EFFECTS OF HYDROCORTISONE ON THE HYPERCALCEMIA AND PLASMA LEVELS OF 13,14-DIHYDRO-15-KETO-PROSTAGLANDIN E_2 IN MICE BEARING THE $HSDM_1$ FIBROSARCOMA

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SUMMARY

In mice bearing the prostaglandin-producing $HSDM_1$ fibrosarcoma, the plasma concentration of 13,14-dihydro-15-keto-PGE_2 was elevated before the development of hypercalcemia, and the magnitude of the rise was greater than that of PGE_2. When hydrocortisone, which inhibits synthesis of PGE_2 by $HSDM_1$ cells in culture, was administered to tumor-bearing mice, the steroid hormone prevented the rises in plasma PGE_2 metabolite and calcium concentrations. At the dose levels used, hydrocortisone did not inhibit the calcium-mobilizing action of parathyroid hormone *in vivo* or the bone resorption-stimulating activity of PGE_2 *in vitro*. These findings are consistent with our hypothesis that the hypercalcemic syndrome in $HSDM_1$ tumor-bearing mice is due to the secretion of PGE_2 by the tumor.

INTRODUCTION

Studies of the $HSDM_1$ fibrosarcoma have led us to conclude that the hypercalcemic syndrome that occurs in mice bearing this transplantable tumor is due to the excessive secretion of prostaglandin E_2 (PGE_2) by the neoplasm (1). This conclusion was derived from the results of several sorts of experiments in tumor-bearing animals and with $HSDM_1$ cells in culture (2-5). Subsequently, it has been found that anti-inflammatory steroids inhibit prostaglandin production by $HSDM_1$ cells (6), rheumatoid synovia (7), and methylcholanthrene-transformed mouse fibroblasts (8). The experiments described in this report were undertaken to examine the effects of hydrocortisone administration on plasma calcium, plasma PGE_2 metabolites, tumor weight, and tumor PGE_2 content and to define the time-courses of the development of hypercalcemia and hyperprostaglandinemia in tumor-bearing mice.

METHODS AND MATERIALS

Animals. The $HSDM_1$ fibrosarcoma was passed serially in mice of the Swiss Albino strain by methods described previously (3). Treatment with hydrocortisone (HC) was begun on the day of tumor transfer or within 48 hr and continued for the duration of each experiment as indicated in RESULTS. HC was administered orally in an average daily dose of 100 µg/mouse/day. The steroid was incorporated in a known amount of ground diet which the mice consumed completely each 24 hr. In experiments in which parathyroid hormone (PTH) was administered, it was given by subcutaneous injection. Three injections were given 29, 23 and 5 hr before bleeding. Each injection contained either 10 or 20 U. The preparation used was Parathyroid Injection U.S.P. (Eli Lilly & Co., 100 USP Units/ml).

Measurement of plasma calcium. Individual mice were bled by cardiac puncture into heparinized syringes. Blood was immediately centrifuged at 4°C, and the plasma separated for measurements of calcium, PGE_2 and PGE_2 metabolite concentrations. Calcium was measured with a Corning calcium analyzer, model 940, by fluorometric titration.

Measurement of 13,14-dihydro-15-keto-PGE_2 in plasma. The major metabolite of PGE_2, 13-14-dihydro-15-keto-PGE_2 (referred to in the text and in the figures as PGE_2 metabolite), was measured by radioimmunoassay. The corresponding $PGF_{2\alpha}$ metabolite, 13-14-dihydro-15-keto-$PGF_{2\alpha}$, cross reacts 5% with the anti-13,14-dihydro-15-keto-PGE_2 (9). Several samples of mouse plasma also were assayed with anti-13,14-dihydro-15-keto-$PGF_{2\alpha}$. The 13,14-dihydro-15-keto-PGE_2 cross reacts with this anti-$PGF_{2\alpha}$ metabolite 3% (10). These simultaneous radioimmunoassays demonstrated that it is the PGE_2 metabolite values, not the $PGF_{2\alpha}$ metabolite values, that are reported in this paper.

Tumors. At the termination of each experiment or experimental interval, the tumors were carefully excised, weighed, and extracted in Gey's balanced salt solution (5 ml/g). Protein was precipitated by heating for 10 min in a boiling water bath, and the final supernatant solution was extracted 3 times with diethyl ether (2.5 to 3 volumes) dried under nitrogen gas, and the residue dissolved in Gey's solution as described previously in detail (3). In some experiments the tumors were frozen immediately in liquid N_2 and then extracted at 4°C in Gey's solution containing indomethacin (100 µg/ml). The concentrations of PGE_2 in the tumor extracts and in unextracted plasma were measured by radioimmunoassay (11).

Bone culture. The effect of HC on PGE_2-stimulated bone resorption was studied using the neonatal mouse calvaria bone culture system previously described in detail (3,12). Bone resorption was monitored by measurement of medium total calcium concentration using the Corning calcium analyzer.

Statistical method. Results of each experiment (6-10 mice per group or 4 bones per group in bone culture experiments) were subjected to an analysis of variance, and the standard errors (SE) were calculated from the residual error term of that analysis. Where appropriate and when variances were not heterogenous, data were pooled from independent experiments of similar design.

RESULTS

The time-course of changes in plasma calcium and PGE_2 metabolite concentrations following tumor cell implantation are shown in Fig. 1. Small tumors were present in all animals by one week, but significant ($p<0.05$) elevation of

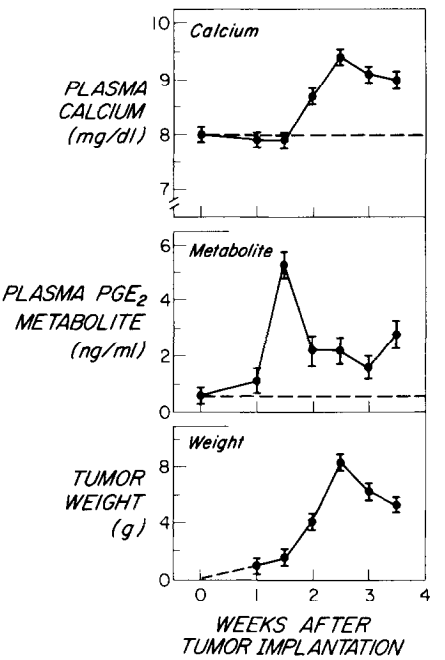

Fig. 1. Changes in plasma calcium and PGE_2 metabolite concentrations and tumor weights as a function of time after tumor cell implantation. Each point gives the mean value of 7-10 mice and the bars give the SE. In most other experiments of similar design, the plasma PGE_2 metabolite concentration in untreated tumor-bearing mice remained in the range of 4.5 to 6.0 ng/ml during the period 2.5 to 3.5 weeks after implantation (for example, see Fig. 2). In this experiment the mean contents of PGE_2 in tumors taken at 1, 1.5, 2, 2.5, 3 and 3.5 weeks after implantation were 35, 38, 90, 95, 140 and 175 ng/g fresh weight, respectively.

plasma calcium was not observed until two weeks after tumor implantation. Plasma PGE_2 metabolite concentrations were elevated at 1.5 weeks and preceded the development of hypercalcemia. Plasma PGE_2 at 1.5 weeks was 1.4 ± 0.24 ng/ml (mean ± SE) as compared to control levels of 0.57 ± 0.24 ng/ml, indicating that measurements of the PGE_2 metabolite reveal a greater incremental increase above basal levels than PGE_2 itself; the rapid conversion of PGE_2 to its more stable 13,14-dihydro-15-keto-metabolite (13) presumably accounts for these findings. It is noteworthy that hyperprostaglandinemia preceded hypercalcemia, a finding that is consistent with our hypothesis (3) that excessive secretion of PGE_2 by the tumor is the cause of the hypercalcemic syndrome and not a secondary phenomenon.

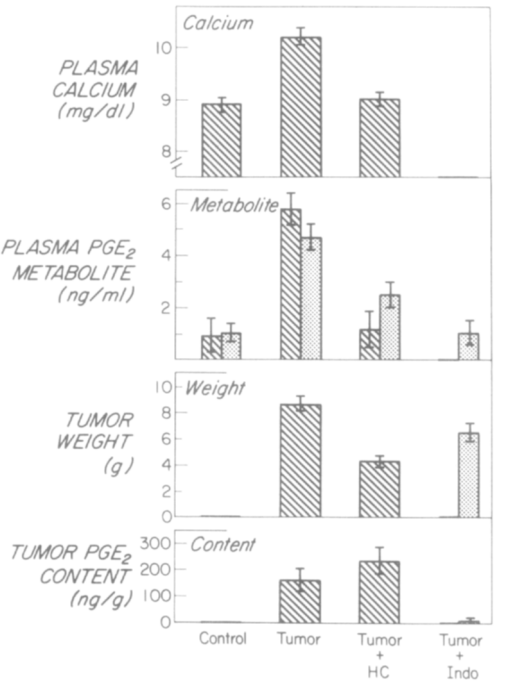

Fig. 2. Effects of HC on plasma calcium and PGE_2 metabolite concentrations, and on tumor weight and PGE_2 content in mice bearing the $HSDM_1$ fibrosarcoma. Wide bars give the pooled results from two separate experiments, and the brackets give the SE. Narrow bars give the mean values for individual experiments. Plasma calcium concentrations were not measured in the tumor plus indomethacin (Indo) group. In these experiments of 19-21 days duration HC (100 µg/mouse/day) were begun on the day of tumor implantation and continued throughout the entire experiment. The values given are those obtained at the end of the experiment. There were 8-17 mice per group.

The effects of HC are shown in Fig. 2. In HC-treated tumor-bearing mice the concentrations of plasma calcium and PGE_2 metabolite were the same or only slightly higher than those of control animals while the untreated mice with tumors had elevated plasma calcium and PGE_2 metabolites. Tumor weight in HC-treated mice was reduced markedly (about 50%), but tumor PGE_2 content per gram of tissue was not affected. Consistent with our previous studies (4), administration of indomethacin reduced tumor PGE_2 content to < 4% of control values and had a small but significant ($p<0.05$) effect on tumor weight. Indomethacin-treated tumor-bearing mice also had plasma PGE_2 metabolite concentrations that were the same as untreated control mice.

Table 1

LACK OF EFFECT OF HYDROCORTISONE (HC) ON PARATHYROID HORMONE (PTH)-INDUCED HYPERCALCEMIA IN MICE

Expt. no.	Treatment*	Body wt.† (g)	Plasma calcium† (mg/dl)
36	None	26 ± 0.98	8.4 ± 0.37
	HC (100 μg/day X 9)	26 ± 0.92	8.5 ± 0.35
	PTH (10 U/dose X 3)	28 ± 0.87	11.1 ± 0.33
	HC + PTH	26 ± 0.82	11.1 ± 0.31
38	None	28 ± 0.60	7.9 ± 0.39
	HC (100 μg/day X 19)	27 ± 0.60	7.8 ± 0.39
	PTH (20 U/dose X 3)	29 ± 0.60	13.8 ± 0.39
	HC + PTH	28 ± 0.57	13.8 ± 0.37

* Note that treatment with hydrocortisone was for 9 days in Expt. no. 36 and for 19 days in Expt. no. 38. Treatment with PTH at the dose levels indicated was given in 3 separate injections during the last 29-30 hr of each experiment as described in Methods and Materials.

† Mean values ± SE for groups of 7-10 and 9-10 mice in Expt. no. 36 and 38, respectively.

The normal plasma calcium concentration in HC-treated, tumor-bearing mice is not due to an effect of HC on basal plasma calcium or to an effect that prevents the action in vivo of a potent bone resorption-stimulating factor, PTH (Table 1), In two experiments basal plasma calcium concentrations were unchanged by HC, and the response to exogenous PTH was identical in control and HC-treated mice. The dose of HC used had no effect on body weight. The effects of HC and PTH on plasma PGE_2 metabolite concentrations were also studied (Fig. 3). HC reduced basal plasma PGE_2 metabolites in both control and PTH-treated mice but did not affect the calcium-mobilizing action of PTH. PTH had no effect on plasma PGE_2 metabolite concentrations.

To rule out the possibility that HC might be acting directly on bone to

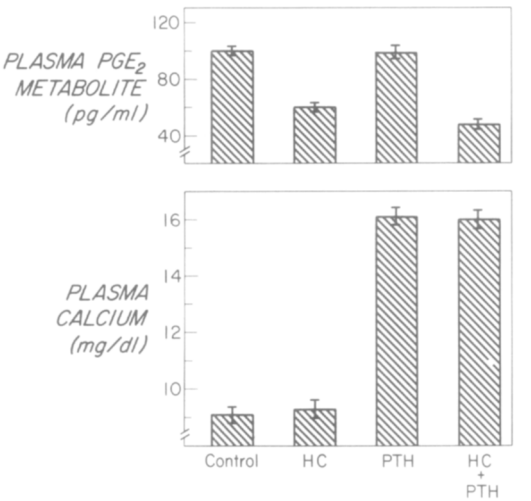

Fig. 3. Effects of HC and PTH alone and in combination on plasma calcium and PGE_2 metabolite concentrations. In this experiment mice were untreated (control), treated with HC (100 µg/mouse/day for 8 days), with PTH alone (40 U/dose X 3 doses), or with HC plus PTH. The bars give the mean values of 8-10 mice per group and the brackets give the SE.

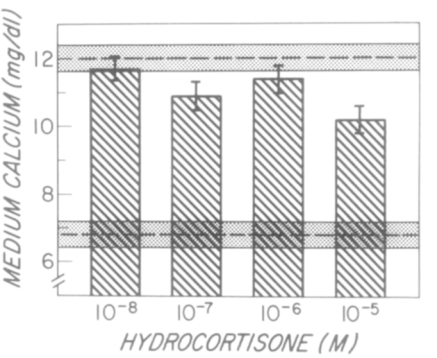

Fig. 4. Effect of HC on PGE_2-stimulated bone resorption in vitro. Groups of 4 bones each were untreated (lower dashed horizontal line at 6.8 mg calcium/dl), treated with 100 ng PGE_2/ml (upper dashed horizontal line at 12.0 mg calcium/dl), or with PGE_2 (100 ng/ml) plus hydrocortisone (10^{-8} to 10^{-5} M) for 48 hr and medium calcium concentration determined. The bars give the mean values and the brackets are the SE. The shaded areas surrounding the control and PGE_2 alone mean values give the SE.

prevent the calcium-mobilizing effect of PGE_2, we tested the bone resorption-stimulating activity of PGE_2 in the absence and presence of HC (Fig. 4). At concentrations up to 10^{-6}M, HC had no significant effect on PGE_2-mediated bone resorption. A small inhibitory effect was observed at 10^{-5}M HC, a concentra-

tion well above the extracellular fluid levels that were achieved in the *in vivo* experiments in mice.

DISCUSSION

Previous studies had shown elevations of plasma concentrations of PGE_2 and calcium 2.5 to 3.0 weeks after implantation of $HSDM_1$ tumor cells in mice (1,3,4) although the increase in PGE_2 was only about two-fold. We now show that PGE_2 metabolite concentrations in plasma are elevated by 1.5 weeks, that this increase precedes the development of hypercalcemia and that the rise, in absolute magnitude, is greater than that of PGE_2 itself. The greater rise in PGE_2 metabolites probably reflects the rapid metabolism of PGE_2 and the relative stability of the metabolite in plasma (13). We conclude that PGE_2 concentrations are elevated sufficiently early in tumor-bearing mice to be causally related to the hypercalcemia. A preliminary report of analogous findings in rabbits bearing the VX_2 carcinoma has been presented (14).

Reversal of the hypercalcemic syndromes that occur in mice bearing the $HSDM_1$ tumor and rabbits carrying the VX_2 carcinoma has been shown (4,15). These effects appear to be due to inhibition by indomethacin of PGE_2 synthesis by the tumor cells mediated through the prostaglandin cyclooxygenase (16) thus preventing the conversion of arachidonic acid to PGE_2. In contrast, it appears that the inhibitory effect of HC on prostaglandin synthesis occurs by prevention of arachidonic acid release from phospholipid precursors in the cell and not by interference with the activity of the cylooxygenase (8,17). These different mechanisms of inhibition may explain our findings on PGE_2 content in tumors from indomethacin-(1,3,4, and Fig. 2) and HC-treated mice (Fig. 2). The low content of PGE_2 in tumors of indomethacin-treated animals probably reflects the true endogenous situation; synthesis could not occur during homogenization because the cyclooxygenase was already inhibited. On the other hand, the PGE_2 content of tumors from HC-treated mice was not statistically different from controls ($p>0.05$) even if the tumors were rapidly frozen in liquid N_2 and

homogenized in buffer containing indomethacin. Presumably, active cyclooxygenase was able to synthesize PGE_2 very rapidly during homogenization before indomethacin could inhibit it. Such rapid synthesis of PGE_2 is known to occur when tissues are processed in aqueous media (18). An alternative explanation, namely that HC prevents the conversion of PGE_2 to its metabolites, seems unlikely from results of previous studies which showed 96% inhibition of PGE_2 production by HC in rheumatoid synovia accompanied by significantly lesser inhibition, 78%, of accumulation of 13,14-dihydro-15-keto PGE_2 (7).

Reversal of the hypercalcemic syndrome by HC in $HSDM_1$ tumor-bearing mice may explain the ability of anti-inflammatory glucocorticoids to moderate or eliminate hypercalcemia in certain patients with cancer. In addition to the known direct cytotoxic actions of the steroid hormones and their effects on calcium absorption from the gut, it is possible in some patients that glucocorticoids may act to inhibit prostaglandin production by the tumor.

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REFERENCES

1. Tashjian, A. H., Jr., Voelkel, E. F., Goldhaber, P., and Levine, L. (1974) Federation Proc. 33, 81-86.
2. Levine, L., Hinkle, P. M., Voelkel, E. F., and Tashjian, A. H., Jr. (1972) Biochem. Biophys. Res. Commun. 47, 888-896.
3. Tashjian, A. H., Jr., Voelkel, E. F., Levine, L., and Goldhaber, P. (1972) J. Exp. Med. 136, 1329-1343.
4. Tashjian, A. H., Jr., Voelkel, E. F., Goldhaber, P., and Levine, L. (1973) Prostaglandins 3, 515-524.
5. Franklin, R. B., and Tashjian, A. H., Jr. (1975) Endocrinology 97, 240-243.
6. Tashjian, A. H., Jr., Voelkel, E. F., McDonough, J., and Levine, L. (1975) Nature 258, 739-741.
7. Kantrowitz, F., Robinson, D. R., McGuire, M. B., and Levine, L. (1975) Nature 258, 737-739.
8. Hong, S. L., and Levine, L. (1976) Proc. Nat. Acad. Sci. USA 73, 1730-1734.
9. Levine, L., to be published.

10. Pong, S-S., and Levine, L., In Prostaglandins, P. W. Ramwell (Ed.), Vol. 3, Plenum Press, N. Y., in press.
11. Levine, L., Gutierrez-Cernosek, R. M., and Van Vunakis, H. (1971) J. Biol. Chem. 246, 6782-6785.
12. Robinson, D. R., Tashjian, A. H., Jr., and Levine, L. (1975) J. Clin. Invest. 56, 1181-1188.
13. Samuelsson, B., and Green, K. (1974) Biochem. Med. 11, 298-303.
14. Seyberth, H. W., Hubbard, W. C., Morgan, J. L., Sweetman, B. J., and Oates, J. A. (1976) Clin. Res. 24, 370A (Abstract).
15. Voelkel, E. F., Tashjian, A. H., Jr., Franklin, R., Wasserman, E., and Levine, L. (1975) Metabolism 24, 973-986.
16. Flower, R. J. (1974) Pharm. Rev. 26, 33-67.
17. Gryglewski, R. J. (1976) Pharm. Res. Commun. 8, 337-348.
18. Samuelsson, B., Granström, E., Green, K., Hamburg, M., and Hammarström, S. (1975) Ann. Rev. Biochem. 44, 669-695.