EVIDENCE THAT THE BONE RESORPTION-STIMULATING FACTOR PRODUCED BY MOUSE FIBROSARCOMA CELLS IS PROSTAGLANDIN E_2

A New Model for the Hypercalcemia of Cancer*

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Goldhaber has described a transplantable mouse fibrosarcoma which enhances the resorption of bone in tissue culture (2, 3). When fragments of the tumor ($HSDM_1$) were placed in the same culture vessel with mouse calvaria, marked resorption of the bone was observed. Fragments of $HSDM_1$ tumor could be cultured alone, and the medium from such explants also stimulated bone resorption when it was added to calvaria in organ culture. These findings suggested that the tumor was synthesizing and secreting a bone resorption-stimulating factor. Results of our previous experiments revealed that the factor could be extracted from the tumor tissue and recovered from the medium of $HSDM_1$ cells grown in monolayer culture (1). Of particular interest was the finding that the $HSDM_1$ factor could be extracted into organic solvents, and that it had several chemical and biological properties of a prostaglandin.

We have recently reported that $HSDM_1$ cells in culture synthesize and secrete large amounts of prostaglandin E_2 (4). In the present report, we wish to present evidence that leads us to conclude that the bone resorption-stimulating factor produced by the $HSDM_1$ tumor is prostaglandin E_2.

Materials and Methods

The $HSDM_1$ Tumor.—A fibrosarcoma was induced in a Swiss albino mouse by subcutaneous implantation of a Millipore filter (5, 6). The tumor has been passed serially in mice of the same strain for more than a decade. Excised tumor from a donor mouse is minced into

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small fragments which are injected subcutaneously in new host animals. At least 75% of injected mice develop palpable tumors within 1 wk, and these are transplanted at approximately 3-wk intervals. In the experiments reported here, tumors were carried exclusively in male mice, although animals of both sexes can serve as host.

Establishment of $HSDM_1$ Tumor Cells in Monolayer Culture.—The procedures used were similar to those reported in detail previously from this laboratory for the establishment of rat pituitary tumor cells in culture (7). In brief, a tumor was removed aseptically, minced with fine scissors, and the cell aggregates dispersed with Viokase and plated in plastic Petri dishes (Falcon Plastics, Division B-D Laboratories, Inc., Los Angeles, Calif.) in Ham's F 10 medium (8) supplemented with 15% horse serum and 2.5% fetal calf serum. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The tumor cells adapted readily to the conditions of primary culture without resort to the serial culture to animal enrichment technique previously described (7). Primary cultures were subcultured by treatment with Viokase, and the cells diluted 1:5 to 1:40 and replated in fresh dishes. Such cultures have been serially propagated, with subculture every 10–20 days, for 41 months without signs of senescence, as evidenced by a decreased rate of cell growth or loss of ability to produce the bone resorption-stimulating factor.

Five clonal strains of $HSDM_1$ cells were derived by plating single cells in Microtest II tissue culture plates (Falcon) with 100 μl of cell culture medium. Such single cells also grew well, and each cell gave rise to a colony which was harvested, subcultured, and propagated in Petri dishes as a clonal strain. The five clones are designated $HSDM_1C_1$, $HSDM_1C_2$, ... $HSDM_1C_5$. They have been in continuous culture for 11 months.

All clonal strains as well as the uncloned cultures secrete the bone resorption-stimulating factor into the culture medium (see below). To demonstrate that the cells in culture (both uncloned and clonal strains) were indeed tumor cells, we harvested cells from monolayers (7, 9) and injected 10^6 cells per animal into groups of four to six male Swiss albino mice. All injected mice developed tumors within less than 10 days. The culture-derived tumors were excised and extracted, and the extracts were assayed biologically (see below) and were found to contain the bone resorption-stimulating factor. The cycle of transfer of tumor cells from mouse to culture was then successfully repeated again.

In certain experiments, $HSDM_1$ cells were maintained in serum-free, synthetic F 10 medium alone.

Extraction and Fractionation of Tumors and Cell Culture Medium.—Excised $HSDM_1$ tumors were homogenized for 30 sec in Gey's balanced salt solution (3 ml/g) in a Waring Blendor at room temperature. The insoluble material was removed by centrifugation (10,000 *g* at 4°C for 20 min) and the supernatant solution was designated, crude extract. The crude extract was placed in a boiling water bath and stirred for 15 min, and the large precipitate which formed was removed by centrifugation (10,000 *g* at 4°C for 30 min). The supernatant solution was fractionated sequentially on columns of Sephadex G-50 and then G-10 using 0.2 M ammonium acetate, pH 4.7, as the solvent (1). Fractions collected from the column eluates were pooled, diluted with distilled water, and lyophilized. The residue was redissolved in water and lyophilized again to eliminate the bulk of the ammonium acetate. The final powder was weighed and dissolved in Gey's balanced salt solution for assay in the bone culture system.

Certain aqueous solutions or extracts were acidified to pH 3.5–3.7 with citric or acetic acid and were extracted three times at room temperature with 1.5 volumes of fresh diethyl ether. The ether extracts were combined and dried under a stream of nitrogen gas at 40°C, and the residue was taken up in Gey's balanced salt solution for bioassay in the bone culture system or for radioimmunoassay for prostaglandins (see below).

Medium from $HSDM_1$ and control cells in culture was extracted with ether exactly as described above.

Assay for Bone Resorption-Stimulating Activity.—Initial experiments were performed using the mouse calvaria bone-resorbing culture system of Goldhaber (10). Groups of four or five bones per treatment were used. In addition to unknown or test substances, each experiment

contained one group of control bones and two groups of bones treated with standard materials, either parathyroid hormone (PTH)[1] (Eli Lilly and Co., Indianapolis, Ind.; parathyroid extract, USP, 100 units/ml) or prostaglandins,[1] at two dose levels. Medium was changed every 2 days, and the extent of bone resorption was estimated semiquantitatively by microscopic examination of the cultures. Each bone was given a resorption score (index of resorption) on a scale of 0–6, where 6 was complete loss of mineral. At the end of each experiment, after 6–10 days of culture, the mineral remaining in the bone was stained by von Kossa's method.

In order to make the bone-resorption assay more efficient and quantitative, it was modified for later experiments as follows. 1 day old mice were injected subcutaneously or intraperitoneally with 10 μCi of ^{45}Ca. 4 days later the calvaria were removed and fixed to cover slips exactly as in the unlabeled bone culture method described above. All bones were placed in culture for an initial 24 hr period without specific treatment. This "washout" period was used to allow release of the most rapidly exchangeable ^{45}Ca in the bone. The washout medium was recovered and counted and the bones were then divided randomly into control, standard, and treatment groups of three to five bones each. This period was designated, zero time. Fresh medium containing control, standard, or test substances was added to appropriate groups of bones. 24 hr later an aliquot (25–50 μl) of medium was taken for determination of ^{45}Ca content by liquid scintillation counting. The experiment was terminated at 48 hr (after 2 days of treatment) and the total accumulated ^{45}Ca radioactivity in medium again determined. The results are expressed as counts per minute ^{45}Ca per milliliter of medium at 24 and 48 hr, and a mean value and standard error were calculated for each time interval for each experiment (see statistical method below). Measurements of ^{45}Ca in the medium from each bone during the washout period and in the residual bone mineral at the end of the experiment (after hydrolysis in 98% formic acid) indicated an acceptable degree of uniformity of labeling between bones ($P > 0.05$ for the difference between bones).

Radioimmunoassay of Prostaglandins.—Prostaglandins were assayed immunologically using the technique of Levine, Gutierrez-Cernosek, and Van Vunakis (11). Where indicated, samples were treated with NaOH immediately before assay (pH 12.0–12.5 at 100°C for 5–10 min) to convert PGE to PGB.[1] All assays were performed with antiserum to PGB_1 (11).

For analysis of prostaglandins in serum, the extraction and concentration procedure of Gutierrez-Cernosek, Morrill, and Levine (12) was used. Blood was drawn from control or $HSDM_1$ tumor-bearing mice (2–3 wk after transfer of tumor) by cardiac puncture with special care to avoid hemolysis or tissue fluids. Samples from individual mice (about 1 ml) were incubated at 37°C for 30–40 min to accelerate clot formation. Clots were removed by forceps, and the serum was separated from residual erythrocytes by centrifugation (5000 *g* at room temperature for 20 min). Calcium levels in serum were determined immediately (see below), and the remaining serum was frozen at −70°C for 1–7 days before extraction for assay of prostaglandins. Sera from individual mice were combined into pools of 3.5–5.0 ml, extracted with methylal-ethanol, and concentrated as previously described for human sera (12).

Analysis of Serum Calcium.—Serum from individual control or tumor-bearing mice was obtained as described above. The concentration of calcium in serum was determined by the method of Copp (13).

Statistical Method.—The serum calcium and ^{45}Ca data in each experiment were subjected to an analysis of variance. The standard errors (SE) were calculated from the residual error term of each analysis.

Materials.—Indomethacin (lot No. 35811) was a gift from Merck, Sharp & Dohme Research Laboratories, West Point, Pa. The nonradioactive prostaglandins were obtained from Dr. John Pike of The Upjohn Co., Kalamazoo, Mich. The prostaglandins-^{3}H and ^{45}Ca were purchased from New England Nuclear Corp., Boston, Mass.

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[1] *Abbreviations used in this paper:* PGE, PGA, and PGB for prostaglandins of the E, A, and B series, respectively; PTH, parathyroid hormone.

RESULTS

Effect of Extracts of the HSDM$_1$ Tumor on Bone Resorption in Tissue Culture.—Crude aqueous extracts of the HSDM$_1$ tumor were tested for bone resorption-stimulating activity initially in the semiquantitative culture system. Fig. 1 shows the results obtained with three dose levels of crude extract. Concentrations of 800 and 2400 μg of solid/ml culture medium clearly stimulated bone resorption. Similar extracts of normal control mouse liver, kidney, and muscle, or of three other transplantable tumors (adrenal, pituitary, and liver) did not stimulate bone resorption. Fractionation of the crude extract by precipitation of protein by heating and sequential gel filtration on columns of Sephadex G-50 and G-10 gave material which was enriched in bone resorption-stimulaing activity by a factor of at least 1000-fold (1). This material stimulated resorption at dose levels of less than 1 μg of solid/ml medium (Fig. 2). The specific bio-

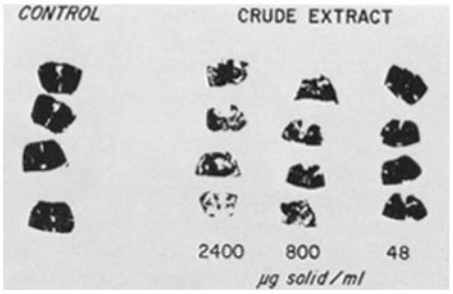

FIG. 1. Appearance of stained (von Kossa) bones (four per group) at the end of a 7 day period in culture. Control bones on the left show little loss of mineral (black stain). On the right is shown the loss of mineral (loss of black staining material) induced by 800 and 2400 μg/ml medium of a crude aqueous extract of HSDM$_1$ tumor tissue.

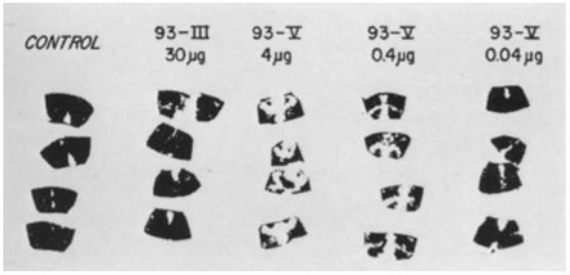

FIG. 2. Effects of fractionated HSDM$_1$ tumor extract on bone resorption. Control bones are on the left. The materials designated 93-III and 93-V are fractions from gel filtration on a column of Sephadex G-10 (1). Fraction 93-III is material from the void volume of the column. It did not stimulate bone resorption at a dose level of 30 μg of solid/ml culture medium. Fraction 93-V was from the retarded portion of the chromatogram. Enhancement of bone resorption was seen clearly at 4.0 and 0.4 μg of solid/ml.

logical activities of several bone resorption-stimulating factors were compared. The results, summarized in Table I, show that the $HSDM_1$ factor is as active as parathyroid horone and prostaglandins of the E series.

In order to demonstrate that the factor extracted from the $HSDM_1$ tumor was produced by viable tumor cells and was not a product of cell necrosis, we established dispersed cell cultures from the tumor and examined the medium for bone resorption-stimulating activity.

TABLE I

Comparison of Specific Activities of Several Bone Resorption-Stimulating Factors

Factor	Concentration needed to give a bone resorption index of 3.0*
	μg/ml medium
Parathyroid hormone	0.05
Vitamin D_2	50
25-OH vitamin D_3	5
Dibutyryl-cAMP	50
PGE_1	0.03
PGE_2	0.03
PGA_1	5
PGA_2	5
$HSDM_1$ factor‡	0.2
$HSDM_1$ factor (ether soluble)§	0.02

* The extent of bone resorption was estimated by visual observation of bones at 48-hr intervals (for 7–8 days). Resorption was scored on a scale of 0–6, where 0 is no resorption and 6 is complete demineralization. A mean index score (four bones per group) of 3.0 is a half-maximum effect.

‡ Active fraction from Sephadex G-10 filtration.

§ Activity from above Sephadex G-10 fraction which was extracted into ether; no detectable residual activity remained in the aqueous phase.

Effect of Medium from Cultures of $HSDM_1$ Cells on Bone Resorption.—Uncloned $HSDM_1$ cell lines secreted the bone resorption-stimulating factor into the culture medium. Fig. 3 shows the appearance of bones cultured with aliquots of medium from $HSDM_1$ cells. As with extracts of $HSDM_1$ tumor tissue, the factor secreted by the cells in culture was extracted into ether (Fig. 3).

Cultures of $HSDM_1$ cells were cloned and five clonal strains were established. Fig. 4 gives the appearance of clone 1, $HSDM_1C_1$. $HSDM_1C_1$ cells have a population doubling time of about 40 hr and secrete the bone resorption-stimulating factor into the culture medium during both the exponential and

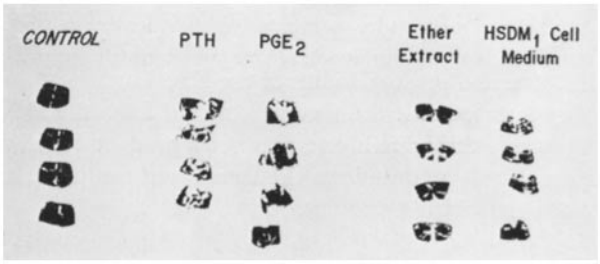

FIG. 3. Effect of $HSDM_1$ cell culture medium on bone resorption. Control bones are on the left. On the far right is shown the effect 0.2 ml of medium which had been on cultured $HSDM_1$ cells for 3 days. The effect of an ether extract of the same $HSDM_1$ medium (dose equivalent to 0.2 ml of original medium) is shown in group 4 (ether extract). Effects of parathyroid hormone (0.10 units/ml) and of PGE_2 (0.01 μg/ml) are also shown. Control medium from five other cell lines did not stimulate bone resorption in this assay system.

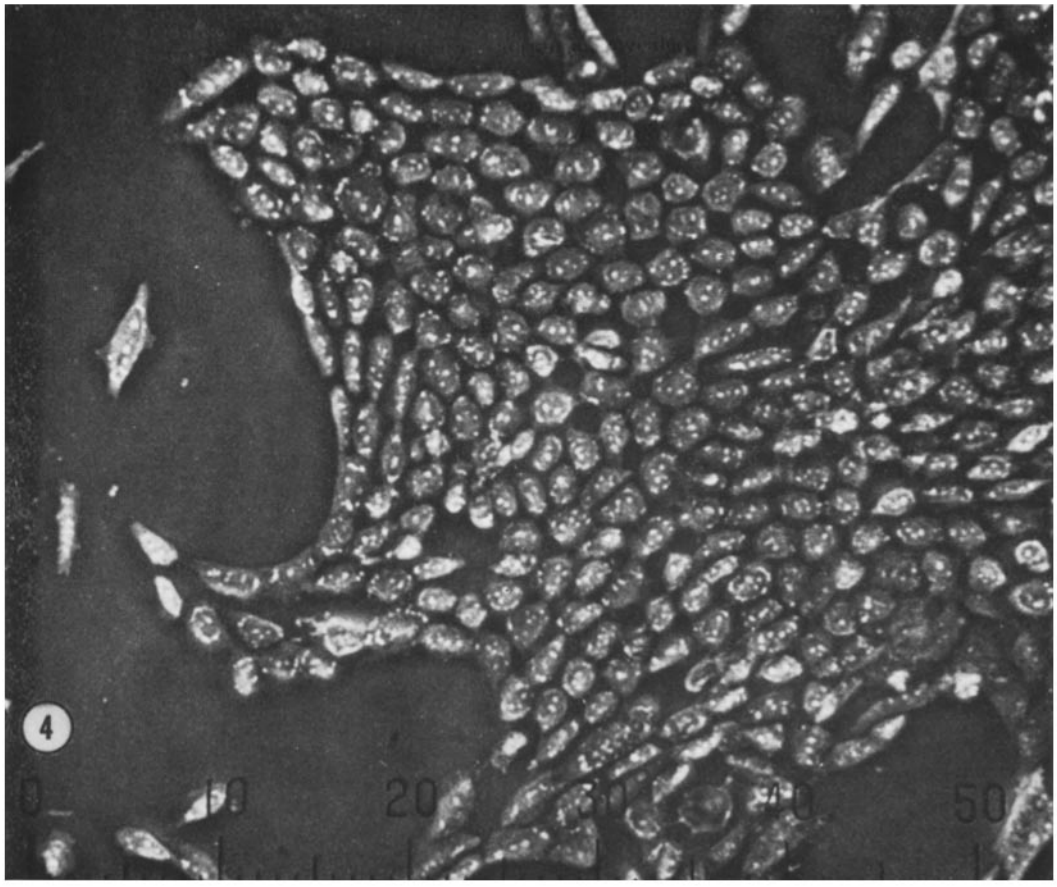

FIG. 4. Appearance of $HSDM_1C_1$ cells 5 days after subculture. (Unstained, phase-contrast microscopy, one scale division is equal to 0.01 mm.)

plateau phases of cell growth (4). Using the ^{45}Ca-release bone culture assay method, the results given in Table II show the stimulation of ^{45}Ca release induced by medium from $HSDM_1C_1$ cells. Similar ether extracts of fresh control medium and of conditioned medium from five other tumor cell lines (three mouse, one rat, and one human) did not stimulate bone resorption or ^{45}Ca release.

In order to show that the cells which were cloned were indeed tumor cells, the cultured cells (10^6 per mouse) were injected into mice. All five injected mice developed tumors within 1 wk. The tumors were removed, extracted, and the extracts were assayed for bone resorption-stimulating activity. Each extract stimulated bone resorption. Portions of the culture-derived tumors were prepared for culture and new dispersed cell cultures were established. Each of these second generation cell cultures also secreted the bone resorption-stimulating factor into the culture medium.

TABLE II

Effects of PTH and Medium from $HSDM_1C_1$ Cells on Bone Resorption

Treatment	Dose	^{45}Ca release from bone*	
		24 hr	48 hr
	per ml	*cpm × 10^{-4}/ml medium*	
Control	—	1.6 ± 0.36	2.3 ± 0.64
PTH	0.01 U	2.5 ± 0.36	4.0 ± 0.64
$HSDM_1C_1$ medium‡	0.20 ml	2.8 ± 0.36	4.8 ± 0.64

* Mean values of four bones per group ± SE.

‡ Medium was on $HSDM_1C_1$ cells for 4 days. It was extracted with ether and tested at a dose level equivalent to 0.20 ml of original medium.

Several lines of evidence led us to postulate that the factor produced by the $HSDM_1$ tumor in vivo and secreted by the tumor cells in vitro might be a prostaglandin (1). In brief, the characteristics of the factor which were consistent with this hypothesis were: (*a*) its low molecular weight (determined by gel filtration, dialysis, and ultracentrifugation; (*b*) its stability at 100°C (pH 7.5 for 15 min); (*c*) its resistance to inactivation by trypsin and pepsin; (*d*) its high specific biological activity (Table I); and (*e*) its solubility in ether, chloroform, and ethyl acetate. However, before determining whether the tumor cells did produce prostaglandins, we tested several of these cyclic, oxygenated C_{20} fatty acids in the ^{45}Ca-release assay. Table III shows that PGE_1 and PGE_2 stimulate ^{45}Ca release at dose levels of 0.01–0.10 μg/ml. PGA_1 and PGA_2 were about 50-fold less active, and $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGB_1, and PGB_2 were inactive at the dose levels tested.

Assay of Medium from $HSDM_1$ Cells for Prostaglandins.—Media from uncloned and cloned $HSDM_1$ cells and from five control cell lines were assayed immunologically for prostaglandins. Based on the results of the serological experiments reported previously (4), the results are expressed in terms of a

TABLE III

Effects of Various prostaglandins on Bone Resorption

Experiment No.	Treatment	Dose	^{45}Ca release from bone*	
			24 hr	48 hr
		$\mu g/ml$	$cpm \times 10^{-4}/ml$ medium	
I	Control	—	1.4	2.1
	PGE_1	0.10	3.5‡	5.8‡
	PGE_2	0.10	3.6‡	6.2‡
	PGA_1	3.0	2.2	3.9
	PGA_2	3.0	2.9	4.0
	$PGF_{1\alpha}$	3.0	1.7	2.8
	$PGF_{2\alpha}$	3.0	2.1	3.6
	PGB_1	10.0	2.2	3.5
	PGB_2	10.0	2.3	3.4
II	Control	—	2.4	3.0
	PGE_1	0.10	4.9‡	8.5‡
	PGE_1	0.01	4.7‡	7.0‡
	PGE_2	0.05	4.6‡	9.0‡
	PGE_2	0.005	3.8‡	5.0‡
III	Control	—	1.5	2.5
	PGE_2	0.10	3.6‡	6.3‡
	PGA_1	5.0	3.4‡	5.4‡
	PGA_1	0.5	2.4	4.5
	PGA_2	5.0	2.6	5.2‡
	PGA_2	0.5	2.4	4.3

* Mean values of three or four bones per group. The range of the SE for the three experiments was ±0.47–0.50 for 24 hr and 0.68–0.91 for 48 hr, respectively.

‡ $P < 0.05$ as compared to control ^{45}Ca release.

PGE_2 standard. Fresh culture medium contained no detectable PGE_2. The results given in Table IV show that both uncloned and cloned $HSDM_1$ cells produced PGE_2, while control cells, which do not produce bone resorption-stimulating factors, do not produce measurable quantities of PGE_2. Both aqueous and ether extracts of $HSDM_1$ tumor tissue which contained bone resorption-stimulating activity also contained PGE_2 by immunoassay.

Taken together, the above results show that $HSDM_1$ cells produce both a

bone resorption-stimulating factor and PGE_2. The data in Tables I and III and in Fig. 3 show that PGE_2 stimulates bone resorption at low dose levels. In order to determine whether PGE_2 and the bone resorption-stimulating factor were produced in parallel, we took advantage of the finding that indomethacin is a potent inhibitor of PGE_2 synthesis by $HSDM_1$ cells (4).

Effect of Indomethacin on the Production of PGE_2 and the Bone Resorption-Stimulating Factor.—The data in Table V show that PGE_2 production was inhibited by indomethacin. When $HSDM_1C_1$ cells were grown in the presence of indomethacin, the medium was also found to lack bone resorption-stimulating

TABLE IV

Prostaglandin Production by $HSDM_1$ Cells in Culture

Cell type	Prostaglandin production*
	μg PGE_2 per mg cell protein per 24 hr
$HSDM_1$ (uncloned)	1.0–1.2
$HSDM_1C_1$	2.0
$HSDM_1C_2$	1.2
$HSDM_1C_3$	2.0
$HSDM_1C_4$	0.80
$HSDM_1C_5$	0.72
Mouse fibroblasts (R_5)	<0.03
Mouse neuroblastoma ($41A_3$)	<0.01
Mouse adrenal (AT_{10})	<0.01
Rat glial (C_6)	<0.01
Human HeLa	<0.01

* Medium samples were 3-day collections which were assayed for prostaglandins after treatment with alkali as described previously (4, 11). When medium was extracted with ether and assayed serologically, the PGE_2 was quantitatively extracted into the ether phase.

TABLE V

Effect of Indomethacin on PGE_2 Production by $HSDM_1C_1$ Cells

Treatment	Dose	Prostaglandin production*
	ng/ml	*μg PGE_2 per mg cell protein per 24 hr*
Control	—	1.94
Indomethacin	100	0.15
Indomethacin	10	0.58
Indomethacin	1.0	0.95
Indomethacin	0.10	1.45

* Medium containing either no additions (control) or indomethacin was added to replicate dishes of $HSDM_1C_1$ cells. 3 days later the medium was collected for prostaglandin assay. The results are the mean of duplicate determinations.

activity (Table VI). Indomethacin does not affect the bone culture assay per se, nor does it inhibit the effect of PGE_2 on ^{45}Ca release (Table VII). Likewise, indomethacin does not affect the growth of $HSDM_1$ cells (4), nor does it cause the cells to produce a substance which inhibits the effect of PGE_2 on bone resorption (Table VIII).

TABLE VI

Effect of Indomethacin on the Production of the Bone Resorption-Stimulating Factor by $HSDM_1C_1$ Cells

Treatment	^{45}Ca release from bone*	
	24 hr	48 hr
	cpm × 10^{-4}/ml medium	
Control	1.8 ± 0.36	2.7 ± 0.60
$HSDM_1C_1$ medium‡	3.3 ± 0.36	6.2 ± 0.60
$HSDM_1C_1$ + indomethacin medium‡	1.5 ± 0.36	2.2 ± 0.60

* Mean values of four bones per group ± SE.

‡ $HSDM_1C_1$ cells were grown for 4 days in medium without or with indomethacin (100 ng/ml). The medium was extracted with ether and tested at a dose level equivalent 0.10 ml of original medium for untreated cells and 0.20 ml of original medium for indomethacin-treated cells.

TABLE VII

Effects of Indomethacin on ^{45}Ca Release from Labeled Bone

Treatment	Dose	^{45}Ca release from bone*	
		24 hr	48 hr
	ng/ml	*cpm × 10^{-4}/ml medium*	
Control	—	2.2 ± 0.34	3.5 ± 0.58
PGE_2‡	100	3.0 ± 0.34	5.0 ± 0.58
Indomethacin‡	500	2.0 ± 0.34	2.9 ± 0.58
PGE_2 + indomethacin‡	100 + 500	2.9 ± 0.34	5.8 ± 0.58

* Mean values of four bones per group ± SE.

‡ Labeled bones were cultured in control medium or in medium containing PGE_2, indomethacin alone, or PGE_2 plus indomethacin at the dose levels shown.

Effect of the $HSDM_1$ Tumor on Serum Calcium Levels in Tumor-Bearing Mice.—The results in Table IX show that mice bearing $HSDM_1$ tumors have higher serum calcium levels than control mice ($P < 0.001$). Examination of undecalcified sections of the long bones of the extremities from 10 tumor-bearing mice showed no gross or microscopic evidence of metastatic tumor cell deposits.

Effect of the $HSDM_1$ Tumor on Serum Prostaglandin Levels in Tumor-Bearing Mice.—The data in Table X show that mice bearing $HSDM_1$ tumors have levels of PGE_2 in serum that are two to three times higher than control mice.

TABLE VIII

Medium from Indomethacin-Treated HSDM$_1$C$_1$ Cells Does Not Inhibit the Effect of PGE$_2$ on Bone Resorption

Treatment	Dose of PGE_2	^{45}Ca release from bone*	
		24 hr	48 hr
	ng/ml	*cpm* $\times 10^{-4}$*/ml medium*	
Control	—	1.5	2.5
PGE_2	100	3.6	6.2
PGE_2	10	2.8	4.4
$HSDM_1C_1$ + indomethacin‡	—	1.4	2.0
$HSDM_1C_1$ + indomethacin + PGE_2	100	4.4	6.8
$HSDM_1C_1$ + indomethacin + PGE_2	10	2.6	4.6

* Mean values of four bones per group. The SE for 24 and 48 hr were ± 0.48 and 0.80, respectively.

‡ $HSDM_1C_1$ cells were treated with indomethacin (100 ng/ml) for 3 days and medium collected and extracted with ether for assay of bone resorption-stimulating activity either without or with added PGE_2. $HSDM_1C_1$ medium was tested at a dose level equivalent to 0.10 ml of original medium.

TABLE IX

Serum Calcium Concentrations in Control and HSDM$_1$ Tumor-Bearing Mice

Experiment No.	Treatment	No. of mice	Serum calcium*
			mg/100 ml
I	Control	22	9.6 ± 0.10
	Tumor bearing‡	24	10.3 ± 0.10
II	Control	23	9.8 ± 0.08
	Tumor bearing‡	35	11.1 ± 0.16

* Mean values ± SE.

‡ Animals were bled 18–24 days after inoculation of tumor cells.

DISCUSSION

Klein and Raisz have shown that prostaglandins are potent stimulators of bone resorption in tissue culture (14). This knowledge together with our finding that the transplantable mouse fibrosarcoma, $HSDM_1$, produced a very active bone resorption-stimulating factor, which was extracted into organic solvents, led us to postulate that the $HSDM_1$ factor might be a prostaglandin (1). $HSDM_1$ tumor cells were established in culture and were shown to synthesize and secrete large quantities of PGE_2 (4). In the present report, we have shown that mice bearing the $HSDM_1$ tumor have higher levels of both calcium (Table IX) and PGE_2 (Table X) in serum than control mice. In addition, the

$HSDM_1$ cells in culture secrete into the medium the bone resorption-stimulating factor which has several chemical and biological properties in common with prostaglandins. The $HSDM_1$ factor is extracted quantitatively into organic solvents, it is of low molecular weight, stable at neutral pH at 100°C, resistant to inactivation by trypsin and pepsin, and active in enhancing bone resorption in vitro at submicrogram per milliliter quantities, comparable to those of PGE_2 (Table III). We have tested five other lines of tumor cells in culture which do not produce prostaglandins, and none of these produce a bone resorption-stimulating factor. Lastly, indomethacin, which inhibits PGE_2 synthesis in

TABLE X

Levels of PGE_2 in the Serum of Control and $HSDM_1$ Tumor-Bearing Mice

Experiment No.	Treatment	Serum PGE_2
		pg/ml
I*	Control	245 ± 35
II‡	Control	185
	Tumor-bearing 1	680
	Tumor-bearing 2	520
	Tumor-bearing 3	500
	Tumor-bearing 4	500
III‡	Control	275
	Tumor-bearing 1	720
	Tumor-bearing 2	840

* Mean value ± SE; six separate pools of control sera; each pool was derived from 5–10 mice.

‡ Each value is the mean of duplicate determinations of a separate pool of serum from 6–20 control or tumor-bearing mice.

$HSDM_1$ cells at low concentrations (50% inhibition at 1 ng/ml, 3×10^{-9} M) (Table V), also inhibits production of the bone resorption-stimulating factor (Tables VI and VIII). At the dose levels used, indomethacin does not inhibit cell growth or protein synthesis in $HSDM_1$ cells (4). Furthermore, indomethacin does not inhibit nonspecifically the effects of PGE_2 or cause the $HSDM_1$ cells to produce an inhibitor of bone resorption in vitro (Tables VII and VIII).

From the results of these experiments, we conclude that the bone resorption-stimulating factor produced by $HSDM_1$ cells is PGE_2. Furthermore, we believe that secretion of PGE_2 by the tumor in vivo can explain the elevated levels of this prostaglandin measured in serum and the relative hypercalcemia which tumor-bearing mice exhibit.

Klein and Raisz (14) and ourselves have not been able to show a calcium-

mobilizing effect of PGE_1 or PGE_2 (up to 500 μg/rat, subcutaneously) in intact or parathyroidectomized rats. The experiment has not yet been done in mice. Failure to observe an effect of a single injection in vivo may well be explained by the rapid metabolism and clearance from plasma known to exist for PGE in the whole animal. On the other hand, the constant high secretory rate of the $HSDM_1$ tumor might give rise to levels of PGE_2 in plasma which were sufficiently high for a sufficient period to enhance bone resorption in vivo.

The $HSDM_1$ tumor and cell culture systems constitute useful models for studying the effects of prostaglandin excess in vivo and in vitro. These findings may even offer an explanation for some of the previously unexplained hypercalcemias associated with malignant tumors in man. The syndrome or syndromes of hypercalcemia in patients with cancer in whom no bone metastases are evident is not uncommon (15). Some of these tumors synthesize PTH (15–18) and this may explain the hypercalcemia in those patients. However, in other cases, with what appears to be a similar tumor-induced syndrome, PTH has not been measured in excess in plasma or tumor tissue (15–19). We would postulate that the etiology of the hypercalcemia in some of these cases could be due to the synthesis and secretion of large quantities of prostaglandins, presumably PGE, by the tumor. It has already been reported in man, for example, that some medullary carcinomas of the thyroid gland secrete prostaglandins (20); and two experimental tumors have recently been shown to contain measurable quantities of intracellular prostaglandins (21). The availability of sensitive radioimmunoassay methods for prostaglandins should now make it possible to test this hypothesis. If confirmed in clinical studies, these results suggest that indomethacin or related drugs which inhibit prostaglandin synthesis might prove beneficial in the medical management of prostaglandin-induced hypercalcemia.

SUMMARY

A transplantable mouse fibrosarcoma, $HSDM_1$, produces a potent bone resorption-stimulating factor. The factor can be extracted from the tumor tissue and harvested from the medium of clonal strains of $HSDM_1$ tumor cells growing in monolayer culture. It has several chemical and biological properties of a prostaglandin. Using radioimmunoassay techniques, we have shown that $HSDM_1$ cells synthesize and secrete large quantities of prostaglandin E_2 (PGE_2). The specific bone resorption-stimulating activity of the $HSDM_1$ factor extracted from the tumor is high and approximately equal to that of PGE_2 as measured in a bone tissue culture system in vitro. Indomethacin, a potent inhibitor of PGE_2 synthesis in $HSDM_1$ cells, also inhibits production by the cells of the bone resorption-stimulating factor, and has no detectable nonspecific effects on the bone culture assay system. Mice bearing the $HSDM_1$ tumor have higher levels of both calcium and PGE_2 in serum than control mice. We conclude that PGE_2 is the bone resorption-stimulating factor pro-

duced by $HSDM_1$ tumor cells, and that secretion of PGE_2 by the tumor in vivo accounts for the relative hypercalcemia observed in tumor-bearing animals. The $HSDM_1$ tumor cell system constitutes a new model for studying the pathogenesis of hypercalcemia associated with certain malignant tumors.

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