, Significantly different from control ($P < 0.01$).

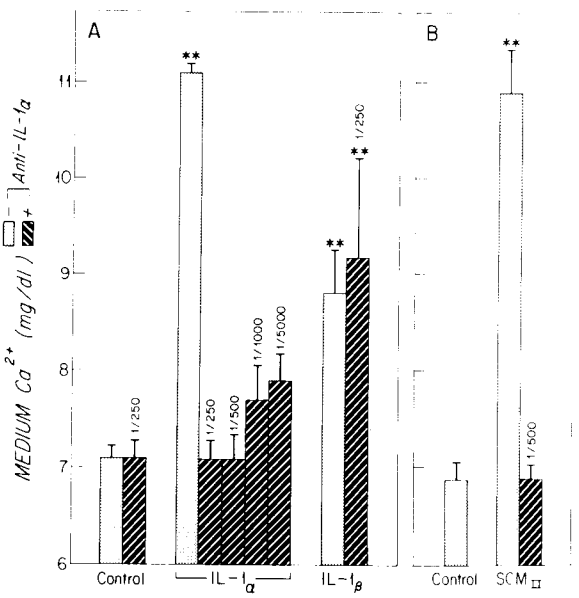

FIG. 7. Antibody to rhIL-1α completely neutralized IL-1α-induced bone resorption. A, Preincubation of rhIL-1α at 1 U/ml with antibody to rhIL-1α (up to a dilution of 1:5000) completely neutralized rhIL-1α-induced increases in medium Ca^{2+}. The rhIL-1α antibody showed no cross-reactivity with rhIL-1β. B, Antibody to rhIL-1α completely neutralized SCM_{II}-induced bone resorption. SCM_{II} (500 ng protein/ml) caused a significant increase in medium Ca^{2+}. Preincubation of SCM_{II} with IL-1α antibody (1:500 dilution) completely inhibited the SCM_{II}-induced increase in medium Ca^{2+}. Each *bar* gives the mean value, and the *brackets* give the SE for five bones per group. **, Significantly different from control ($P < 0.01$).

gous IL-1s. SCM_{II}-induced bone resorption was completely neutralized by anti-IL-1α antibody (Fig. 7B). Nonimmune rabbit serum (diluted 1:250) did not inhibit SCM_{II}-induced bone resorption (data not shown). Thus, the neutralization was specific and not due to protease activity in rabbit serum. These results are consistent with the conclusion that most, if not all, of the BRSA in SCM_{II} is due to IL-1α. In results not shown, the bone culture media obtained from the experiments shown in Fig. 7 were also assayed for PGE_2 and 6-keto-$PGF_{1\alpha}$; anti-IL-1α (1:250 and 1:500) completely inhibited the production of PGE_2 and PGI_2 induced by IL-1α and SCM_{II}.

TNF has been shown to stimulate secretion of IL-1 from monocytes (29) and endothelial cells (30). Moreover, there is evidence that IL-1 can induce the secretion of TNF by monocytes (31). TNF has also been shown to stimulate bone resorption in mouse calvariae (32). Although TNF is characteristically produced by monocytes/macrophages, its multiple biological similarities with IL-1 and bidirectional interaction with IL-1 prompted us to investigate a possible role for TNF in SCM_{II}-induced bone resorption. Antibody to human TNF inhibited completely TNF-induced bone resorption, but it failed to inhibit the BRSA of SCM_{II} (Fig. 8A). In addition, anti-TNF did not cross-react with rhIL-1α (Fig.

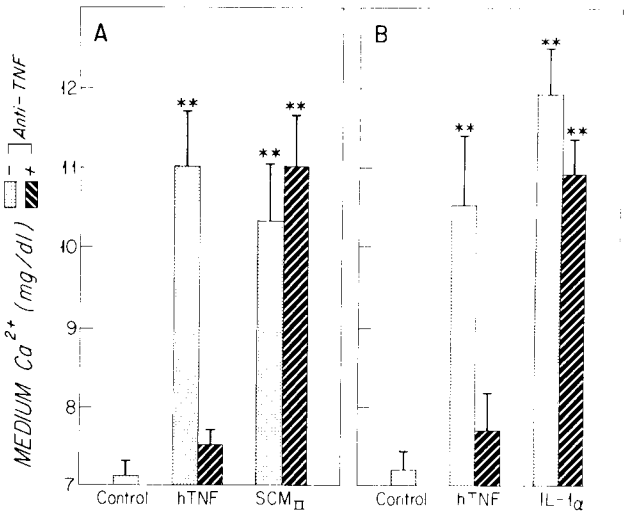

FIG. 8. Antibody to TNF did not inhibit SCM_{II}-induced bone resorption. hTNF (10 ng/ml) caused a significant increase in medium Ca^{2+} that was completely inhibited by preincubation with 5 μg/ml TNF antibody. In contrast, preincubation with TNF antibody (5 μg/ml) had no effect on either the SCM_{II}-induced (500 ng protein/ml) or the IL-1α-induced (1 U/ml) increases in medium Ca^{2+}. Each *bar* gives the mean value, and the *brackets* give the SE for five bones per group. **, Significantly different from control ($P < 0.01$).

8B). These data show that TNF is not the bone resorption-stimulating factor in SCM_{II} and strongly suggest that SCM_{II}, which contains IL-1α, does not act via enhanced TNF production by mouse calvariae.

It is possible that the unfractionated SCC-9 CM contained multiple factors having the potential to cause bone resorption, and that in the process of isolation of SCM_{II}, some of these factors may have been separated. To test this hypothesis, immunoneutralization studies were performed. Antibody to IL-1α completely inhibited the BRSA of crude SCC-9 and SCC-13 CM (Fig. 9), but had no effect on the activity of hPTHrP-(1–34). These data are consistent with the conclusion that the BRSA produced by these two squamous cell carcinoma cell lines is IL-1α and not PTHrP.

Effects of SCC-9 and SCC-13 CM on cAMP production

Other investigators have shown that CM from squamous cell carcinomas contains PTHrP (2, 5) and that PTHrP can cause bone resorption (6, 7, 33). Because PTHrP causes an increase in cAMP production in PTH target cells, we studied the actions of various preparations of squamous cell carcinoma CM on cAMP production in ROS 17/2.8 bone cells.

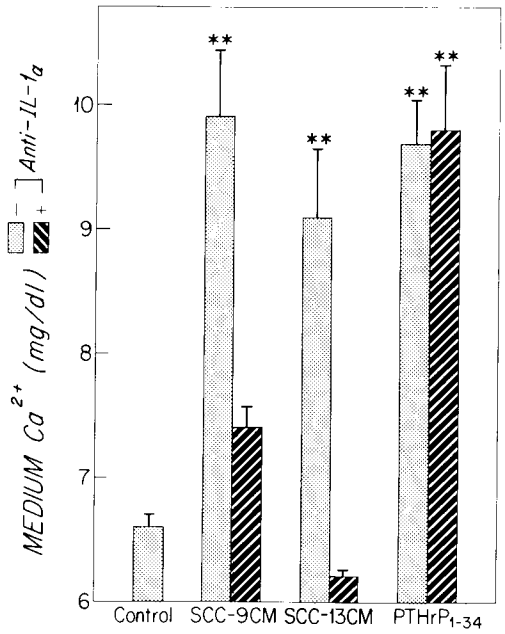

FIG. 9. Effects of IL-1α antibody on the BRSA of crude SCC-9 and SCC-13 CM. Crude SCC-9 and SCC-13 CM increased medium Ca^{2+} at dilutions of 1:10 and 1:5, respectively. Preincubation of SCC-9 and SCC-13 CM with IL-1α antibody (1:500 dilution) completely neutralized the increases in medium Ca^{2+}. The small residual effect of SCC-9 CM in the presence of antibody was not statistically significant and was not seen in a repeat experiment. IL-1α antibody had no effect on hPTHrP-(1–34) (5 ng/ml). Each *bar* gives the mean value, and the *brackets* give the SE for five bones per group. **, Significantly different from control ($P < 0.01$).

Neither crude SCC-9 CM nor SCC-13 CM increased cAMP production in ROS 17/2.8 cells (Table 2). In addition, the more purified forms of SCC-9 CM, SCM_I and SCM_{II}, also failed to enhance cAMP production at concentrations that had marked BRSA in the bone culture assay (Table 2). rhIL-1α did not stimulate cAMP production. These data indicate that SCC-9, SCC-13 CM, SCM_I, and SCM_{II}, which all contain marked BRSA, do not contain measurable quantities of the PTH-like factor, or if present, the concentrations are below the level of detection of our assay methods.

IL-1 biological activity in squamous cell carcinoma CM

Using a thymocyte proliferation bioassay, IL-1-like activity was detected in both SCM_I and SCM_{II} (Table 3). Unpurified SCC-9 CM and SCC-13 CM did not demonstrate detectable IL-1-like activity with this bioassay. It is likely that the crude CM interfered with the bioassay, and thus, IL-1-like activity was not detected.

Measurement of IL-1α and -β in squamous cell carcinoma CM

Measurement of IL-1-like bioactivity by a thymocyte proliferation bioassay alone has certain disadvantages. The most relevant drawbacks are that other factors or cytokines contained in crude samples can interfere with the assay by either inhibiting or enhancing the biological response, and the bioassay does not discriminate between IL-1α and IL-1β. Direct quantitation of IL-1α and IL-1β in squamous cell carcinoma CM was accomplished with

TABLE 2. Effects of squamous cell carcinoma CM on cAMP production by ROS 17/2.8 bone cells

Treatment	Medium cAMP (pmol/ml)[a]
None	3.4 ± 0.13
PTHrP-(1–34) (10 ng/ml)	19 ± 0.72[b]
Unconditioned SCC-9 medium[c] (1:5 dilution)	4.1 ± 0.67
Unconditioned SCC-13 medium[d] (1:5 dilution)	1.7 ± 0.39
3T3 CM[b] (1:5 dilution)	1.4 ± 0.22
SCC-9 CM (1:5 dilution)	3.6 ± 0.19
SCC-13 CM (1:5 dilution)	3.6 ± 0.26
SCM_I (5 μg protein/ml)	2.6 ± 0.10
SCM_{II} (0.5 μg protein/ml)	2.4 ± 0.16
rhIL-1α (1 U/ml)	3.2 ± 0.43

Rat osteosarcoma cells (ROS 17/2.8) were grown to confluence in 35-mm dishes and fed 24 h before the experiment. The cells were washed twice with serum-free F-10. Test and control agents in serum-free F-10 with 100 μM IBMX were added. The cells were then incubated for 60 min at 37 C in 5% CO_2-95% air, and the medium was collected, boiled for 5 min, and then frozen at −20 C until RIA for cAMP.

[a] Values are the mean ± SE of triplicate samples, each assayed in duplicate.

[b] Significantly greater than no treatment control ($P < 0.01$)

[c] Serum-free.

[d] Serum-containing.

TABLE 3. IL-1-like biological activity in squamous cell carcinoma CM

Treatment	Dose/culture	[^{3}H]Thymidine incorporation (cpm × 10^{-3})[a]	IL-1-like activity (U/ml)[a]
Unconditioned SCC-9 medium	40 μl	<1	<1
Unconditioned SCC-13 medium[b]	40 μl	Inhibitory[c]	
3T3 CM	40 μl	<1	<1
SCC-9 CM	100 μl	<1	<1
SCC-13 CM[b]	40 μl	Inhibitory[c]	<1
SCM_I	50 ng	25 ± 0.37	143 ± 2.16
SCM_{II}	2.5 ng	30 ± 0.96	353 ± 11.2

The bioassay for IL-1-like activity measured thymocyte proliferation as described in *Materials and Methods*. A unit of IL-1 was defined as that amount which resulted in half-maximal incorporation of [^{3}H]thymidine, which was determined to be 17,235 cpm.

[a] Mean ± SE for four samples.

[b] Contained serum, other samples did not.

[c] Medium alone inhibited thymocyte proliferation.

specific ELISAs. SCC-9 CM, SCC-13 CM, and the purified fractions all contained measurable concentrations of IL-1α (Table 4). Unconditioned medium controls and 3T3 fibroblast CM did not contain detectable amounts of either IL-1α or IL-1β. The concentration of IL-1α in SCM_I was 17-fold higher than that in the crude SCC-9 CM, while that in SCM_{II} was 1.6-fold higher than the level in SCM_I. SCM_I did contain measurable immunoreactive IL-1β, but at the concentrations used in the bone resorption assay, these low amounts would not have caused bone resorption. Using the results of the immunoassay to calculate the amounts of IL-1α in the crude squamous cell carcinoma CM, it can be determined that there was sufficient IL-1α present to account for all of the BRSA detected in the bone resorption bioassay (34).

Discussion

We have shown that CM from two squamous cell carcinoma cell lines, which were derived from tumors not associated with HHM at the time of biopsy, contained potent BRSA before chromatographic processing and failed to increase cAMP production in bone cells. SCC-9 CM and SCC-13 CM both contained high concentrations of immunoreactive IL-1α. In addition, SCC-9 and SCC-13 CM-induced bone resorption was neutralized completely by preincubation with antibody to IL-1α. In contrast, little or no immunoreactive IL-1β was found in SCC-9 and SCC-13 CM, nor was the BRSA inhibited by anti-IL-1β. These results lead us to conclude that IL-1α was released by these squamous carcinoma cells and is the primary mediator of the enhanced bone resorption found in the CM of cultures of these cell lines.

TABLE 4. Measurement of IL-1α and -β in squamous cell carcinoma CM by ELISA

Sample	IL-1β (ng/ml)	IL-1α (ng/ml)
Unconditioned SCC-9 medium	<0.1	<0.1
Unconditioned SCC-13 medium	<0.1	<0.1
3T3 CM	<0.1	<0.1
SCM-9 CM	<0.1	1.5 ± 0.04
SCM-13 CM	<0.1	1.4 ± 0.23
SCM_I	1.5 ± 0.1	25 ± 1.0
SCM_{II}	<0.1	40 ± 1.7

Concentrations of IL-1α and -β were measured by ELISA as described in *Materials and Methods*. Values are the mean ± range of duplicate samples, each assayed in duplicate.

To characterize further the BRSA, CM was fractionated by gel filtration and subsequent HPLC ion exchange chromatography. The active fractions contained a major peak of BRSA that was coincident with PGE_2-stimulating activity. SCM_{II} increased bone resorption in a dose-dependent manner from 20 to 500 ng/ml, but it failed to increase cAMP in either calvariae or bone cells. Although SCM_{II} contained PGE_2-stimulating activity, SCM_{II}-induced BRSA was only partially inhibited when PGE_2 synthesis was inhibited completely. This is consistent with the mechanism of action we have found for recombinant human IL-1α (34). SCM_{II} contained high concentrations of immunoreactive and biologically active IL-1α, and antibody to IL-1α completely inhibited SCM_{II}-induced bone resorption. The antibody showed no cross-reactivity with either hPTHrP or IL-1β. Although cross-reactivity of this antiserum with other potential bone resorption-stimulating factors cannot be ruled out entirely, we consider that possibility unlikely. Taken together these results are consistent with the conclusion that the BRSA in SCM_{II} is IL-1α. Moreover, the concentration of IL-1α present in SCM_{II} was sufficient to account for all of the BRSA present.

Both crude CM and CM fractions in several states of increasing purity, which exhibited BRSA, did not stimulate cAMP production in bone cells. PTHrP, if present, should have been detected in these fractions, as they were in the proper mol wt range. Even if other unassayed fractions contained PTHrP, the protein was not present in sufficient quantity to stimulate bone resorption.

HHM is a syndrome associated with a variety of malignant neoplasms and has often been linked with tumors of squamous cell origin (1, 35). Several squamous cell carcinomas associated with HHM have been found to produce PTHrP and/or IL-1α. Sato *et al.* (8–10) have found that IL-1α alone or together with a PTH-like factor is produced by some squamous cell carcinomas associated with HHM. The relative importance of PTHrP and IL-1α in HHM *in vivo* is still not clear. In fact, it has been shown that normal human keratinocytes produce multiple bone resorption-stimulating factors (36), including PTH-like (37) and IL-1-like (38–41) factors. We have found potent BRSA from two squamous cell carcinomas derived from patients lacking the clinical signs of HHM. We have shown that these tumor cells produce IL-1α and that all of the BRSA could be accounted for by IL-1α production. Accurate quantification of IL-1α was established by a newly developed ELISA for IL-1α. It is clear, therefore, that squamous cell carcinomas not associated with HHM produce IL-1α. Thus, since production of IL-1α is not exclusive to tumors producing HHM, it is probable that an additional mechanism(s) is needed to cause HHM. Whether these mechanisms include synergism of IL-1α with other factors, such as PTHrP, or an increase in the quantity of secreted IL-1α is presently unknown. Sato *et al.* (8), however, have preliminary evidence supporting the concept of synergism between IL-1α and a PTH-like factor. In addition, because IL-1α does not cause an increase in cAMP production, it is unlikely to be the sole mediator of HHM.

Tumor-derived PTHrP was isolated and purified solely on the basis of its ability to stimulate cAMP production in PTH target cells. Thus, other potential stimulators of bone resorption that do not act via enhanced cAMP production, such as IL-1α, could have been overlooked. Nevertheless, it is important to recognize that IL-1α production by squamous cell carcinomas does not correlate directly with clinical HHM.

Acknowledgments

The authors thank Dr. J. G. Rheinwald for providing SCC-9 and SCC-13 cell lines; Dr. Anthony Cerami for the anti-TNF; Yolanda Santo, Joan Joseph, Xianhui Rong, and Thomas Bosma for expert assistance; and Ms. Jean Foley for her excellent help in the preparation of this manuscript.

References

1. Martin TJ 1988 Humoral hypercalcemia of malignancy. Bone Mineral 4:83
2. Moseley JM, Kubota M, Diefenbach-Jagger H, Wettenhall REH, Kemp BE, Suva LJ, Rodda CP, Ebeling PR, Hudson PJ, Zajac JD, Martin TJ 1987 Parathyroid hormone-related protein purified from a human lung cancer cell line. Proc Natl Acad Sci USA 84:5048
3. Burtis WJ, Wu T, Bunch C, Wysolmerski JJ, Insogna KL, Weir EC, Broadus AE, Stewart AF 1987 Identification of a novel 17,000-dalton parathyroid hormone-like adenylate cyclase-stimulating protein from a tumor associated with humoral hypercalcemia of malignancy. J Biol Chem 262:7151
4. Suva LJ, Winslow GA, Wettenhall REH, Hammonds RG, Moseley JM, Diefenbach-Jagger H, Rodda CP, Kemp BE, Rodriguez H, Chen EY, Hudson PJ, Martin TJ, Wood WI 1987 A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression. Science 237:893
5. Strewler GJ, Stern PH, Jacobs JW, Eveloff J, Klein RF, Leung SC, Rosenblatt M, Nissenson RA 1987 Parathyroid hormone-like protein from human renal carcinoma cells. Structural and functional homology with parathyroid hormone. J Clin Invest 80:1803
6. Horiuchi N, Caulfield MP, Fisher JE, Goldman ME, McKee RL, Reagan JE, Levy JJ, Nutt RF, Rodan SB, Schofield TL, Clemens TL, Rosenblatt M 1987 Similarity of synthetic peptide from human tumor to parathyroid hormone *in vivo* and *in vitro*. Science 238:1566
7. Kemp BE, Moseley JM, Rodda CP, Ebeling PR, Wettenhall REH, Stapleton D, Diefenbach-Jagger H, Ure F, Michelangeli VP, Simmons HA, Raisz LG, Martin TJ 1987 Parathyroid hormone-related protein of malignancy: active synthetic fragments. Science 238:1568
8. Sato K, Fujii Y, Kasono K, Tsushima T, Shizume K 1988 Production of interleukin-1α and a parathyroid hormone-like factor by a squamous cell carcinoma of the esophagus (EC-GI) derived from a patient with hypercalcemia. J Clin Endocrinol Metab 67:592
9. Sato K, Mimura H, Han DC, Kakiuchi T, Ueyama Y, Ohkawa H, Okabe T, Kondo Y, Ohsawa N, Tsushima T, Shizume K 1986 Production of bone-resorbing activity and colony-stimulating activity *in vivo* and *in vitro* by a human squamous cell carcinoma associated with hypercalcemia and leukocytosis. J Clin Invest 78:145
10. Sato K, Fujii Y, Ono M, Nomura H, Shizume K 1987 Production of interleukin-1α-like factor and colony-stimulating factor by a squamous cell carcinoma of the thyroid (T3M-5) derived from a patient with hypercalcemia and leukocytosis. Cancer Res 47:6474
11. Rheinwald JG, Beckett MA 1981 Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. Cancer Res 41:1657
12. Allen-Hoffman BL, Rheinwald JG 1984 Polycyclic aromatic hydrocarbon mutagenesis of human epidermal keratinocytes in culture. Proc Natl Acad Sci USA 81:7802
13. Majeska RJ, Rodan SB, Rodan GA 1980 Parathyroid hormone-responsive clonal cell lines from rat osteosarcoma. Endocrinology 107:1494
14. Robinson DR, Tashjian Jr AH, Levine L 1975 Prostaglandin-stimulated bone resorption by rheumatoid synovia. A possible mechanism for bone destruction in rheumatoid arthritis. J Clin Invest 56:1181
15. Ivey JL, Wright DR, Tashjian Jr AH 1976 Bone resorption in organ culture. Inhibition by the divalent cation ionophores A23187 and X-537A. J Clin Invest 58:1327
16. Levine L, Van Vunakis H 1970 Antigenic activity of prostaglandins. Biochem Biophys Res Commun 41:1171
17. Levine L, Gutierrez Cernosek RM, Van Vunakis H 1971 Specificities of prostaglandin B_1, $F_{1\alpha}$ and $F_{2\alpha}$ antigen-antibody reactions. J Biol Chem 246:6782
18. Gaudet RJ, Alam I, Levine L 1980 Accumulation of cyclooxygenase products of arachidonic acid metabolism in gerbil brain during reperfusion after bilateral common cortid artery occlusion. J Neurochem 35:653
19. Levine L 1986 Measurement of arachidonic acid metabolites by radioimmunoassay. In: Fahey JL, Friedman H, Rose NR (eds) Manual of Clinical Laboratory Immunology, ed 3. American Society of Microbiology, Washington DC, p 685
20. Boland CJ, Fried RM, Tashjian Jr AH 1986 Measurement of cytosolic free Ca^{2+} concentrations in human and rat osteosarcoma cells: actions of bone resorption-stimulating hormones. Endocrinology 118:980
21. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC

1985 Measurement of protein using bicinchoninic acid. Anal Biochem 150:76
22. Gaffney EV, Koch GA, Tsai SCJ, Lingenfelter SE, Piscitelli J 1987 Quantitation of human interleukin-1β with polyclonal antibodies. J Immunol Methods 101:271
23. Gaffney EV, Chu CW, Lingenfelter SE, Lisi PJ, Koch GA 1987 Enzyme linked immunoassay with monoclonal antibody for human interleukin-1β. Biotechniques 5:652
24. Dewhirst FE, Stashenko PP, Mole JE, Tsurumachi T 1985 Purification and partial sequence of human osteoclast-activating factor: identity with interleukin-1 β. J Immunol 135:2562
25. Lorenzo JA, Sousa SL, Alander C, Raisz LG, Dinarello CA 1987 Comparison of the bone-resorbing activity in the supernatants from phytohemagglutinin-stimulated human peripheral blood mononuclear cells with that of cytokines through use of an antiserum to interleukin-1. Endocrinology 121:1164
26. Sato K, Fujii Y, Kasono K, Saji M, Tsushima T, Shizume K 1986 Stimulation of prostaglandin E2 and bone resorption by recombinant human interleukin 1 alpha in fetal mouse bones. Biochem Biophys Res Commun 138:618
27. Sato K, Fujii Y, Asano S, Ohtsuki T, Kawakami M, Kasono K, Tsushima T, Shizume K 1986 Recombinant human interleukin-1 alpha and beta stimulate mouse osteoblast-like cells (MC3T3-E1) to produce macrophage-colony stimulating activity and prostaglandin E2. Biochem Biophys Res Commun 141:285
28. Dinarello CA 1985 An update on human interleukin-1: from molecular biology to clinical relevance. J Clin Immunol 5:287
29. Dinarello CA, Cannon JG, Wolff SM, Bernheim HA, Beulter B, Cerami A, Figari IS, Palladino Jr MA, O'Connor JV 1986 Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J Exp Med 163:1433
30. Nawroth PO, Bank I, Handley D, Cassimeris J, Chess L, Stern D 1986 Tumor necrosis factor/cachectin interacts with endothelial cell receptors to induce release of interleukin 1. J Exp Med 163:1363
31. Philip R, Epstein LB 1986 Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin-1. Nature 323:86
32. Tashjian Jr AH, Voelkel EF, Lazzaro M, Goad D, Bosma T, Levine L 1987 Tumor necrosis factor-α (cachectin) stimulates bone resorption in mouse calvaria via a prostaglandin-mediated mechanism. Endocrinology 120:2029
33. Fukayama S, Bosma TJ, Goad DL, Voelkel EF, Tashjian Jr AH 1988 Human parathyroid hormone (PTH)-related protein and human PTH: comparative biological activities on human bone cells and bone resorption. Endocrinology 123:2841
34. Bosma T, Levine L, Tashjian Jr AH 1988 Recombinant human interleukin-1α and -1β: Comparative activities and actions on neonatal mouse calvariae. J Bone Mineral Res [Suppl 1] 3:S196 (Abstract 510)
35. Stewart AF, Insogna KL, Burtis WJ, Aminiafshar A, Wu T, Weir EC, Broadus AE 1986 Frequency and partial characterization of adenylate cyclase-stimulating activity in tumors associated with humoral hypercalcemia of malignancy. J Bone Mineral Res 1:267
36. Fried RM, Voelkel EF, Rice RH, Levine L, Tashjian Jr AH 1988 Evidence for multiple bone resorption-stimulating factors produced by normal human keratinocytes in culture. Endocrinology 122:2467
37. Merendino Jr JJ, Insogna KL, Milstone LM, Broadus AE, Stewart AF 1986 A parathyroid hormone-like protein from cultured human keratinocytes. Science 231:388
38. Luger TA, Stadler BM, Katz SI, Oppenheim JJ 1981 Epidermal cell (keratinocyte)-derived thymocyte-activating factor (ETAF). J Immunol 127:1493
39. Sauder DN 1985 Biologic properties of epidermal cell thymocyte-activating factor (ETAF). J Invest Dermatol 85:176s
40. Kupper TS, Ballard DW, Chua AO, McGuire JS, Flood PM, Horowitz MC, Langdon R, Lightfoot L, Gubler U 1986 Human keratinocytes contain mRNA indistinguishable from monocyte interleukin 1α and β mRNA. Keratinocyte epidermal cell-derived thymocyte activating factor is identical to interleukin 1. J Exp Med 164:2095
41. Bell TV, Harley CB, Stetsko D, Sauder DN 1987 Expression of mRNA homologous to interleukin 1 in human epidermal cells. J Invest Dermatol 88:375