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Evidence for Multiple Bone Resorption-Stimulating Factors Produced by Normal Human Keratinocytes in Culture*

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ABSTRACT. Conditioned medium from cultured normal human foreskin keratinocytes enhanced the release of calcium from neonatal mouse calvaria in organ culture. Unfractionated keratinocyte-conditioned medium (KCM) stimulated bone resorption in a dose-dependent manner, but it did not increase the concentration of prostaglandin E₂ (PGE₂) in the bone culture medium until a maximal dose of KCM for resorption was used. Furthermore, inhibitors of PGE₂ synthesis, indomethacin, ibuprofen, and piroxicam, did not inhibit KCM-induced calcium release. High concentrations of KCM increased cAMP production by calvaria in the presence of isobutylmethylxanthine, but the increase was small compared with that produced by a dose of bovine PTH that caused a similar level of bone resorption. The bone resorption-stimulating activity of KCM was not lost after incubation at 56 C for 60 min, but it was lost after heating at 100 C for 10 min. Fractionation of KCM by gel filtration chromatography revealed two distinct peaks of bone resorption-stimulating activity. One peak, KCM_I, caused a significant in-

crease in bone resorption at 2 µg protein/ml. KCM_I did not increase medium PGE₂, and inhibition of PGE₂ synthesis in bone had no effect on KCM_I-induced bone resorption. KCM_I failed to increase cAMP production by human osteosarcoma SaOS-2 cells. Another peak, KCM_{II}, caused a dose-dependent increase in bone resorption, and a significant increase in medium calcium was noted at a 20-fold lower concentration (0.1 µg protein/ml) than with KCM_I. In contrast to KCM_I, the increase in bone resorption stimulated by KCM_{II} was accompanied by a parallel increase in the production of PGE₂, and inhibition of PGE₂ synthesis completely inhibited the bone resorption-stimulating activity of KCM_{II}. KCM_{II} also caused an increase in cAMP production by SaOS-2 cells. We conclude that KCM contains at least two distinct bone resorption-stimulating factors, one of which acts via a PG-mediated mechanism and the other by a PG-independent pathway. (*Endocrinology* 122: 2467-2475, 1988)

KNOWLEDGE of the multiple immune and endocrine functions of epidermal keratinocytes has been evolving rapidly. Keratinocytes secrete various cytokines that may play a role in local immune responses. Among the first of these cytokines from keratinocytes to be studied was an interleukin-1-like (IL-1-like) molecule termed epidermal thymocyte-activating factor (ETAF) (1, 2). ETAF is pyrogenic and has chemotactic activity for neutrophils (3, 4). Keratinocytes also express mRNAs that are homologous or identical to monocyte-derived IL-1α and IL-1β (5, 6). In addition, keratinocytes have been shown to produce a natural killer cell activity-augmenting factor (7), a T cell growth factor (8), and an IL-3-like factor (9), suggesting a complex role for these

cells in immunological responses. Although IL-1 has been shown to cause bone resorption *in vitro* (10, 11), ETAF-induced bone resorption has not been documented.

Recently, normal human epidermal keratinocytes in culture have been found to produce a PTH-like protein (12) that has been characterized by its ability to increase cAMP in PTH target tissues and to be inhibited by the PTH antagonist PTH₍₃₋₃₄₎. An essential characteristic of any suspected PTH-like protein would be its ability to enhance bone resorption; however, keratinocyte-conditioned medium (KCM) has not been evaluated for bone resorption-stimulating activity. It is possible that factors released by keratinocytes may have a profound effect on local and systemic bone metabolism. Therefore, we have characterized the action of KCM on bone resorption. We have found that conditioned medium from normal human epidermal keratinocytes in culture markedly stimulates bone resorption *in vitro*. Furthermore, we have partially separated two distinct factors released by keratinocytes that cause bone resorption. One of these factors induces bone resorption via a prostaglandin (PG)-

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dependent mechanism, while the other mediates resorption by a PG-independent pathway.

Materials and Methods

Cell culture

Normal human foreskin keratinocytes were grown by standard procedures (13). Briefly, the cells were cultivated on an irradiated 3T3 feeder layer in Dulbecco-Vogt Eagle's Medium supplemented with hydrocortisone (0.4 μ g/ml), epidermal growth factor (10 ng/ml), insulin (5 μ g/ml), transferrin (5 μ g/ml), adenine (0.1 mM), T₃ (20 pM), and fetal bovine serum (5%). Four-day-old medium was collected from the cell cultures that were near or at confluence. This KCM was pooled and frozen at -20 C for later experimentation. Serum-free KCM (without fetal bovine serum or epidermal growth factor) as well as serum-containing KCM without epidermal growth factor were collected as described above.

The human osteosarcoma cell line SaOS was grown in monolayer culture, as described previously (14, 15).

Organ culture of bone

Neonatal mouse calvaria were placed in organ culture as free-floating bones, as previously described (16, 17). The medium was Dulbecco's Modified Eagle's Medium supplemented with 15% heat-inactivated (60 C for 1 h) horse serum. Incubation of half-calvaria (by sagittal sectioning into two symmetrical portions) was performed at 37 C in a roller drum apparatus under an atmosphere of 50% O₂, 5% CO₂, and 45% N₂. Calvaria 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 the release of calcium (⁴⁰Ca²⁺) from the calvaria into the culture medium 72 h after addition of the test agents.

Measurement of calcium

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

Measurement of PGE₂

PGE₂ produced by the bones and released into the culture medium was measured by RIA, as described previously in detail (18).

Measurement of cAMP

Calvaria were prepared and cultured as described above. After incubation for 24 h, the medium was removed, and calvaria were rinsed in serum-free Dulbecco's Medium. Washes were discarded. Serum-free control medium or medium containing specific test agents was added, and incubations were continued for 1 h (37 C; 50% O₂, 5% CO₂, and 45% N₂). Serum-free medium used for the 1-h incubation contained 3 μ M iso-

butylmethylxanthine (IBMX). After the 1-h incubation, medium samples were collected, placed in a boiling water bath for 5 min, and stored at -20 C. The cAMP concentration was measured by RIA (14, 19).

SaOS-2 cells were grown to confluence in 16-mm culture wells. Growth medium was aspirated, and the cells were washed twice with Hank's Balanced Salt Solution (HBSS). HBSS with 400 μ M IBMX in the absence or presence of various treatment agents was then added to the cells. The cells were incubated for 20 min (37 C; 95% air and 5% CO₂). After incubation, the medium was collected, boiled for 5 min, and stored at -20 C until measurement of cAMP by RIA (14, 19).

Gel chromatography

KCM was concentrated 10- to 15-fold by lyophilization and then chromatographed at 4 C on a siliconized Bio-Gel P-60 (2.5 $\times$ 110 cm; Bio-Rad Laboratories, Richmond, CA) column. The column was eluted using a 0.15-M NaCl solution 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.

Measurement of protein

The protein concentration in all samples was determined using a bicinchoninic acid (Pierce Chemical Co., Rockford, IL) assay (20).

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.

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 Dulbecco's Modified Eagle's Medium which was from M. A. Bioproducts (Walkersville, MD). Indomethacin, ibuprofen, and IBMX were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine PTH (bPTH; 190 U/mg) was used as a partially purified material from Wilson Laboratories (Chicago, IL). Piroxicam was a gift from Pfizer Pharmaceuticals (Groton, CT). Radioactive tracer for cAMP, adenosine 3',5'-cyclic phosphoric acid [¹²⁵I]2'-O-succinyl (>150 Ci/mmol) was purchased from ICN (Irvine, CA). Anti-cAMP was obtained from Miles (Elkhart, IN).

Results

Actions of KCM on bone in organ culture

KCM from confluent cultures of normal human foreskin keratinocytes stimulated bone resorption in neonatal mouse calvaria in organ culture. Unfractionated KCM elicited a large increase in calcium release from calvaria, but there was no associated increase in PGE₂ accumu-

lation in the bone culture medium (Fig. 1A). To control for the effects of EGF and other factors present in the keratinocyte growth medium, conditioned medium from keratinocytes maintained at confluence without EGF or without serum was tested for bone resorption-stimulating activity. The activity of KCM without added EGF did not differ quantitatively from that prepared in the presence of EGF (Fig. 1A). The results in Fig. 1B show that serum-free KCM lacking EGF also contained bone resorption-stimulating activity. Thus, the activity in KCM is not derived from EGF or serum, and these medium supplements are not essential for the production and release of bone resorption-stimulating factors from keratinocytes. KCM diluted in bone culture medium is not acidic; therefore, the effect of KCM on calcium release from bone is not due merely to solubilization of mineral at low pH.

KCM, KCM without EGF, and serum-free KCM did not enhance the release of PGE_2 by mouse calvaria (Fig. 1), suggesting that KCM-induced bone resorption does not proceed via a PG-mediated mechanism. However, it was possible that local production of PGE_2 by certain bone cells that could not be detected by measurement of PGE_2 accumulation in bulk medium might still be the mechanism by which KCM stimulates bone resorption. Such a mechanism has indeed been described recently in this culture system for tumor necrosis factor by Tashjian, Jr., *et al.* (21). To test this possibility the following experiments were performed. Indomethacin, a well characterized inhibitor of PGE_2 synthesis in mouse calvaria, was added to the bone culture medium before treatment with KCM. Indomethacin had no inhibitory effect on the bone resorption-stimulating activity of KCM (Fig. 2).

Furthermore, two other inhibitors of PG synthesis (ibuprofen and piroxicam) also failed to decrease KCM-induced bone resorption despite the fact that they all prevented the small increase in PGE_2 production induced by KCM in this experiment (Table 1).

Several stimulators of bone resorption, such as PTH, vasoactive intestinal peptide, and exogenous PGE_2 , act via a cAMP-mediated mechanism. Therefore, KCM was tested for its ability to acutely increase cAMP accumulation in the medium and mouse calvaria. KCM at near-maximal concentrations for bone resorption-stimulating activity did cause a small but significant ($P < 0.01$) increase in cAMP production by mouse calvariae (Fig. 3). Bones treated with PTH, at a dose that caused comparable bone resorption, induced a large increase in medium cAMP. At a lower concentration (150 μg protein/ml) of KCM no increase in cAMP accumulation above the control value was noted. This lower concentration, however, caused significant bone resorption (Fig. 4). In addition, KCM did not contain a factor that inhibited the production of cAMP induced by PTH (Fig. 3, last column).

Dose response of KCM

Crude KCM increased bone resorption in a dose-dependent manner, reaching a maximum at 500 μg protein/ml (Fig. 4). KCM caused a significant increase in bone resorption at 75 μg protein/ml. An increase in medium PGE_2 induced by KCM was not noted until bones were incubated with 500 μg protein/ml KCM, a concentration that caused maximal stimulation of bone resorption. However, lower concentrations of KCM in-

FIG. 1. Conditioned medium from normal human foreskin keratinocyte contains bone resorption-stimulating activity. KCM was used at a concentration of 300 μg protein (P)/ml bone culture medium. Addition of KCM to mouse calvariae caused a significant increase in the medium calcium concentration in 3 days compared to the control value (medium conditioned by incubation with 3T3 feeder cells only). The amount of epidermal growth factor (EGF) contained in 300 μg protein/ml is equivalent to 2 ng/ml EGF (assuming no loss or degradation during the culture period). KCM lacking EGF (A) and serum-free KCM at a 1:5 dilution (B) also stimulated bone resorption. A and B represent two separate experiments. No significant increase in medium PGE_2 concentration was observed. Each bar gives the mean value, and the brackets give the SE for four bones per group. **, Significantly different from control ($P < 0.01$).

FIG. 2. KCM-induced bone resorption is not inhibited by indomethacin (INDO). KCM at a concentration of 300 μ g protein/ml bone culture medium caused a significant increase in the medium Ca²⁺ concentration (□) compared with the control value in a 3-day incubation period. Addition of 400 ng/ml indomethacin did not inhibit the KCM-induced increase in medium Ca²⁺. The medium PGE₂ concentration (▨) did not vary from the control value in any of the treatment groups. Each bar gives the mean value, and the brackets give the SE for four bones per group. **, Significantly different from control ($P < 0.01$).

TABLE 1. Actions of inhibitors of PG synthesis on KCM-induced bone resorption

Treatment	Medium Ca ²⁺ (mg/dl)	Medium PGE ₂ (ng/ml)
None	7.5 $\pm$ 0.26	0.34 $\pm$ 0.11
KCM	12.9 $\pm$ 1.2 ^a	1.04 $\pm$ 0.14 ^a
KCM + indomethacin	12.7 $\pm$ 1.5 ^a	0.07 $\pm$ 0.01 ^b
KCM + ibuprofen	12.5 $\pm$ 1.8 ^a	0.59 $\pm$ 0.27 ^c
KCM + piroxicam	12.4 $\pm$ 2.0 ^a	0.10 $\pm$ 0.01 ^b

Values are the mean $\pm$ SE (three or four bones per group; 72-h treatment). Neonatal mouse calvariae were incubated with crude KCM at 300 μ g protein/ml. Indomethacin, ibuprofen, and piroxicam were used at 200 ng/ml.

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

^b Significantly less than control ($P < 0.05$).

^c Significantly less than KCM alone ($P < 0.05$).

duced bone resorption without causing a measurable increase in the medium PGE₂ content.

Stability of bone resorption-stimulating activity

The bone resorption-stimulating activity in crude KCM was not lost after repeated freezing and thawing or after heating at 56 C for 60 min (data not shown). However, the activity was decreased markedly when KCM was heated for 10 min at 100 C (Table 2).

Maximal concentrations of KCM had no effect on medium calcium in devitalized bones, while the same concentration of KCM caused a significant increase in medium calcium in vital bones (Table 3). These results indicate that the bone resorption-stimulating activity in

FIG. 3. Effects of KCM on cAMP release by mouse calvariae. After a 24-h preincubation, calvariae were washed twice in serum-free medium containing 3 μ M IBMX. Test and control media with IBMX were then added to the bones and incubated for 60 min at 37 C. Medium was then removed, boiled for 5 min, and assayed for cAMP by RIA. KCM at 300 μ g protein(P)/ml increased cAMP significantly, but the increase was small compared with the large increase in cAMP induced by 2 U/ml PTH. KCM (150 μ g protein/ml) did not increase medium cAMP. Addition of both KCM (300 μ g protein/ml) and PTH (2 U/ml) to mouse calvaria did not alter medium cAMP levels from those observed with PTH alone. Each bar gives the mean value, and the brackets give the SE for three bones per group. **, Significantly different from control ($P < 0.01$).

crude KCM was not due to a nonspecific physicochemical influence on calcium mobilization.

Partial purification of KCM

KCM was fractionated by gel filtration using Bio-Gel P-60. Fractions (5 ml each) were collected and pooled; protein concentrations were determined and then assayed for bone resorption-stimulating activity at a standard protein concentration. In addition, these fractions were assayed for their ability to stimulate PGE₂ production by bone. The biological activity did not elute with the majority of the protein present in KCM. In fact, the active fractions contained less than 1% of the total protein. Two distinct peaks of bone resorption-stimulating activity were obtained (Fig. 5). Fractions 51–60 (termed KCM_I), with an approximate mol wt of 10,000–20,000, contained substantial activity. The bone resorption stimulated by KCM_I was not associated with an increase in medium PGE₂ (Figs. 5 and 6). Fractions 81–90 (KCM_{II}), of lower mol wt range (estimated 3,000–6,000), also increased bone resorption. In contrast to the activity of KCM_I, KCM_{II} markedly increased the production of PGE₂ (Figs. 5 and 7). KCM_I elicited bone resorption in a dose-dependent manner, showing significant activity at 2 μ g protein/ml. No increase in medium PGE₂ was noted at any concentration of KCM_I used (Fig.

FIG. 4. Dose response for KCM on bone resorption and PGE₂ production. KCM was concentrated by lyophilization and then dialyzed (3500 mol wt cut-off) against 0.02 M HEPES buffer before addition to the bone culture medium. KCM increased bone resorption significantly above control at 75 µg protein (P)/ml, while a significant increase in PGE₂ was first observed only at 500 µg protein/ml KCM. Each point gives the mean $\pm$ SE for five bones per group. SE bars for the control point in the lower panel is contained within the symbol. **, Significantly greater than control ($P < 0.01$).

TABLE 2. Heat stability of the bone resorption-stimulating activity of KCM

Treatment	Medium Ca ²⁺ (mg/dl)	Medium PGE ₂ (ng/ml)
None	7.5 $\pm$ 0.29	<0.12
KCM	11.2 $\pm$ 1.0 ^a	0.29 $\pm$ 0.09
KCM (100 C; 10 min)	7.7 $\pm$ 0.08	0.15 $\pm$ 0.01

Values are the mean $\pm$ SE (four bones per group; 72-h treatment). Neonatal mouse calvaria were incubated with crude KCM at 300 µg protein/ml either fresh or heated to 100 C for 10 min.

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

6). KCM_{II} elicited a dose-dependent increase in bone resorption, with significant activity at a concentration as low as 100 ng protein/ml. Thus, KCM_{II} is 10- to 20-fold more potent than KCM_I at this stage of partial purification. In addition, KCM_{II} caused a dose-dependent increase in medium PGE₂ (Fig. 7). A slight increase in

TABLE 3. KCM does not cause calcium release from devitalized bone

Treatment	Medium Ca ²⁺ (mg/dl)	
	Vital bones	Devitalized bones
None	7.7 $\pm$ 0.32	6.6 $\pm$ 0.1
KCM	9.8 $\pm$ 0.47 ^a	6.5 $\pm$ 0.1
PTH	11.0 $\pm$ 0.93 ^a	6.8 $\pm$ 0.15

Values are the mean $\pm$ SE (five bones per group; 72-h treatment). Neonatal mouse calvaria were devitalized by repeated freezing and thawing and then treated in the same manner as the vital bones. The calvaria were incubated with crude KCM (600 µg protein/ml) or PTH (2 U/ml).

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

FIG. 5. Gel chromatography of KCM. KCM was concentrated by lyophilization and chromatographed on a Bio-Gel P-60 column at 4 C. Five-milliliter fractions were collected, and absorbance (A_{280}) was measured at 280 nm (· · · ·). Fractions were pooled into eight groups, and protein concentrations were determined. The fractions were pooled as follows: group 1, fractions 10-24; group 2, fractions 25-35; group 3, fractions 36-50; group 4, fractions 51-60; group 5, fractions 61-70; group 6, fractions 71-80; group 7, fractions 81-90; and group 8, fractions 91-105. Each pooled group was assayed for bone resorption-stimulating activity (●) and PG synthesis-stimulating activity (○). The values obtained are plotted at the midpoint of the fraction number representing each pool. Pooled fractions were assayed at 5 µg protein/ml, except for the first pool (<200 ng/ml) and peak I (800 ng/ml). Two peaks of bone resorption-stimulating activity were clearly noted, and those fractions were designated KCM_I and KCM_{II}. KCM_I caused elevation of the medium Ca²⁺ concentration, but failed to increase PGE₂ production. KCM_{II} raised medium Ca²⁺ and also caused an increase in medium PGE₂.

bone resorption-stimulating activity was noted in fractions 10-24, but the increase was not significant (Fig. 5).

To determine if the bone resorption-stimulating activity in KCM_I and KCM_{II} was mediated by PGE₂, indomethacin was used to inhibit the synthesis of PGs. When bones were treated with KCM_I plus indomethacin, the activity was unaffected (Fig. 8). In contrast, the bone resorption-stimulating activity of KCM_{II} was inhibited completely by indomethacin (Fig. 8). These results suggest that the activity of KCM_{II} is mediated via a PG-

FIG. 6. KCM_I stimulated bone resorption independently of PGE₂ production. KCM_I caused a dose-dependent increase in release of Ca²⁺ from bone; a significant ($P < 0.05$) increase in bone resorption was obtained at 2.0 μg protein/ml and above. KCM_I did not increase medium PGE₂ at any concentration tested. Each point gives the mean $\pm$ SE for groups of four bones each. *, $P < 0.05$; **, $P < 0.01$ (vs. control).

dependent pathway, while KCM_I acts via a PG-independent mechanism. Moreover, these findings indicate that KCM_I and KCM_{II} represent two different bone resorption-stimulating factors. Neither KCM_I nor KCM_{II} contains immunoreactive epidermal growth factor (assay generously performed by Dr. David Orth).

Actions of KCM on the production of cAMP

Both KCM_I and KCM_{II} were studied for their ability to enhance cAMP production by the human osteosarcoma cell line SaOS-2. These osteoblast-like cells increase cAMP in response to agents such as PTH and vasoactive intestinal peptide (14, 22). At concentrations that caused maximal bone resorption, KCM_I did not increase cAMP release by SaOS-2 cells (Fig. 9A). In contrast, KCM_{II} (0.5 μg protein/ml) elicited a 5-fold increase in medium cAMP. KCM_{II}, at concentrations as low as 0.1 μg protein/ml, caused a significant increase in cAMP release from SaOS-2 cells (Fig. 9B). Addition of indomethacin to KCM_{II} completely inhibited the stimulated release of cAMP from the cells (Fig. 9B). This finding suggests that KCM_{II}-induced stimulation of cAMP production was mediated indirectly via the production of PGs. Indomethacin does not inhibit PTH-stimulated production of cAMP.

FIG. 7. KCM_{II} stimulated bone resorption and PGE₂ production. KCM_{II} caused a dose-dependent increase in Ca²⁺ release from bone; a significant ($P < 0.05$) increase in bone resorption was obtained at 100 ng protein/ml and above. The increase in Ca²⁺ was associated with a dose-dependent increase in PGE₂. Each point gives the mean $\pm$ SE for groups of four bones each. *, $P < 0.05$; **, $P < 0.01$ (vs. control).

Discussion

Keratinocytes, the predominant proliferating cell type in the epidermis, have been shown to produce several cytokines that may play an important role in the immunological function of the skin. The results of the present study have shown that cultured normal human keratinocytes also produce at least two distinct factors that cause bone resorption. These bone resorption-stimulating factors are produced by keratinocytes even in the absence of serum. The bone resorption-stimulating activity in the crude KCM was not dependent on PG production and only increased cAMP in bones to a small degree compared with PTH. Fractionation of KCM revealed two distinct peaks of biological activity, one at 10,000–20,000 daltons called KCM_I and one at 3,000–6,000 daltons called KCM_{II}. Although both KCM_I and KCM_{II} cause bone resorption, they do so by different mechanisms. These findings suggest the presence of two separate factors, rather than KCM_{II} being a degradation product of KCM_I.

KCM_I stimulates bone resorption in neonatal mouse calvariae via a PGE₂-independent pathway. KCM_I does not increase cAMP production in SaOS-2 cells, unlike certain other PGE₂-independent bone resorption-stimulating agents, such as PTH and vasoactive intestinal

FIG. 8. Indomethacin (INDO) inhibited the bone resorption-stimulating activity of KCM_{II} but not of KCM_I. Although KCM_I failed to increase bulk medium PGE₂, it was still possible that KCM_I increased PGE₂ locally, which, in turn, caused bone resorption. Indomethacin (200 ng/ml) was used to inhibit PG synthesis. Medium Ca²⁺ and PGE₂ were assayed as described in *Materials and Methods*. Inhibition of PGE₂ synthesis had no effect on the bone resorption-stimulating activity of KCM_I (5 μ g protein/ml). In contrast, inhibition of PGE₂ synthesis by indomethacin inhibited completely the bone resorption-stimulating activity of KCM_{II} (0.8 μ g protein/ml). Each point gives the mean $\pm$ SE for groups of six bones each. *, $P < 0.05$; **, $P < 0.01$ (vs. control).

peptide. In contrast, KCM_{II} stimulates an increase in the production of PGE₂ in mouse calvaria, and the bone resorption-stimulating activity of KCM_{II} is inhibited completely by doses of indomethacin that are not non-specific inhibitors of resorption (22). These results indicate that KCM_{II} induces bone resorption via a PG-mediated mechanism. In addition, these data suggest that the majority of the bone resorption-stimulating activity measured in crude KCM is derived from KCM_I. At high concentrations of crude KCM, bone cell PGE₂ production was markedly increased, suggesting that KCM_{II} was now being detected. Thus, KCM_I appears to be released from keratinocytes in greater quantity (or with less degradation) than KCM_{II}, although KCM_{II} at the present

FIG. 9. Actions of KCM_I and KCM_{II} on cAMP release by SaOS-2 cells. SaOS-2 cells were grown to confluence in 16-mm culture wells and fed 24 h before the experiments. The cells were washed twice with HBSS. Test and control agents in HBSS with 400 μ M IBMX were then added. The cells were incubated for 20 min at 37 C in 5% CO₂-95% air, and the supernatant solutions were collected and boiled for 5 min. The samples were stored at -20 C until assayed for cAMP. KCM_{II} [0.5 μ g protein (P)/ml] caused a significant increase in medium cAMP, while KCM_I (2 μ g protein/ml) failed to increase medium cAMP above control levels (A). B, Stimulation of cAMP production by KCM_{II} and complete inhibition of the cAMP response of 0.5 μ g protein/ml KCM_{II} by indomethacin (INDO; 200 ng/ml). Each bar gives the mean $\pm$ SE for groups of four wells each. **, Significantly different from control ($P < 0.01$).

stage of purification is the more potent bone-resorbing factor. KCM_{II} also stimulated a significant, although small, increase in cAMP production by SaOS-2 cells, similar to the increase in cAMP in bones produced by the crude KCM. The increase in cAMP was inhibited completely by indomethacin, indicating that KCM_{II} causes the increase in cAMP indirectly via the local production of cyclooxygenase products, presumably PGs.

Other investigators have reported that medium conditioned by human keratinocytes causes an increase in cAMP levels in the rat osteosarcoma cell line ROS 17/2.8 (12). This increase in cAMP was inhibited by the PTH antagonist PTH-(3-34), suggesting that PTH and PTH-(3-34) the PTH-like molecule may act on the same receptor; however, antibody inhibition studies show the PTH-like protein to be distinct from native PTH (12). It is possible, but unlikely, that KCM_{II} is responsible for this PTH-like activity, as it does cause an increase in cAMP levels in cultured osteosarcoma cells. However, KCM_{II}-induced increases in cAMP are PG mediated while, in contrast, PTH-induced increases in cAMP are not mediated by PGs (22). If the PTH-like protein acts through the native PTH receptor, it is unlikely that the PTH-like protein-induced increase in cAMP is mediated via PG production. Therefore, at this stage of purification, it appears that KCM_{II} is distinct from the PTH-like protein. Investigators studying tumors associated with the humoral hypercalcemia of malignancy have recently purified and sequenced a PTH-related protein (25-28). As many of these tumors appear to be keratin-

ocyte-like (29), it is possible that the PTH-related peptide is also expressed in normal epidermal cells. Additional studies are needed to determine if KCM_{II}, which is produced by normal human keratinocytes, is related to this PTH-like molecule or whether it is a separate and distinct bone resorption-stimulating factor produced by keratinocytes.

Alternatively, it has been suggested that human squamous cell carcinomas associated with humoral hypercalcemia of malignancy produce an IL-1 α -like molecule that is responsible for *in vitro* bone resorption but does lack PTH-like activity (30, 31). It is possible that KCM_I, which is produced by normal human keratinocytes, is an IL-1 α -like molecule. The two factors derived from normal human keratinocytes, KCM_I and KCM_{II}, might be expressed to varying degrees by neoplastic keratinocytes. This could account for the findings of PTH-like and/or IL-1-like factors derived from different squamous cell carcinomas. Recently, human keratinocytes were shown to produce and release (32) transforming growth factor- α (TGF α). TGF α has been shown to cause bone resorption in neonatal mouse calvaria in organ culture by a PG-mediated mechanism (33). Therefore, a portion of the bone resorption-stimulating activity of KCM_{II} could be due to TGF release by keratinocytes.

It is now clear that keratinocytes are a very dynamic cell type that produce not only immunologically active cytokines but also a PTH-related peptide and other bone resorption-stimulating factors. These factors are of different mol wt and act by two different mechanisms to enhance bone resorption. Although the pathophysiological role keratinocytes may play in bone metabolism and systemic calcium homeostasis is yet to be determined, the fact that keratinocytes produce and release multiple bone resorption-stimulating factors suggests that these cells may play a significant role in bone physiology.

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