Control of VX2 Carcinoma Cell Growth in Culture by Calcium, Calmodulin, and Prostaglandins

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ABSTRACT

Based on our in vivo observation that growth of VX2 carcinoma transplanted in rabbits paralleled development of hypercalcemia, we studied the regulation of VX2 tumor growth using a clonal cell line isolated from VX2 tumor (VX2-L). VX2-L cell growth was dependent on prostaglandins released by the cultured cells into the medium, since indomethacin suppressed VX2-L growth, and prostaglandins A2, E1, E2, F1α, and F2α stimulated VX2-L proliferation. Prostaglandins D2 and I2 inhibited VX2-L proliferation. In contrast, prostaglandins D3 and I3 inhibited VX2-L proliferation. In contrast to previous reports, increases in extracellular calcium concentration promoted VX2-L growth not only directly but indirectly through augmentation of prostaglandin E synthesis. Antagonists of the intracellular calcium binding protein calmodulin inhibited cell replication.

Increases in extracellular calcium also stimulated production of a nonprostaglandin macromolecular bone-resorbing factor. This factor may account for the hypercalcemia which we were unable to block by indomethacin.

These results suggest a close relationship between VX2-L growth, prostaglandin production, and hypercalcemia. It is proposed that calcium blockers and anticalmodulin drugs might be powerful anticancer and/or antihypercalcemic agents for malignant cells such as VX2-L.

INTRODUCTION

One of the animal models used to study the mechanism of hypercalcemia associated with cancer has been the VX2 carcinoma which occurs in rabbits. Hypercalcemia seen in VX2 tumor-bearing rabbits has several interesting characteristics. It is associated with increased osteoclastic bone resorption but occurs in the absence of metastases to and destruction of bone by tumor cells (23, 59). Excision of the tumor results in reversal of hypercalcemia (58), but hypercalcemia occurs in thyroparathyroidectomized rabbits bearing the tumor (52). It is therefore plausible that VX2 tumor cells release a humoral bone-resorbing factor which is unrelated to the function of the parathyroid glands. Voelkel et al. (51) demonstrated that a PG3 synthesis inhibitor indomethacin (16) prevented the hypercalcemia in VX2-bearing rabbits and suggested that PGE2, a potent stimulator of osteoclastic bone resorption (28), released by the tumor cells accounts for the hypercalcemia.

We were able to reproduce similar hypercalcemia in rabbits by transplantation of clonal VX2 cells (VX2-L) established as described in the text and found that increases in serum calcium occurred in parallel with tumor enlargement, which led us to speculate that there may be a close correlation between serum calcium level and tumor growth. Furthermore, we confirmed that VX2-L released significant amounts of PGE into the culture medium. There is accumulating evidence which suggests that PGs are involved in the growth of a variety of human tumors (2, 10, 24, 43) and animal tumors (17, 39, 41, 42).

In the present study, we first studied the relationship between growth of VX2-L in culture, extracellular calcium concentration, and PGs. We found that VX2-L growth is dependent on PGs and calcium, and PG production by the cells is influenced by calcium. We also found that the intracellular calcium receptor calmodulin is involved in calcium stimulation of growth and PG production of VX2-L. In addition, we report that VX2-L cells produce a macromolecular bone-resorbing factor. These results suggest that several factors, PGs, and this macromolecular factor are responsible for the hypercalcemia in VX2-bearing rabbits.

MATERIALS AND METHODS

VX2 Carcinoma. VX2 tumor was kindly supplied by Dr. Yohei Ito, Department of Microbiology, Faculty of Medicine, Kyoto University. The tumor has been serially passaged in male albino rabbits weighing between 0.8 and 1.2 kg in our laboratory. Aseptically excised tumor was cut into pieces of approximately 5 mm in diameter by scalpel, and one piece for each animal was transplanted i.m. in the thigh muscles by trocar (37). The transplantation was repeated every 4 weeks.

Establishment of VX2-L Cell Line. The tumor tissue was minced with scissors into pieces smaller than 1 mm in diameter and was placed in 35-mm plastic culture dishes (Linbro; Flow Laboratories, Inc., McLean, VA) with a drop of culture medium (see below) to initiate explant outgrowth cultures according to the method of Jensen et al. (27). After 3 weeks when the cells grew out from the tumor pieces, the primary cultures were harvested with 0.05% trypsin-0.01% EDTA solution. Five x 10⁶ cells were plated onto 35-mm plastic culture dishes in DMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 5% (v/v) FBS (Grand Island Biological Co., Grand Island, NY) and 1% (v/v) penicillin-streptomycin solution (Flow Laboratories) and maintained in culture at 37° in a humidified atmosphere of 5% CO2 and 95% air. The culture medium was changed every 2 days. The VX2-L clone was established by single-cell cloning in microwell plates as described (33). The VX2-L clone established in this manner was grown in a similar culture condition to that described above. In each experiment, FBS was dialyzed before use in order to eliminate small molecular substances including PGs and calcium.

Measurement of DNA Synthesis. VX2-L cells were inoculated and cultured for 24 hr in DMEM supplemented with 5% FBS. The cultures were washed with ice-cold calcium- and magnesium-free PBS 3 times, and the culture medium was changed into DMEM supplemented with 0.2% dialyzed FBS and containing drugs and/or calcium as indicated. After 44 to 68 hr of culture, the culture medium was changed into fresh serum-free DMEM. The cells were then incubated with [3H]thymidine (1

1 This study was supported in part by a grant from the Ministry of Education of Japan.
2 To whom requests for reprints should be addressed.
3 The abbreviations used are: PG, prostaglandin; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; W-5, N-(6-aminohexyl)naphthalenesulfonamide; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; VX2-L, clonal cell line isolated from VX2 carcinoma; PGI2, prostaglandin F1α (other prostaglandins defined similarly).

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CANCER RESEARCH VOL. 45 JANUARY 1985 398

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DNA synthesis was determined by the incorporation of the isotope into the acid-precipitable fraction of the cells as described (62). Measurement of Cell Number. After the VX2-L cells were cultured in the presence of 0.2% dialyzed FBS and drugs as indicated for 44 to 68 h, they were harvested with 0.05% trypsin-0.01% EDTA solution and stained with 0.5% trypan blue. The cells which excluded trypan blue were counted on a hemocytometer.

PGE Determination. PGE content in the medium harvested from the cultures treated with drug and/or calcium as indicated in the presence of 0.2% dialyzed FBS for 72 hr was determined, using radioimmunoassay kits (Clinical Assays, Cambridge, MA). Samples for PGE assay were prepared according to instructions provided by the manufacturer. Although the antiserum used in the kit were directed against PGE1, the antibody cross-reacts about 23% with PGE2. Thus, the data in the text were expressed as PGE including PGE1 and PGE2.

Measurement of 45Ca Entry into Cells. Confluent VX2-L cells were washed with calcium-free Earle’s solution (14) 3 times and reincubated in the same solution containing drugs tested for 15 min. The cultures were then incubated with 45CaCl2 (1 µCi/ml) (8.15 mCi/mg calcium; New England Nuclear) for 5 min. Incubation was terminated by the addition of ice-cold Earle’s solution (calcium containing), and the cells were gently scraped with a rubber policeman and washed with 15 ml of ice-cold Earle’s solution on TM-2 membrane filters (0.45 µm; Toyo Roshi Co., Tokyo, Japan) under vacuum as described in Ref. 30. The radioactivity remaining on the filters was counted at 30% efficiency in a liquid scintillation spectrophotometer (LKB).

Assay for Bone-resorbing Activity. The culture supernatants from confluent VX2-L cells to which drugs were added in serum-free DMEM for 72 hr were dialyzed against PBS (NaCl, 8.0; KCl, 0.2; CaCl2, 0.1; MgSO4·7H2O, 0.06; MgCl2·6H2O, 0.1; Na2HPO4·2H2O, 1.44; KH2PO4, 0.2 g/liter, pH 7.2) using Spectrapor 6 dialysis membranes with a nominal molecular weight cutoff of about 10,000 (Spectrum Medical Industries, Inc., Los Angeles, CA). Bone-resorbing activity in dialyzed culture supernatants was assessed by the release of 45Ca incorporated previously from fetal rat long bones in organ culture according to the methods described previously (61). Bone resorption was expressed as the percentage of release of 45Ca from individual bones into the culture medium during 72 hr of culture. Four bones were used in each treatment group.

Serum Calcium Analysis. Heparinized blood was drawn from the ear vein of VX2 tumor-bearing rabbits at 7-day intervals. Serum calcium concentrations were determined by the method of Morin (36).

Protein Determination. Protein was determined by the method of Lowry et al. (31) using bovine serum albumin (Fraction V; Sigma Chemical Co., St. Louis, MO) as a standard.

Statistics. Statistical differences were analyzed by Student’s t test.

Reagents. PGs, nifedipine, trifluoperazine dihydrochloride, and ionophore A23187 were kindly provided by Ono Pharmaceutical Co. (Osaka, Japan), Bayer Pharmaceutical Co. (Osaka, Japan), Yoshitomi Pharmaceutical Co. (Osaka, Japan), and Eli Lilly Co. (Indianapolis, IN), respectively. W-5 and W-7 were purchased from Rikaiken Co. (Nagoya, Japan). Indomethacin and reagents used for assay for serum calcium level were obtained from Sigma. Other reagents were all analytical grade.

RESULTS

Established VX2 Clone. VX2-L cells, established as described in "Materials and Methods," demonstrated an epithelial shape and proliferated rapidly (doubling time at log phase, 28 to 32 hr) with piling up to form a dense cell layer, and they have been maintained for more than 2 years in our laboratory (Fig. 1). VX2-L cells demonstrated anchorage-independent growth by forming colonies in soft agar (number of colonies/5 × 105 cells plated, 363 ± 189 (S.E.) for 6 experiments) when assayed by the method of Hamburger and Salmon (21). These results suggest that VX2-L cells retain cancerous property even after long cultivation in vitro. Furthermore, transplantation of 1 to 3 × 107 VX2-L cells in the thigh muscles of young male rabbits resulted in formation of tumors histologically similar to VX2 carcinoma. Although we obtained several VX2 clones other than VX2-L, and each clone showed epithelial shape and anchorage-independent growth in vitro and produced PGE similarly to VX2-L, none of them, for unknown reason, formed tumors in animals. Therefore, in the following experiments, only VX2-L cells were used. In a preliminary experiment, we examined the effect of serum concentration in the culture medium on VX2-L growth and found that the cells were capable of growing, although the rate of proliferation was positively dependent on serum concentration added in the culture medium, under the culture conditions used in each experiment.

PGE Production and Growth of VX2-L. VX2-L cultured in DMEM supplemented with 0.2% dialyzed FBS for 72 hr released PGE (21 ng/106 cells) into the medium. Addition of 1 µM indomethacin to the cultures resulted in a dramatic decrease in PGE production, which was accompanied by a marked reduction of DNA synthesis and cell division (Table 1). Growth of other VX2 clones was also inhibited by 1 µM indomethacin (data not shown).

Skin fibroblasts isolated from VX2 tumor-bearing rabbits released much less PGE into the culture medium (2.1 ng/106 cells) than did VX2-L, which was inhibited by indomethacin. However, in contrast to VX2-L, indomethacin has no effect on DNA synthesis and cell division of skin fibroblasts (Table 1). Since the number of VX2-L cells or skin fibroblasts which had been treated with 1 µM indomethacin for 68 hr was higher than that of the cells at plating, it is unlikely that the effects of indomethacin seen in this experiment are due to cytotoxicity.

Effects of Various PGs on VX2-L Growth. PGA2, PGF1α, and PGF2α, significantly stimulated VX2-L growth at a concentration of 10−6 M. PGE1 and PGE2 markedly promoted VX2-L growth at a concentration as low as 10−8 M, and PGE2 was more effective than PGE1 (Chart 1). On the other hand, both PGD2 and PGF2α inhibited VX2-L growth (Chart 1).

Tumor Growth and Hypercalcemia. Tumors formed by transplantation of VX2-L were palpable after 7 days and visible after 10 to 14 days. Tumor volume calculated according to the method described previously by Ito et al. (26) began to increase within 2 weeks after transplantation and reached approximately

### Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>PGE production (ng/10⁶ cells)</th>
<th>Cell no. (10⁶/35-mm dish)</th>
<th>[3H]Thymidine incorporation (dpm/mg protein × 10⁻¹⁰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VX2-L</td>
<td>None</td>
<td>21 ± 3³</td>
<td>58 ± 3</td>
<td>14 ± 3</td>
</tr>
<tr>
<td></td>
<td>Indomethacin (1 µM)</td>
<td>0.5 ± 0.1</td>
<td>21 ± 2</td>
<td>6 ± 1⁴</td>
</tr>
<tr>
<td>Skin fibroblast</td>
<td>None</td>
<td>2.1 ± 0.2</td>
<td>30 ± 3</td>
<td>7 ± 1</td>
</tr>
<tr>
<td></td>
<td>Indomethacin (1 µM)</td>
<td>Undetectable</td>
<td>29 ± 1</td>
<td>7 ± 1</td>
</tr>
</tbody>
</table>

*Mean ± S.E. (n = 6). ³Significantly smaller than untreated group (p < 0.01).
VX₂ CELL GROWTH AND HYPERCALCEMIA

Chart 1. Effects of various PGs on VX₂-L growth. VX₂-L cells (1 x 10⁶/35-mm dish) were cultured in DMEM supplemented with 5% FBS for 24 hr. Then, the cells were grown in DMEM supplemented with 0.2% dialyzed FBS for 72 hr in the presence of PGA₂ (○), PGD₂ (△), PGE₁ (□), PGE₂ (•), PGF₁α (■), PGF₂α (▲), or PGF₃α (●). The cell number of untreated VX₂-L after 72-hr culture was 3.8 ± 0.4 x 10⁶. Points, mean of 6 cultures.

Chart 2. Growth of VX₂ carcinoma transplanted in rabbits and development of hypercalcemia. The tumor was transplanted as described in the text. Tumor volume (○) was calculated by the formula volume = length x width x thickness (25). Serum calcium level (□) was determined using o-cresolphthalein complexone (36). Points, mean of 6 animals; bars, S.E. Inset, linear regression of the chart.

100 cu cm at 5 weeks of transplantation (Chart 2). In parallel with this, serum calcium concentrations also began to increase after 2 weeks and reached 16.8 ± 0.24 mg/dl (S.E.; n = 6) at 5 weeks (Chart 2). A highly significant correlation existed between serum calcium concentration and tumor volume (Chart 2, inset). Serum calcium concentrations in sham-operated control animals were unchanged (11.8 ± 0.3 mg/dl and 12.1 ± 0.3 mg/dl, pre- and 5 weeks of posttransplantation, respectively).

Calcium Stimulation of VX₂-L Growth. The results demonstrated in Chart 2 suggest that elevations in extracellular calcium may influence VX₂ carcinoma growth. An increase in calcium concentration in the culture medium (DMEM contains 1.8 mm calcium) from 2.5 to 5 mm resulted in a dramatic stimulation of VX₂-L DNA synthesis in a dose-dependent manner (Chart 3). However, in the presence of 1 μM indomethacin, calcium stimulation of DNA synthesis by VX₂-L was greatly impaired. Addition of 1 μM PGE₂ markedly promoted calcium stimulation of VX₂-L DNA synthesis when the calcium concentration was in the range of 1.8 to 3.5 mm. However, when the calcium concentration was above 3.5 mm, no promotion by PGE₂ of calcium stimulation of VX₂-L DNA synthesis occurred. PGE₂ completely restored indomethacin-reduced calcium stimulation of VX₂-L DNA synthesis.

Experiments in which VX₂-L cells were treated with EDTA to further confirm that calcium is necessary for VX₂-L to proliferate were unsuccessful, since the cells detached from the bottom of dishes 3 hr after the addition of EDTA.

Effects of Nifedipine and Ionophore A23187 on VX₂-L Growth. We next studied the effects of drugs known to alter the entry of extracellular calcium into cells on VX₂-L growth. The calcium blocker nifedipine (4) and divalent cation ionophore A23187 (55) were tested. As shown in Table 2, nifedipine inhibited VX₂-L growth in conjunction with inhibition of ⁴⁵Ca entry into the cells in a concentration-dependent manner. In contrast, ionophore A23187 demonstrated a stimulatory effect on VX₂-L growth as well as on ⁴⁵Ca entry into the cells.

Inhibition of VX₂-L Growth by Calmodulin Antagonists. The results shown on Table 2 suggest that changes in intracellular calcium level due to alteration of calcium entry into cells influence VX₂-L growth. It is therefore of particular interest to study the role of the intracellular calcium receptor calmodulin (8, 34) in control of VX₂-L growth. W-7, a newly synthesized anticalmodulin...
drugs (22, 46), significantly inhibited cell division and DNA synthesis of VX2-L at concentrations of 30 to 50 μM. W-7 had no effect on 45Ca entry into the cells (Table 3). In a separate experiment, W-5, a chlorine-deficient analogue of W-7 which is known to bind to and inhibit calmodulin action (29), suppressed calcium stimulated DNA synthesis of VX2-L at concentrations of 30 to 50 μM. The phenothiazine trifluoperazine, which binds to calmodulin only weakly (22), did not inhibit VX2-L growth at a concentration of 30 μM. The phenothiazine trifluoperazine, which is known to bind to and inhibit calmodulin action (29), suppressed VX2-L DNA synthesis (data not shown).

Effects of Calcium and Various Drugs on VX2-L PGE Production. From the results described above, it is likely that calcium and other drugs tested control VX2-L growth through modulating PG production by the cells. We next examined the effects of these agents on PG synthesis by VX2-L. PGE synthesis by VX2-L was dependent on the calcium concentration in the culture medium. An increase in calcium concentration above 2.5 mM resulted in a marked stimulation of VX2-L PGE production (Chart 4). Maximal stimulation was obtained at 4 mM calcium. Addition of indomethacin completely blocked calcium stimulation of VX2-L PGE synthesis. The calcium blocker nifedipine markedly reduced a stimulatory effect of calcium on VX2-L PGE production. The anticalmodulin drug W-7 also suppressed calcium stimulation of PGE production, but to a lesser extent than did nifedipine.

In contrast, the calcium ionophore A23187 enhanced calcium stimulation of VX2-L PGE synthesis.

Bone-resorbing Activity in VX2-L Culture Supernatants. In contrast to a previous paper (51) that indomethacin prevented hypercalcemia in VX2-L-bearing rabbits, we as well as Doppelt et al. (12) were unable to block an elevation of serum calcium level in rabbits transplanted with VX2-L clone by indomethacin, suggesting that an as yet unidentified factor may be involved in the hypercalcemia. Since it is generally believed that increases in serum calcium are due to an increase in bone resorption, we tested if VX2-L releases a bone-resorbing factor into the culture medium in the presence of indomethacin. As depicted in Chart 5, VX2-L cultured in the medium containing 3.5 to 5 mM calcium released a nondialyzable factor(s) with powerful bone-resorbing activity. Treatment of VX2-L with indomethacin failed to inhibit the production of this factor despite the fact that indomethacin completely blocked PG synthesis by VX2-L cells as demonstrated in Chart 4. Nifedipine suppressed the production of the factor when the calcium level in the medium was below 4 mM. However, above 4 mM, VX2-L cells released the factor even in the presence of nifedipine. Similarly, W-7 reduced the production of the factor at a calcium level below 4.5 mM but failed when the calcium level was 5 mM.

DISCUSSION

There is accumulating evidence which indicates a significant role of calcium in the mitotic process of a variety of cells (3, 32, 57). In contrast to normal cells, it has been demonstrated that neoplastic cells require much less calcium for their growth (1, 56). Swierenga et al. (50) have shown that neoplastic cells with high tumorigenicity are able to proliferate in a low-calcium me-
diurn and suggested that the relationship between calcium concentration and growth is a simple and sensitive indicator of tumorigenicity in vivo. However, in VX2-L cells, although the cells showed anchorage-independent growth in vitro and profound tumorigenicity in vivo, growth was stimulated by increases in extracellular calcium (Chart 3) or in the intracellular calcium level caused by stimulation of extracellular calcium influx (Table 2) and was inhibited by decreases in intracellular calcium levels caused by inhibition of extracellular calcium influx (Table 2). These results indicate that VX2-L growth is dependent on calcium, despite its highly tumorigenic property, and thus, VX2-L tumors are different from other neoplastic cells in this respect.

Subsequently, we have found that the intracellular calcium receptor calmodulin is involved in VX2-L growth (Table 3; Chart 3). Involvement of calmodulin in CHO-K1 cell proliferation has been reported (6, 22, 46). In Swiss 3T3 or normal rat kidney cells transformed by oncogenic viruses, increased intracellular calmodulin levels were found compared to their nontransformed counterparts, suggesting that calmodulin may play a role in the expression of one characteristic of transformed cells, uncontrollable growth (7). In fact, Wei and coworkers (53, 54) and Ito and Hidaka (25) have demonstrated that there is a positive correlation between the intracellular calmodulin level and hepatoma growth in vivo. Very recently, however, there are accumulating reports that indicate that calmodulin is not always implicated in the growth of neoplastic (35) and transformed (9, 13) cells. Therefore, the role of calmodulin in cell proliferation is controversial. In the present study, we demonstrated clearly that a new synthetic and specific calmodulin antagonist W-7 inhibited VX2-L growth, suggesting a pivotal role for calmodulin in VX2-L proliferation. It is likely that the influence of calcium and calmodulin on VX2-L growth at least in part, may be related to its hypercalcemia-producing action. Thus, the hypercalcemia provides an environment favorable for VX2-L to proliferate which, in turn, may produce further severe hypercalcemia. In addition to this, we have shown that VX2-L released significant amounts of PGE, which were stimulated by the elevation of the calcium level in the culture medium and required calmodulin function (Chart 4). PGE and other families of PGs are potent bone resorbers (11, 28) and a presumable cause of hypercalcemia in VX2-bearing rabbits. Another well-recognized action of PGE is the negative regulation of humoral and cellular immunity (20, 41). Thus, the hypercalcemia might be an essential event for the VX2 tumor to grow and escape from the host immunosurveillance system. Finally, these results raise the possibility that calcium blockers and anticalmodulin drugs might be potent anticancer drugs in some hypercalcemia-producing tumors, such as the VX2 tumor and many other types of malignant tumor (38), as suggested (25, 53, 54). In a preliminary experiment, we have found that nifedipine administered together with bleomycin, which has been shown recently to be a potent antihypercalcemic agent (40), prevented enlargement of the VX2 tumor and development of the hypercalcemia, and it resulted in increased survival (data not shown).

Inhibition of VX2-L PG production by indomethacin resulted in decreased DNA synthesis and division of VX2-L but not of skin fibroblasts isolated from VX2-bearing rabbits (Table 1), indicating that PG production may be specifically necessary for VX2-L proliferation. This is supported further by the results that PAG1, PGE1, PGF2a, and PGF2a promoted VX2-L growth (Chart 1). At present, it is not known if PG stimulation of VX2-L growth is mediated by specific PG receptors on the plasma membrane. If so, VX2-L cells proliferate in response to PGs secreted from the cell itself, thus providing an example of an autocrine system (48). Studies to prove this are in progress.

It is of interest that PGD2 and PGF2a inhibited VX2-L growth (Chart 1). Recently, antineoplastic (18, 47) and antimetastatic actions (15, 49) of PGD2 both in vivo and in vitro have been reported. Our results support these previous reports. As far as PGF2a is concerned, this is the first demonstration to our knowledge that PGF2a is effective in directly inhibiting malignant cell growth. It is generally believed that the effects of PGD2 and PGF2a (5) as well as PGE and PGF (45) are mediated through cyclic AMP accumulation in cells. Growth of VX2-L cells treated with the phosphodiesterase inhibitor isobutylmethylxanthine and the nonspecific adenylate cyclase stimulator forskolin was suppressed (data not shown). It therefore seems likely that inhibition of VX2-L growth by PGD2 and PGF2a might be mediated through a mechanism associated with cyclic AMP. Mechanism(s) involved in VX2-L growth stimulation by PGE, PGE2, or PGF2a are unknown but probably are not associated with cyclic AMP. Further studies are needed to elucidate the mechanism.

Our result that indomethacin suppressed VX2-L growth is inconsistent with previous reports in which indomethacin had no effect on VX2 growth (51). This discrepancy is likely to result from the difference of VX2 strain used and the culture condition. In the previous experiments, VX2 cells were cultured in the presence of high concentrations of sera (15% horse and 2.5% fetal calf serum), which supply enough PGs for VX2 cells to proliferate. In contrast, in our experiments, VX2-L was cultured in DMEM supplemented with 0.2% dialyzed serum. In these culture conditions, endogenous PGs are the major source of PGs for VX2-L growth.

VX2-L released nondialyzable macromolecular factor(s) with profound bone-resorbing activity at high calcium concentration (Chart 5). Failure of indomethacin to inhibit the production of this factor suggests that a mechanism independent of PGs but directly dependent upon calcium might be involved in the production of this factor, and this factor might be responsible for the hypercalcemia which is not prevented by administration of indomethacin. In fact, the calcium blocker nifedipine suppressed the release of this factor at calcium levels below 4 mm (Chart 5). Recently, Doppelt et al. (12) demonstrated that the hypercalcemia in their variant of VX2-bearing rabbits was not affected by treatment of indomethacin, and elevated plasma calcium levels were reduced rapidly to normal when the diet was changed from the normal to a calcium-free diet. They proposed that the hypercalcemia is dependent on dietary calcium but independent of PG. Our results fit in well with their observations, since production of the macromolecular bone-resorbing factor was dependent on extracellular calcium concentrations. Of particular interest is the relationship between PGs and this factor in producing the hypercalcemia in VX2-bearing rabbits. At low calcium levels, no production of this factor occurred (Chart 5), although concentrations of PGE sufficient to cause bone resorption were secreted into the medium (Chart 4). We speculate that the hypercalcemia in VX2-bearing rabbits might involve 2 steps. PGs first secreted by VX2 tumors elevate serum calcium by causing increased bone

| J. T. Potts, personal communication. |
resorption, and then, elevated serum calcium induces PG-independent but calcium-dependent production of this factor that further elevates the serum calcium level. This idea is supported by the result of Galasko et al. (19), who found that administration of indomethacin before or within 7 days of VX2 tumor transplantation (no hypercalcemia in animals) was effective in inhibiting bone destruction, whereas indomethacin administered 14 or 21 days after transplantation (when the animals were already hypercalcemic) was unable to block osteolysis.

Calcium stimulation of VX2-L DNA synthesis was greatly impaired in the presence of indomethacin (Chart 3). This result, together with the finding that calcium stimulates VX2-L PGE production (Chart 4), suggests that the stimulatory effect of calcium on VX2-L growth is mediated through PG production. Restoration by PGE2 of indomethacin-inhibited calcium stimulation of VX2-L DNA synthesis further supports this (Chart 3). However, since indomethacin failed to completely block calcium stimulation of VX2-L (Chart 3), it appears that VX2-L growth is not only dependent on PGs but directly dependent on calcium or partly on a mechanism unrelated to PGs.

The result that the anticalcinomul drug W-7 suppressed calcium stimulation of PGE production by VX2-L (Chart 4) indicates calmodulin involvement in VX2-L PGE production. It is generally accepted that the effect of calcium on many cellular functions is mediated by the ubiquitous cytosolic calcium-binding protein calmodulin (8, 34). Calmodulin is known to stimulate phospholipase A2 activity (60) that is a rate-limiting enzyme in the generation of the PG precursor arachidonic acid. Taken together, it is proposed that, in the control of VX2-L growth, calcium activates calmodulin, which stimulates phospholipase A2 activity. Elevated enzyme activity then increases production of PGE which, in turn, promotes VX2-L growth.

In conclusion, the present study demonstrates that VX2-L has a variety of capabilities including production of PGE and a macromolecular bone-resorbing factor causing the hypercalcemia and increased responsiveness to calcium and PGE. These influences may be important factors in tumor cell replication and lead to unrestrained tumor growth.

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CANCER RESEARCH VOL. 45 JANUARY 1985

403
Fig. 1. Phase-contrast microscopy of VX2-L. The cells were cultured in DMEM supplemented with 5% FBS for 10 days. Note that the cells demonstrate epithelial shape and are piling up to form a dense cell layer.
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