Breast Cancer Growth Is Inhibited by Vasoactive Intestinal Peptide (VIP) Hybrid, a Synthetic VIP Receptor Antagonist

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ABSTRACT

Breast cancer vasoactive intestinal peptide (VIP) receptors were characterized. Using in vitro autoradiographic techniques, 125I-labeled VIP bound with high affinity to breast biopsy sections. 125I-labeled VIP bound specifically to five breast cancer cell lines examined using receptor-binding techniques. Specific 125I-labeled VIP binding to MDA-MB-231 cells was inhibited with high affinity by VIP and pituitary adenylate cyclase-activating polypeptide (IC50 = 2 nM) and with moderate affinity by the VIP hybrid (IC50 = 0.5 μM). VIP elevated the cAMP in a dose-dependent manner, and VIP hybrid (10 μM) inhibited the increase in cAMP caused by VIP. Using Northern blot analysis, VIP (10 nm) stimulated c-fos and c-myc mRNA, and the increase caused by VIP was reversed by the VIP hybrid. The VIP hybrid inhibited breast cancer growth in vitro and in vivo using nude mice bearing breast cancer xenografts. These data suggest that the VIP hybrid is a breast cancer VIP receptor antagonist.

INTRODUCTION

VIP2 is a 28-amino acid peptide initially isolated from porcine stomach (1). It is structurally similar to the 27-amino acid PACAP, GRF, PHI, secretin, and helodermin (2). The receptors for VIP and PACAP have been cloned and have seven transmembrane domains. The VIP1 and VIP2 receptors contain 459 and 430 amino acids, respectively (3, 4) and bind VIP as well as PACAP with high affinity. The PACAP type I receptor contains 495 amino acids and binds PACAP but not VIP with high affinity (5, 6).

When VIP or PACAP bind to their receptors, a stimulatory guanine nucleotide-binding protein is activated (Gs), increasing adenylyl cyclase activity (7). Nanomolar concentrations of VIP or PACAP elevate intracellular cAMP and activate protein kinase A (8). In addition, PACAP but not VIP elevates cysolic Ca2÷ (9). PACAP receptors may also interact with a guanine nucleotide-binding protein (Gq) causing PI turnover. Phospholipase C metabolizes PI-4,5-bisphosphate to inositol-1,4,5-trisphosphate which elevates cytosolic Ca2÷ and diacylglycerol which activates protein kinase C.

VIP alters the proliferation of numerous cells including neuroblasts, keratinocytes, glial cells, and lung cancer (10—14). In NSCLC, VIP increased colony number and the actions of VIP were reversed by the synthetic VIP receptor antagonist VIPhyb (15, 16). VIPhyb inhibits 125I-labeled VIP binding to NSCLC cells, the elevation of cAMP caused by VIP, and the proliferation of NSCLC cells induced by VIP. Also, VIPhyb inhibits basal growth of NSCLC in vitro and in vivo.

Because VIP is also produced in NSCLC cells (17), VIP is an autocrine growth factor for NSCLC.

VIP receptors may also be active in other epithelial cancers. VIP binding sites have previously been detected in gastric and colon cancer as well as melanoma and neuroblastoma (18—21). In this study, the effects of VIPhyb were investigated on breast cancer cells.

MATERIALS AND METHODS

Cell Culture. Breast cancer cells (BT-20, MCF-7, MDA-MB-231, SKBR-3, and ZR-75—1) were purchased from American Type Culture Collection and cultured in DMEM containing 10% heat-inactivated FBS. The cells were cultured in 5%CO2/95% air at 37°C and were used in the exponential growth phase. Routinely, the cells, which are adherent, were split 1:20 weekly. The cell lines were Mycoplasma free. For in vivo labeling with [32P]methionine, MDA-MB-231 or MCF-7 cells were cultured in methionine-free DMEM containing 10% FBS. After 16 h at 37°C, the conditioned medium was removed, and the cells were washed three times with PBS. The cells were treated with 0.2% NaOH (250 μl), and the contents were added to a scintillation vial. Then 250 μl of 0.2% HCl were added followed by 10 ml Aquasol. The samples were shaken and counted in a beta counter.

VIP Receptor Assays. For receptor-binding studies, the cells were rinsed three times in SIT medium (DMEM containing 3 x 10—8 M Na2SO4, 5 μg/ml insulin, and 10 μg/ml transferrin). The binding studies were conducted using breast cancer cells in 24-well plates (0.5 x 105 cells/well). The cells were incubated with 125I-labeled VIP (0.2 nm) in the presence or absence of competitor; the receptor-binding medium was SIT containing 1% BSA and 1 mg/ml bacitracin. After incubation at 37°C for 30 min, free 125I-labeled VIP was removed by washing three times in receptor-binding medium. The cells that contained bound peptide were dissolved in 0.2 N NaOH and counted in a gamma counter (14).

VIP receptors were identified in biopsy specimens using in vitro autoradiographic techniques (22). Breast biopsy sections (10 μm) were incubated with 125I-labeled VIP (2200 Ci/mmol) in the presence or absence of competitor (1 μM VIP) using 130 mM NaCl, 5 mM MgCl2, 5 mM KCl, 1 mM MnCl2, 1 mM EGTA, and 10 mM HEPES/NaOH (pH 7.4) containing 1% BSA and 1 mg/ml bacitracin (23). After 90 min at 25°C, free radiolabeled peptide was removed by washing in buffer three times for 15 min. After a brief dip in water followed by drying, the sections containing bound 125I-labeled VIP were apposed to Hyperfilm. After 2 weeks at —80°C, the films were developed.

Adenylate Cyclase. cAMP was assayed using a RIA (8). Cell lines MDA-MB-231 or MCF-7 were washed twice in SIT medium and resuspended in SIT medium containing 1% BSA, 1 mg/ml bacitracin, and 100 μM isobutyl-methylxanthine. After 5 min, the reaction was quenched by the addition of an equal volume (0.5 ml) of ethanol. The samples were vortexed and frozen at —80°C until assayed.

Nuclear Oncogenes. MDA-MB-231 cells in 15-cm dishes were treated with ST medium (10 ml) containing 0.5% FBS for 4 h. Then appropriate stimuli were added, e.g., 10 nM VIP for 60 min. The medium was removed, and the total RNA was isolated using the guanidinium isothiocyanate method (24). Ten μg of denatured RNA were separated in a 0.66 M formaldehyde-1% agarose gel (25). The gel was treated with ethidium bromide to assess RNA integrity. The RNA was blotted onto a Nylon membrane overnight, and the membrane was hybridized with cDNA probes for c-fos, c-jun, or c-myc (26) labeled with [32P]dCTP using a Bethesda Research Laboratories random priming kit. The membrane was exposed to Kodak XAR-2 film at —80°C, and the autoradiogram was developed.

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2 The abbreviations are: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; GRF, growth hormone-releasing factor; PHI, peptide histidine isoleucine; PI, phosphatidylinositol; NSCLC, non-small cell lung cancer; hyb, hybrid; FBS, fetal bovine serum; GIT, guanidinium isothiocyanate; PMA, phorbol 12-myristate 13-acetate.

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**Growth Assays.** Growth assays in vitro were conducted using MDA-MB-231 cells or MCF-7 cells and the agarose cloning system (27). The base layer consisted of 3 ml of 0.5% agarose in SIT medium containing 5% FBS in 6-well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, the peptide, and $3 \times 10^4$ single viable cells. For each cell line and peptide concentration, triplicate wells were plated. After 2 weeks, 1 ml of 0.1% p-iodonitrotetrazolium violet was added, and after 16 h at 37°C the plates were scored for colony formation; the number of colonies larger than 100 μm in diameter were counted using an Omnicron image analysis system.

The ability of VIPhyb to inhibit xenograft formation in nude mice was investigated (14). Female athymic BALB/c nude mice, 4–5 weeks old, were housed in a pathogen-free temperature-controlled isolation room, and the diet consisted of autoclaved rodent chow and autoclaved water given ad libitum. MCF-7 cells ($1 \times 10^7$) were injected into the right flank of four mice by s.c. injection after administration of an estradiol pellet (0.75 mg/2 months). Also, MDA-MB-231 cells were injected into nude mice. Palpable tumors were observed in approximately 90% of the mice after 1 week. Then PBS (100 μl) or VIPhyb (10 μg/day s.c.) was administered. The tumor volume (height × width × depth) was determined twice weekly using calipers and recorded. When the tumor became necrotic, the growth studies were terminated.

**RESULTS**

**Receptor Binding.** Table 1 shows that all breast cancer cell lines examined had $^{125}$I-labeled VIP-binding sites. The density varied 20-fold, being lowest in BT-20 and highest in SKBR-3. Because MDA-MB-231 was a representative cell line, it was used in subsequent experiments. The structure-activity relationships of $^{125}$I-labeled VIP binding were investigated. Fig. 1 shows that specific $^{125}$I-labeled VIP binding was inhibited in a dose-dependent manner by VIP. Little specific binding was inhibited by 0.1 nM VIP whereas almost all specific binding was inhibited by 1 μM VIP. Specific $^{125}$I-labeled VIP binding was half maximally inhibited (IC₅₀) by 2 nM VIP. Similarly, PACAP had an IC₅₀ value of 2 nM but VIPhyb was less potent with an IC₅₀ value of 0.5 μM. Similar binding data were obtained using MCF-7 cells.

VIP binding sites were detected on breast cancer tumors (Fig. 2). Using in vitro autoradiography techniques, high densities of total $^{125}$I-labeled VIP-binding sites were detected in three breast cancer biopsy specimens (Fig. 2, A, C, and E), whereas nonspecific binding in the presence of 1 μM VIP was greatly reduced (Fig. 2, B, D, and F). These data suggest that receptors which bind PACAP and VIP with high affinity are present on breast biopsy specimens.

**cAMP.** VIP elevated the cAMP levels in MDA-MB-231 cells in a dose-dependent manner. Using a RIA, Fig. 3 (top panel) shows that the basal cAMP was 1 fmol, and 0.1, 1, and 10 nM VIP elevated the cAMP to approximately 2, 12, and 11 fmol, respectively. The ED₅₀ was 0.3 nm. VIPhyb itself had little effect on the basal cAMP levels, but inhibited the increase in cAMP caused by VIP. Fig. 3 (bottom panel) shows that VIPhyb inhibited the increase in cAMP caused by 10 nM VIP and the half maximal dose was approximately 10 μM. If 10 μM VIPhyb was added to MDA-MB-231 cells in the presence of increasing amounts of VIP, the dose response curve was shifted to the right such that the ED₅₀ value for VIP was 4 nM. These data suggest that VIPhyb functions as a breast cancer VIP receptor antagonist.

**Nuclear Oncogenes.** VIP caused a concentration and time-dependent increase in c-fos mRNA. Fig. 4 shows that 10 nM VIP or 1 nM PACAP caused a 4-fold increase in c-fos mRNA after 1 h. Similarly, rat GRF, PHI, or helodermin, which bind with moderate affinity to VIP receptors (IC₅₀ = 50, 200, and 100 nm, respectively), elevated c-fos mRNA. The positive control was PMA, which activated protein kinase C, and increased c-fos mRNA 25-fold. The negative control was glucagon (IC₅₀ > 10 μM), which does not bind to VIP receptors and did not increase c-fos mRNA. Equal amounts of RNA were loaded onto the gel based on ethidium bromide staining of the 28S and 18S rRNA bands. Fig. 5 shows that VIP increased c-myc mRNA. VIPhyb had no effects on basal c-myc mRNA but inhibited the

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**Table 1: VIP receptor density in breast cancer cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$^{125}$I-labeled VIP bound specifically</th>
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<tbody>
<tr>
<td>BT-20</td>
<td>604</td>
</tr>
<tr>
<td>MCF-7</td>
<td>1310</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>4863</td>
</tr>
<tr>
<td>SKBR-3</td>
<td>12027</td>
</tr>
<tr>
<td>ZR75-1</td>
<td>5491</td>
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</tbody>
</table>

*Mean value of four determinations is shown. SE was approximately 10% of the mean value.*

**Fig. 1.** VIP breast cancer receptor binding. The percentage of specific $^{125}$I-labeled VIP (0.2 nm) bound to MDA-MB-231 cells was determined as a function of unlabeled VIP (●), PACAP (○), or VIPhyb (▲) concentration. The mean value ± SE of three determinations, each repeated in quadruplicate, is indicated. Similar results were obtained using MCF-7 cells.

**Fig. 2.** In vitro autoradiography. $^{125}$I-labeled VIP binding to sections from lobular breast carcinoma 0403–89 (A and B), lobular breast carcinoma 0724–89 (C and D), and ductal breast carcinoma 0307–89 (E and F) biopsy specimens was determined as a function of no additions (A, C, and E) and 1 μM VIP addition (B, D, and F). The difference between the two represents specific binding.
increase in c-myc mRNA caused by 10 nM VIP. Also, VIPhyb antagonized the increase in c-fos mRNA caused by VIP. In addition to c-fos and mRNA, VIP stimulated c-jun mRNA (data not shown).

**Proliferation.** The effects of VIPhyb on breast cancer growth were investigated in vitro and in vivo. Using a colony-counting assay in vitro, VIPhyb significantly reduced proliferation in vitro using a 1 but not 0.1 μM dose (Table 2). As a control, VIPhyb had little effect on the uptake of amino acids into the cell, suggesting that it is not cytotoxic. Palpable tumors formed 1 week after injection of MDA-MB-231 cells into nude mice. Nude mice were then given daily s.c. injections of PBS, and Fig. 6 shows that control tumors grew rapidly, reaching a volume of 3993 mm³ after 2.5 weeks. Nude mice given s.c. injections of 0.1 μg of VIPhyb daily grew rapidly, similar to the control. Nude mice given injections of 1 μg/day VIPhyb showed significant inhibition of tumor growth by 60%. Ten μg/day VIPhyb significantly slowed MDA-MB-231 tumor proliferation as well as MCF-7 tumor growth (data not shown). Therefore, VIPhyb slows the growth of estrogen-dependent and -independent breast cancer tumors.

**DISCUSSION**

Functional VIP receptors are present on breast cancer cells. The receptors bind VIP and PACAP with high affinity and a synthetic VIP receptor antagonist, VIPhyb, with moderate affinity. High-affinity 125I-labeled VIP-binding sites were detected on five of five breast cancer cell lines examined. Similarly, high-affinity VIP-binding sites were detected on 17 of 17 breast cancer biopsy specimens (28, 29). Because some breast cancer cells such as T-47D (30) have a high density of VIP-binding sites (293 fmol/mg protein), it may be possible to image breast cancer tumors using VIP ligands of high specific activity. 125I-labeled VIP was used to image colon carcinomas (18). Previously, 111In-labeled ocreotide, a somatostatin analogue, was used to image breast cancer tumors (28). Unfortunately, only approximately 50% of the breast cancer tumors bound ocreotide with high affinity, whereas 100% of the breast biopsies bind VIP with high affinity (29). Other growth factor receptors associated with breast cancer include epidermal growth factor, c-erbB-2, and insulin-like growth factor I (31–34).

Using a full-length cDNA probe for the VIP receptor, Northern blot autoradiograms yielded 5.5-, 2.4-, and 1.3-kb bands on all breast cancer cell lines examined.3 Previously, a major 5.5-kb band was detected in rat lung, liver, and colon but 5.5-, 2.4-, and 1.3-kb bands were detected in the rat brain (3). The 5.5-kb band may represent the VIP receptor, whereas the 2.4- and/or 1.3-kb band may represent subtypes of the VIP receptor mRNA. The order of peptide potency to inhibit specific 125I-labeled VIP binding to MDA-MB-231 cells was VIP = PACAP-27 > helodermin > PHI > VIPhyb. Previously using T-47D cells, the IC50 values for VIP, helodermin, and human GRF were 1, 40, and 100 nM, respectively (30). It remains to be determined whether breast cancer cells have VIP2 and PACAP type I receptors in addition to VIP1 receptors.

VIP stimulated breast cancer second messenger production. VIP (10 nM) increased cysotolic cAMP 10-fold in cell line MDA-MB-231 in a dose-dependent manner. Similarly, 0.7 nM VIP half maxmally elevated cAMP in T-47D cells (30). The increase in cAMP in MDA-MB-231 cells caused by VIP was reversed by VIPhyb, whereas...
VIPhyb had no effect on basal cAMP. These data suggest that VIPhyb is a breast cancer VIP receptor antagonist. VIP transiently increased nuclear oncogene expression. Ten nm VIP and structurally related peptides increase c-fos mRNA 5-fold after 1 h. In contrast, PMA, which strongly stimulates protein kinase C, increased c-fos mRNA 31-fold. These data suggest that the breast cancer c-fos gene may have PMA- and cAMP-responsive elements in the 5' upstream regulatory region. The increase in c-fos, c-jun, or c-myc mRNA caused by VIP was reversed by VIPhyb. Because c-fos and c-jun were increased by VIP, heterodimers may form activating AP-1 sites. VIP may alter gene transcription, especially of breast cancer growth factor genes.

VIPhyb inhibited breast cancer growth in vitro and in vivo. VIPhyb reduced basal MDA-MB-231 growth and appeared to be cytostatic but not cytotoxic. MDA-MB-231 tumors, which are estrogen independent (35, 36), developed in nude mice and VIPhyb (1 µg/day) significantly slowed tumor growth. VIPhyb did not appear to be toxic, since only the tumor but not body weights were significantly reduced in VIPhyb-treated animals. In some of the nude mice with MCF-7 tumors, which are estrogen dependent, VIPhyb (10 µg) caused regression of tumors. The regression was not due to apoptosis because the tumors from nude mice treated with VIPhyb did not have oligonucleosome induction. It is possible that VIPhyb may impair angiogenesis or interfere with the action of estrogen in the MCF-7 tumors. Similarly, tamoxifen treatment of nude mice with MCF-7 tumors caused tumor regression. Tamoxifen can have side effects in breast cancer patients, however, including induction of secondary tumors (37).

VIPhyb may inhibit breast cancer growth because VIP acts as an autocrine growth factor. Preliminary data indicate that breast cancer cells make and secrete immunoreactive VIP. Furthermore, breast cancer cells have VIP mRNA. Therefore, VIPhyb may antagonize the action of endogenous breast cancer VIP-like peptides. The development of more potent VIP receptor antagonists will permit a potential antitumor strategy using selective VIP antagonists to reduce the proliferation of breast cancer cells.

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REFERENCES

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