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GRANT NUMBER: DAMD17-94-J-4288

TITLE: Vasopressin Gene-Related Products in the Management of Breast Cancer

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REPORT DATE: October 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1997	Annual (1 Oct '96 - 30 Sep'97)

4. TITLE AND SUBTITLE
Vasopressin Gene-Related Products in the Management of Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4288

6. AUTHOR(S)
William G. North, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Dartmouth College
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200* The goal of this project is to improve the detection and treatment of breast cancer by characterizing vasopressin gene expression by this disease and determining the nature and role of products generated through this expression. We have now demonstrated that the vasopressin gene is expressed by seemingly all carcinoma in situ, and this new information added to an absence of vasopressin gene-related products from fibrocystic disease should provide us with a new screening test for distinguishing carcinoma in situ from atypical intraductal hyperplasia. Our recent studies on cell trafficking in breast cancer of vasopressin gene-products have shown that almost all protein processing is outside of conventional secretory vesicles, and components of glycopeptide-related cell surface antiger (GRSA) comprise both the 20 KDa and 40 KDa vasopressin-related proteins we have been characterizing. Therefore both of these proteins are potential targets for immunotherapy. Partial structures for these proteins have been determined, and Abs against tumor-specific structures are now being generated. Additional evidence has been gathered supporting the mitogenic actions of vasopressin on breast cancer, and a structure obtained by us for the complete open reading frame of a new putative hVACM vasopressin receptor expressed by breast cancer cells. Breast cancer expression of all other vasopressin receptor subtypes has been confirmed, and we have commenced DNA sequencing of these structures. Planned studies are to complete the characterization of GRSA and vasopressin receptor subtypes of breast cancer, and ascertain the effectiveness of our Abs to target breast cancer cells in vivo.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, vasopressin gene-related products, receptors, growth	34
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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(5) Introduction

The overall objective of this project is to improve the detection and treatment of breast cancer by evaluating vasopressin gene-related products as tumor marker substances in hyperplastic breast disease, by characterizi the nature and regulation of the vasopressin gene and its products in breast cancer, and by determining the potential usefulness of vasopressin gene-related products on tumor membranes as targets for immunotherap seeks to test the hypothesis that all breast tumors produce vasopressin as an autocrine growth factor, in situ, that this property can be effectively utilized not only to elucidate the pathobiology of this cancer, but also to identify precancerous tissue and develop more successful treatments.

In hypothalamic neurons, vasopressin gene expression leads to the formation of a 750 bp mRNA and the subsequent generation of a 20 KDa precursor that undergoes intragranular enzymatic processing to form vasopressin (VP), vasopressin-associated human neurophysin (VP-HNP), and vasopressin-associated glycopeptide (VAG). All three of these products are released into the circulation by exocytosis. **None** of the products become components of the plasma membrane of neurons.

We have shown that the **vasopressin gene** of chromosome 20 appears to be **expressed by all breast tumors, but not by normal breast tissue** (*North et al., 1995). This indicates that in the mammary gl the expression of the vasopressin gene is a feature unique to tumor cells, a feature common to all hyperplas tissues, or a feature shared only by tumor cells and their progenitors. The first and third of these possibilitie raised the potential use of this expression as a marker of carcinogenesis, and/or forecaster of imminent disea We therefore conducted a survey of the incidence of vasopressin gene expression in fibrocystic disease, and work has been accepted for publication in Endocrine Pathology. No evidence for gene expression could be f for all cases of fibrocystic disease examined, including atypical intraductal hyperplasia. In our study, three individuals with benign breast disease went on to develop breast cancer. Taken together, these findings indic vasopressin gene expression is not a marker of cellular proliferation in the breast, nor a marker of cancer progenitor cells in benign breast disease (*Fay et al., 1997). This leads us to the conclusion that vasopressin gene expression in the breast is likely to be solely associated with the process of carcinogenesis. Therefore, i would seem **the vasopressin gene is an oncogenic marker of breast cancer. We have recentl confirmed this through studying vasopressin gene expression in cases of carcinoma in si** (see Body of this report).

Expression of the vasopressin gene in breast cancer leads to the formation of unique gene related products, some of which become associated with the plasma membrane of tumor c Because these membrane-associated products react with antibodies raised against human vasopressin-associa glycopeptide (VAG), we have referred to them as **GRSA** (Glycopeptide Related cell Surface Antigen). Bec they are located at the cell membrane of the tumor cells, we have demonstrated they can be targeted, in *vitro* with antibodies to VAG. This raises the possibility they can be utilized for targeting tumors in patients throu immunotherapy. We have excellent indirect evidence that strengthens this possibility. Breast cancer uniquel shares the feature of membrane expression of vasopressin gene-related products with small-cell carcinoma o lung (SCCL) , and we have shown we can successfully target these products in SCCL patients using radioiodinated and Indium-labeled antibodies (*North et. al, 1989, *North and Yu, 1993).

What is the nature of GRSA? The VP mRNA and protein products that arise in breast cancer through expression of the vasopressin gene appear to be both structurally normal and abnormal (see Body of this rep We had anticipated this possibility because we (and others) have earlier shown that abnormal and normal for co-exist in SCCL (*North et. al, 1983; Rosenbaum et. al, 1990; *North and Yu, 1993). There appear to be t VPmRNAs in both breast cancer and SCCL. One of these is sequentially almost identical to that in human hypothalamic neurons, while the other is extended by 600 base pairs at the 5' end of the reading frame. The VPmRNAs of both types of tumors give rise to proteins of 40 KDa and 20 KDa as prominent forms, althou the proteins of breast cancer appear to show some structural differences to those of SCCL (*North et al., 19 The 20 KDa form of SCCL is almost identical to the provasopressin of hypothalamic neurons. Both 40 KDa 20 KDa proteins of SCCL become incorporated into the cell membrane as cell-surface antigens. Studies to fu characterize the two VPmRNAs of breast cancer are still in being performed. **We have recently shown t**

both 40 KDa and 20 KDa proteins of this tumor type represent GRSA at tumor cell surfac (see Body of this report).

In normal hypothalamic neurons, 20 KDa provasopressin is processed by proteolysis that is thought to invol least four enzymes. That such proteolysis also occurs in breast cancer is evidenced by our preliminary findin that most patients with breast cancer have inappropriately high plasma levels of vasopressin, and elevated le of VAG (**unpublished data**). Breast cancer can therefore be classified as neuroendocrine in nature. Becau this, we performed studies that demonstrated the presence of the key processing enzymes, carboxypeptidase and prohormone convertases PC2 or PC1/3, and PAM, in the two breast cancer cell lines MCF7 and ZR-75

Why is vasopressin produced by breast cancer? One answer to this question is that vasopressin ser as an autocrine growth factor for these tumors. Vasopressin is already known to act as a growth factor/grow modulating agent in SCCL lines where it promotes calcium mobilization and clonal growth (Hong and Mooc 1991; Sethi and Rozengurt, 1991, Cassoni et al., 1994,1996,1997). Over the last two years we reported tha vasopressin can promote calcium mobilization in two breast cancer cell lines, ZR-75-1 and T47D, and can dramatically influence the cytoskeleton of ZR-75-1. These finding are supported by previous studies on a dimethylbenzathrene-induced rat mammary tumor (Monaco et al., 1978; Monaco et al., 1980; Guilon et al., 1986; Kirk et al., 1986; Woods and Monaco, 1988), human MCF7 breast cancer cells (Taylor et al., 1990), on another breast cancer cell line (Bunn et al., 1992). Choi et al. (1994) were also able to show that vasopre promotes growth of mammary tumors in transgenic mice. These actions of vasopressin have prompted us to investigate the nature of vasopressin receptors on breast cancer cells. Four vasopressin receptors have been indentified in other cells and have been cloned (Birnbaumer et al., 1992; Hirasawa et al., 1994; Sugimoto et 1994; Thibonnier et al., 1994; Burnatowska-Hledin et al., 1995, *Fay et al., 1994,1996; *North et al., 1997a,1997b). These are known as vasopressin V_{1a}, V_{1b}, and V_2, receptors plus vasopressin-activated calcium-mobilizing receptor (VACM1). Although an investigation of vasopressin receptors and the growth promotional activities of vasopressin may seem to fall outside of intentions enunciated in the original propos we believe they nevertheless address the body of the hypothesis advanced in the proposal and fall within the goals of Technical objectives 2 and 3. It is believed that such an investigation could not only explain the seemingly universal expression of the vasopressin gene in breast tumors, but also lead to an additional numb effective therapies.

(6) Body

Technical Objective 1: Vasopressin gene-expression in breast hyperplasia as a predictor cancer (Task 1 in Statement of Work).Breast Cancer/Carcinoma in situ/hyperplasia.

This objective has been satisfied. We now report on our discovery that the vasopressin gene is expressed by carcinoma in situ examined and the implications of this finding. **These most recent findings have not been published.** Also, for the sake of clarity, we include below a summary of earlier reported findings. O findings taken together show that **vasopressin gene expression is a marker of oncogenic transformation in breast tissues.**

Breast Cancer: We performed immunohistochemistry on 19 breast cancers representing a variety of tumo subtypes using antibodies directed against different moieties of the vasopressin precursor structure as indicat Figure 1, below. These comprised rabbit polyclonal antibodies that recognize arginine vasopressin (anti-VP tripeptide bridge region of the precursor (anti-ProVP), and the carboxyl region of vasopressin-associated hu glycopeptide (anti-VAG); and mouse monoclonal antibodies that recognize an amino terminal portion of vasopressin-associated human neurophysin (anti-VP-HNP). Western Blot analysis was performed on protei extracts from an additional 12 breast tumors.

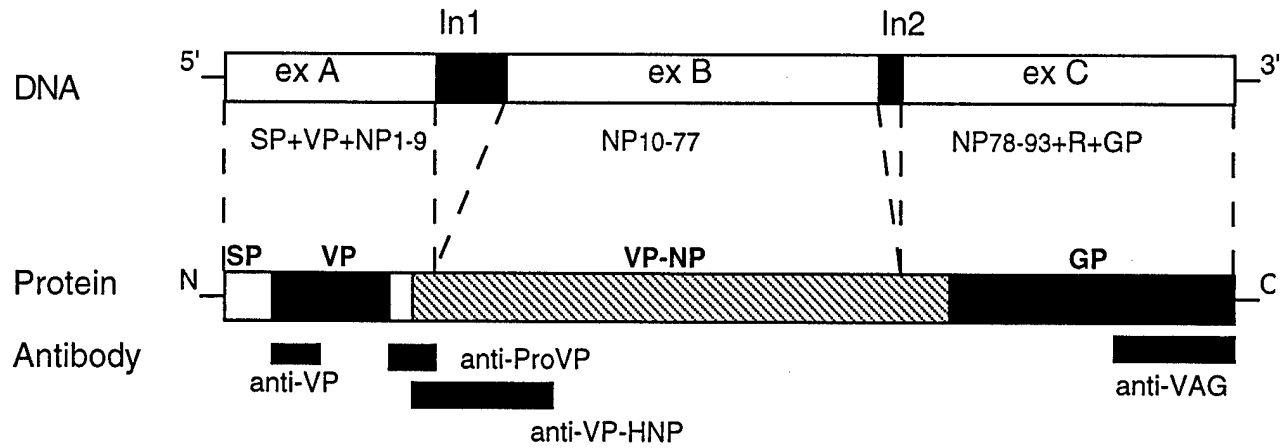


Figure 1. Illustration depicting the structures of the vasopressin gene and protein precursor. Regions of the precursor are blocked out against which Abs, used in immunohistochemistry of breast cancer, are directed.

As shown in Table 1, while VP-related proteins were not detected in normal breast tissues, immunohistochemistry revealed the presence of VP and VAG in all neoplastic cells of all tumor tissues exam ProVP was evident in 11 of 14 tumors while VP-HNP was evident in only one of 19 tumors examined.

Table 1. Presence of vasopressin gene related products in human breast cancer

cancer subtype	VP gene related antigens*			
	VP	ProVP	VP-HNP	VAG
Infiltrating ductal	na	na	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	+	+	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	-	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	na	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	+	-	+
Infiltrating ductal	+	-	-	+
Infiltrating ductal	+	-	-	+
Colloid	+	+	-	+
Colloid	+	na	-	+
Colloid	+	+	-	+
Colloid	+	+	-	+
Infiltrating tubular	+	na	-	+
Infiltrating tubular	+	+	-	+
Infiltrating lobular	+	na	-	+
Total positive	18/18	11/14	1/19	19/19

*Positive (+) or negative (-) immunoreactivity using antibody preparations and the ABC procedure.
na = not attempted.

However, Western blot analysis for all 12 fresh-frozen tumor samples showed the presence of two proteins 42 KDa and 20 KDa, that were both immunoreactive with, not only antibodies against VP and VAG, but als those against VP-HNP (anti-ProVP were not used). The vasopressin precursor of hypothalamic tissues is 2(

KDa in size. These findings provided evidence that the vasopressin gene is expressed as a selective feature o breast cancers. This expression apparently gives rise to an abnormally large vasopressin-related protein, and protein of normal size with possible modifications in the neurophysin region making it less immunoreactive anti-VP-HNP. Both proteins represent potential markers for tumor detection and potential targets for immunotherapy.

Fibrocystic Disease: In order to examine if vasopressin gene expression was a possible predictor of dise we performed a survey of the incidence of vasopressin gene expression in fibrocystic disease, and this work now been accepted, pending revision, for publication in Endocrine Pathology. In this study, we used immunohistochemistry and antibodies against vasopressin (anti-VP) and vasopressin-associated glycopeptid (anti-VAG) to examine formalin-fixed biopsy specimens taken from 17 patients, with various forms of beni breast disease, who were seen at Dartmouth Hitchcock Medical Center between 1975 and 1984. These specimens were selected without any knowledge of follow-up, and included 4 cases of atypical ductal hyperplasia, 6 cases of fibrocystic disease with intraductal hyperplasia, 2 cases of fibrocystic disease with papilloma, 1 case of fibrocystic disease with bilateral mammary hyperplasia, and 4 cases of typical fibrocysti disease. Diagnosis from pathology reports was confirmed by examining hematoxylin- and eosin-stained sec The results of these studies are illustrated in Table 2, and demonstrate that in all cases of benign breast disea examined there was negative staining for both vasopressin and vasopressin-associated glycopeptide. They indicate that the vasopressin gene is <u>not</u> expressed in benign breast disease, and this is in dramatic contrast t what was found for human breast carcinoma using these same antibodies (Table 1). At the completion of the study, it was discovered that three of the individuals with benign breast disease went on to develop breast carcinoma. Although preliminary, these data taken together indicate that (i) expression of vasopressin gene related products is not a marker of cellular proliferation in the breast, (ii) expression of vasopressin gene-rela products is associated with the process of carcinogenesis, and (iii) expression of vasopressin gene-related products is not a marker of precancerous cells in benign breast disease.

Table 2. Absence of vasopressin gene-related products from benign breast fibrocystic conditions

Subtype	VP gene-related antigens*	
	VP	VAG
Fibrocystic Disease	-	-
Fibrocystic Disease	-	-
Fibrocystic Disease	-	-
Fibrocystic Disease	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Hyperplasia	-	-
Atypical Intraductal Hyperplasia	-	-
Atypical Intraductal Hyperplasia	-	-
Atypical Intraductal Hyperplasia	-	-
Fibrocystic Disease with Intraductal Papilloma	-	-
Fibrocystic Disease with Intraductal Papilloma	-	-
Fibrocystic Disease with Bilateral Mammary Hyperplasia	-	-
Total Positive	0/16	0/16

*Positive (+) or negative (-) immunoreactivity using ABC immunohistochemistry

Carcinoma in situ: We have now used immunohistochemistry with anti-VAG antibodies to examine vasopressin gene expression in pre-invasive carcinoma. Blocked out biopsy samples of twelve cases of carcinoma in situ, six of which have been clearly identified as being of the **comedo** variety with abnormal c and extensive necrotic areas, were investigated. All twelve cases (Table 3) showed positive staining with ant VAG demonstrating for this small sampling that vasopressin gene expression is commonly associated with breast carcinoma in situ (Figure 2). Of the DCIS samples, the comedo variety gave the most intense staining

Table 3. Presence of vasopressin gene-related products in carcinoma in situ

Subtype	VP gene-related antigen* VAG
Carcinoma in situ, non-comedo	+
"	+
"	+
"	+
"	+
"	+
Carcinoma in situ, comedo	+
"	+
"	+
"	+
"	+
"	+
Total Positive	12/12

*Positive (+) or negative (-) immunoreactivity using ABC immunohistochemistry

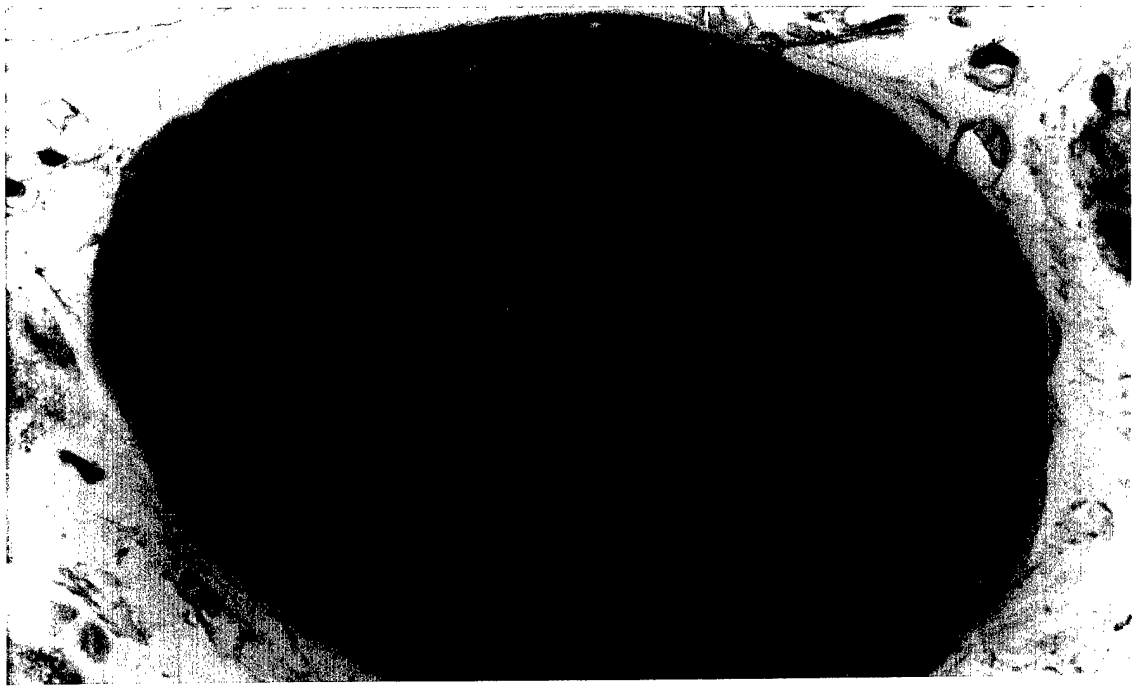
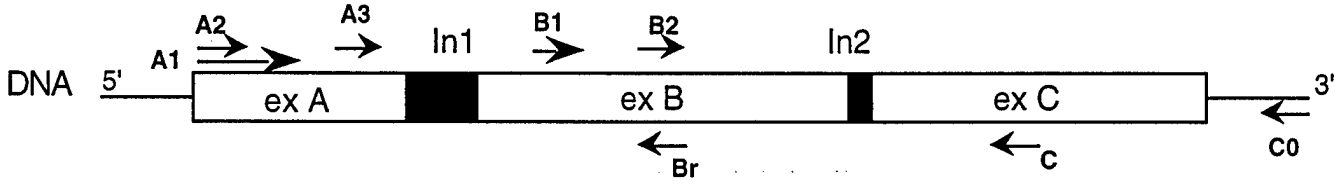


Figure 2. Carcinoma in situ stained using the ABC immunohistochemical method with Abs against VAG

The above results indicate that ABC immunohistochemistry with our antibodies to VAG can clearly distinguish atypical ductal hyperplasia from carcinoma in situ, a distinction currently difficult to make using other available methods. This distinction is important because a diagnosis of atypical hyperplasia has no follow-up, while carcinoma in situ is generally followed-up with ablative surgery. We are therefore intending to further test this finding by enbarking on a screening study that will compare evaluation by histochemical analysis alone with an evaluation that uses both histochemistry and VAG immunohistochemistry.

Technical Objective 2: Characterization of vasopressin gene expression by breast cancer cells (Tasks 2 and 3 in Statement of Work).

The data discussed in this section are largely unpublished.



Structure of Human vasopressin gene and locations of some designed PCR primers

Figure 3

We have established for breast cancer cells that there is abnormal, in addition to normal, production of vasopressin. Abnormal protein forms constituting GRSA might to be generated from one normal and one abnormal gene. RT-PCR, cloning, and sequencing studies on messages from the vasopressin gene of MCF7, T47D, and ZR-75-1 cells have now shown that there appear to be at least two VPmRNAs expressed in breast cancer, one from a 'normal' gene and the product of normal splicing, the second either from a 'normal' gene and the product of alternate splicing or from an abnormal gene having insertions in exon A . The ten primers used in studies conducted this and last year are illustrated in the figure above and described in the following table :.

Table 4. Forward and reverse primers designed for RT-PCR amplification of human vasopressin gene fragments from human breast cancer cells

Forward primer	Length	Nucleotides	Exon	Sequence
A1	21	269-289	1	5'-cttctcctccgcgtgctactt-3'
A2	18	269-286	1	5'-cttctcctccgcgtgcta-3'
A3	21	321-341	1	5'-atgtccgacctggagctgaga-3'
IN	21	1504-1524	intron 1	5'-gtcatccaagaaaccaaggtg-3'
B1	25	1751-1775	2	5'-tgcttcgggcccagcatctgctgcg-3'
B2	22	1830-1851	2	5'-tgccaggaggagaactacctgc-3'
Reverse primer				
INR	20	1517-1536	intron 1	5'-agatctgctcggcaccttgg-3'
Br	22	1830-1851	2	5'-gcaggtagttctcctcctggga-3'
C	22	2152-2173	3	5'-agcaacgccacgcagctggacg-3'
C0	25	2231-2255	3	5'-taggcgtcgggctgggcgggctcga-3'

Normal-sized VPmRNA fragments of 313 bp using **A3C** were obtained from three cell lines. These have been partially sequenced and shown to have a sequence very similar to the VPmRNA found in hypothalamic neurons. We also isolated, and successfully reamplified (but have not yet sequenced) an RT-PCR product(s), from all three cell lines using the specific primers **A1** and **C**, that is 600 bases larger than that predicted from the structure of VPmRNA. Such a structure could represent a VPmRNA that have retained a 600 base portion of intron 1 through alternative splicing (the entire intron 1 segment contains 1373 bases). If the 5' sequence of the product confirms it translates a protein with the N-terminus of provasopressin, it will offer one explanation for the 40,000 dalton species of breast cancer because an extra 600 bases represents an additional 200 amino acid residues. Adding 200 amino acid residues to the 20,000 dalton provasopressin would give a protein of 40,000 daltons. Since antibodies recognize the exon B (at least in Western analyses) and exon C regions of the protein (North et al:, 1995) the intronic insertion would not apparently cause a reading frame shift. The structure of the enlarged form will now be checked through reamplification using both **A2** and **C** primers, and **A3** and **C** primers. If the additional 600 bases in **A1C** are from intron 1, we expect in all cases, reamplified products that are approximately 600 bases larger than predicted from normal VPmRNA. However, if products of normal size are produced this will suggest the enlarged form represents an abnormal vasopressin gene having a 600 base insertion in the exon A region. This insertion would be between bases corresponding to the vasopressin and neurophysin structures. Structures **A2** and **A3** are only separated by 35 bases in normal VPmRNA. While a definitive answer regarding the enlarged form will be best provided through cloning and DNA sequencing, the planned exercise will enable us to eliminate the possibility of alternative co-existing forms. Use of primers **B1, B2, C** and **Co** will likewise enable us to discover if forms extended in the exon B and/or exon C region exist is breast cancer cells (as found by us in SCCL), while use of the forward **IN** and reverse **INR** primers will allow us, when used with **B** and **A** primers, to obtain shortened RT-PCR products for sequencing if regions of intron 1 are indeed included in the abnormal VPmRNA structure. All of these primers have already be used somewhat successfully by us in sequencing VPmRNA forms from SCCL (unpublished). However, two abnormal VPmRNA structures found by us for SCCL have recently been entered into the GENEbank with accession numbers

Despite the exciting prospect that sequences could soon be available for the VPmRNA form(s) that give rise to 40 KDa and 20 KDa GRSA of breast cancer, we have now initiated efforts to perform Edman sequencing on purified samples of these proteins. We have decided to concentrate our studies on protein obtained from the cell line ZR-75-1 and will use both cultured cells and tumor xenografts in nude mice as the protein sources. Purification will employ pH-salt separations, molecular sieve chromatography, and affinity chromatography on columns of Antivasopressin-Sepharose. Our antivasopressin monoclonal antibody, DEN1, has already been used to generate the affinity resin. Protein mixes from affinity chromatography will be S-alkylated and then separated. We intend separating the 20 KDa and 40 KDa protein forms using SDS-PAGE, and either eluting the them directly into dialysis sacks or performing Western transfer to PVDF, and performing solid-phase sequencing.

PCR studies on DNA preparations from breast cancer cell lines have also been conducted using a mixture of specific primers for the vasopressin gene and oxytocin genes. This is because a published study (Morris et al., 1995) has indicated that some hypothalamic neurons in rats can express protein products that are a composite of provasopressin and pro-oxytocin through a cross-over between the vasopressin and oxytocin genes on chromosome 20. We have now established that there is no evident cross-over between the vasopressin and oxytocin genes in breast cancer.

Studies have now been performed that examined sub-cellular trafficking in ZR-75-1 breast cancer cells (unpublished data).

Sucrose-gradient sub-fractionation of these cells (10^8 cells/batch) was carried out and an evaluation conducted by Western analysis and by RIA (VP, VP-HNP, VAG). This evaluation revealed that approximat 80% of **both** the 20 KDa and 40 KDa proteins are located in the plasma membrane. Of the remaining 20%, most (90%) is found outside secretory granules, and approximately 10% is within these granules. The procedures employed were found by us to preserve granules from hypothalamic neurons with >90% of

vasopressin gene-related products located in the granular fraction. Hence, either the granules of breast cance: more susceptible to rupture, or only a small percentage (< 2%) of translated protein is potentially processed t active hormone within these granules and then secreted. This implies that packaging is limited and most prot in breast cancer cells is destined for agranular targeting to the plasma membrane. Both 20 KDa and 40 KDa proteins were found in the granular fraction of cells. This indicates that the 40 KDa product shows a capacit similar to the 20 KDa product to be packaged in the Golgi apparatus. This study indicates that the limited processing of 20 KDa and 40 KDa vasopressin gene-related proteins in breast cancer is largely due to limitec packaging of translated material, rather than to an absence of processing enzymes. An almost identical trafficking pattern was found for SCCL cells in culture and reported on last year.

The breast cancer cell lines MCF7 and ZR-75-1 were examined for the expression of mRNAs for the processing enzymes carboxypeptidase E (CPE), and prohormone convertases PC2 and PC1 (or PC3) using RT-PCR, cloning, and sequencing. The primer pairs used in these studies are depicted in Table 5 below.

Table 2: Primers designed for amplification cDNA fragments of prohormone convertases(PC) and carboxypeptidase E(CPE) from breast cancer cells

Subject	Forward primer	Length	Position	Reverse primer	Length	Position
PC1/PC3	5'tacttgcaagataccaggatg3'	21	540-600	5'gatggagatggtgtagatgct3'	21	1162-1182
PC2	5'gatcctctttttacaaagcagtgg3'	24	454-477	5'ggtgagcacagtcagatgctgcat3'	24	1312-1335
CPE	5'atgggaatgaggctgttgggac3'	21	631-651	5'catggagattggcagaaagca3'	21	1015-1035

RT-PCR studies on CPE provided amplified products of the size predicted from previously published studies on anterior pituitary cells using polyA$^+$RNA from both cell lines. These products were reamplified, cloned and sequenced to provide structures identical to those published for this enzyme. In RT-PCR studies on PC2, we have so far only been able to amplify a product using polyA$^+$RNA from MCF7. This cDNA fragment was shown by us to have the normal base sequence of the enzyme. We subsequently investigated if mRNA for PC1/3 was expressed in MCF7 and ZR-75-1. However, RT-PCR failed to show that this mRNA was expressed in either cell line. **The expression of these enzymes by breast cancer can now be confirmed using available antibodies against PC1 and PC2. The presence of PAM enzyme has recently been demonstrated with anti-PAMs. These antibodies were provided to us through the generosity of Drs. Betty Eiper and Richard Mains of Johns Hopkins.** Our results therefore show that at least **three** of the enzymes necessary for processing provasopressin to active hormone, neurophysin, and glycopeptide, are present in some breast cancer cell lines. We still intend following up these studies on the processing enzymes by performing Western analysis for CPE and additionally determining if substrate-converting enzymatic activities are present in protein extracts from breast cancer cells and tumors.

Technical Objectives 3: Identification of factors regulating the production of GRSA by br cancer; and
4: Determination of the binding properties for antibodies of GRSA and other vasopressin gene-products at tumor cell surfaces (Tasks 4 and 5 of Statement of Work).

We have still not yet commenced studies designed to satisfy these technical objectives, and expect they will largely occupy our efforts during the last twelve months of this award (Year 4). We have already performed studies on the regulation of vasopressin gene-expression in SCCL as part of another ongoing project, so all the methods are at hand to enable us to proceed without pause. We are currently generating xenografts of the breast cancer cell line ZR-75-1 in nude mice in an effort to generate GRSA proteins for sequencing (see abo and this is expected to serve as a springboard for examining binding of VAG antibodies to breast tumors in

vivo. Determination of the protein sequences of GRSA proteins is also expected to lead to production of spe antibodies against unique sequences in these proteins for targeting. **We are currently producing antibodies to one such unique structure.**

Cloning of a novel calcium-mobilizing receptor from cancer cells (NCI H146 SCCL cells MCF7 breast cancer cells)

Last year we described our ability to demonstrate, for breast cancer cells, the expression of mRNA for the n vasopressin receptor, called VACM, using RT-PCR and primers designed from the structure of the rabbit fo of this protein. Since that time the structure of a human clone of VACM from placenta was published by a British research team (Byrd et al., 1997, Stankovic et al., 1997). In order to study the role of this putative receptor in breast cancer, we have now generated a cDNA clone from human cancer cells. Initial efforts wer focused on the small-cell carcinoma cell line NCI-H146, because we had obtained signal transduction data th presence of functional VACM protein in these cells. However, we have now obtained 5' and 3' RACE prod covering the entire open reading frame of mRNA for the protein from MCF7 breast cancer cells. The primer employed in RACE are given in Table 6, below:

TABLE 6: Primers used for 5' and 3' RACE of VACM from MCF7 breast cancer cells

3'-PCR RACE primers	
1432(forward)	**5' gaa-tgg-cta-aga-gaa-gtt-ggt-atg 3'**
138 (reverse)	**5' ttg-ttt-ttg-taa-ggt-aag-gca-gag 3'**
5'-PCR RACE primers	
5' ATG (forward)	**5' tcc-aag-tta-aag-aac-atg-gcg 3'**
2082 (reverse)	**5' tct-tct-ctc-atc-ctt-tct-gta-gtg 3'**

The isolated VACM clone for NCI-H146 contains an open reading frame of 2,343 nucleotides and encodes protein of a predicted size of 781 amino acids. Analysis programs failed to identify hydrophobicity regions c sufficient confluence to classify them as transmembrane regions. The following motifs were identified to be present in the protein structure: two protein kinase A phosphorylation domains (Thr 427 and Ser 731); 15 protein kinase C phosphorylation domains; a Tyrosine phosphorylation domain (Tyr 207); two myristoylatic sites between residues 180 and 185, and 664 and 669; and three glycosylation sites at Asn 145, Asn 289, an Asn 566. Although **these findings are unpublished** we are currently in the process of preparing a manuscript we intend submitting by the end of November, 1997. **Our cDNA sequence for what we ar now referring to as HVACM from human cancer cells has recently been submitted to the GENEbank by us and has been assigned the accession number AF017061.** A complete copy o the GENEbank submission is included in the appendix of this progress report. The availability of cloned HVACM should now allow us to examine in detail the expression of this putative vasopressin receptor, and determine its role in the vasopressin-induced mitogenesis of breast cancer. We are currently having antibodie HVACM generated, and these will be used to examine the incidence and distribution of the protein in breast cancer from our archival library.

Vasopressin-induced phosphorylation (activation) of mitogen-activated protein kinase (MAPK).

We earlier reported that vasopressin can activate MAPK in breast cancer cells, and we have recently tried to provide quantitative data on MCF7 breast cancer cells using a fluorescence Western Blot procedure from EC with a Molecular Dynamics Fluorimager. Two antibodies preparations employed recognize dually phosphorylated MAPK p42/p44 (activated MAPK), and MAPK regardless of phosphorylation status. Treatments with vasopressin and a vasopressin V1 antagonist for 5 and 15 minutes were compared with con using Imagequant software. Data obtained support an increase in MAPK activation at 5 minutes, but not at 1

minutes, and this increase could only be demonstrated for the p44 MAPK isoform. **These data have not been published.**

Expression of vasopressin V1 and V2 receptor subtypes and oxytocin receptors by breast cancer cells.

We earlier reported on the presence of vasopressin V_{1a}, V_{1b}, and V_2 receptors, and oxytocin receptors in breast cancer cell lines BT 549, MCF7, MDA, MB-231, T47D, and ZR-75 using specific primers and RT-PCR. We are currently engaged in obtaining sequences for these receptors for the cell lines MCF7 and T47D. We recently obtained complete sequence information on the open reading frames of all of these receptors produced by small-cell carcinoma of the lung. **These structures have been submitted to the Genebank with accession numbers AF030625, AF030512, AF030626, and AF032388.**

(7) Conclusions

The studies conducted over this year of the granting period have further confirmed our original hypothesis that all breast cancers produce vasopressin as an autocrine growth factor, and that this property c: be utilized to develop more successful treatments. Expression of the vasopressin gene seems to be associatec with all oncogenic transformaion of breast tissue as evidenced by the presence of vasopressin gene products all breast cancers examined, the absence of these products from all varieties of fibrocystic disease, and now demonstration that all carcinoma in situ examined expressed these same products. We believe this most recer finding can have short-term clinical application in providing earlier detection of breast cancer as an effective to distinguish atypical intraductal hyperplasia from carcinoma in situ. There is no other method available for making this distinction.

When vasopressin gene(s) are expressed by breast cancers, they give rise to normal and abnormal products. Studies conducted by us on trafficking of vasopressin gene-related products by breast cancer cells now revealed that about nine-tenths of the proteins become components of the plasma membrane able to pro targets for antibodies in patients. These proteins comprise both the 40 KDa and 20 KDa forms described in earlier reports. Antibodies against one of the recognized abnormal structures in these vasopressin gene-relate proteins are now being produced. Such new or already available antibodies should be potentially useful in la planned immunodiagnosis and immunotherapies.

Vasopressin seems to have a multifaceted role on the growth and physiology of breast cancer cells because we have demonstrated that all known forms of vasopressin receptor subtypes are expressed by these cells. The complete sequence of one putative receptor named hVACM has been determined by us and enterec the Genebank. Structures for vasopressin V_{1a}, V_{1b}, V_2, and abnormal V_2 are currently being determined. Pa sequence data suggest they will have the same sequences submitted to the Genebank by us for vasopressin receptor subtypes produced by small-cell cacinoma cells. We have further demonstrated that through one or more of these receptors, vasopressin is able to alter calcium homeostatasis and activated MAPK kinase in br cancer cells.

Studies anticipated over the next twelve months will include those relating to regulation of vasopress: gene expression and the binding of antibodies to cancer cells in vitro and in vivo, as designed to satisfy task: and 6 in the original statement of work. However, we also expected to complete our sequencing of the vasopressin receptor subtypes we have discovered to be present in breast cancer because knowlege of these structures can provide additional avenues for successful treatment of the diseass, These include inhibition of growth with antagonists, the delivery of toxin attached to specific agonists, or the direction of antibodies ag: abnormal receptor structures (e.g. of some tumor V_2 receptors).

8) References

Altschul, S.F., Warren, G., Miller, W., Myers, E.W., Lipman. D.J. J. Mol. Biol., **215:**410 (1990).

Birnbaumer, M., Seibold, A., Gilbert, S. Ishido, M., Barberis, C., Antaramian, A., Brabet, P., Rosenthal Nature, **357**:333 (1992).

Bunn, P.A., Dienhart, D.G., Chan, D., Tagawa, M., and Jewett, P. Monogr. Natl. Cancer Inst., **13:**145 (1992).

Bradford, M.M. Analytical Biochem., **72:**248 (1976).

Burnatowska-Hledin, M.A., Spielman, W.S., Smith, W.L., Shi, P., Meyer, J.M., Dewitt, D.L. Am. J. Physiol., **268**:1198 (1995).

Bussolati, G., Cassoni, P., Ghisolfi, G., Negro, F., and Sapino, A. Am. J. Pathol., **148**:1895 (1996).

Byrd, P.J., Stankovic, T., McConville, C.M., Smith, A.D., Cooper, P.R., and Taylor, A.M. Gemome Re 7:71-75 (1997).

Cassoni, P., Sapino, A., Negro, F., and Bussolati, G. Virchows Archiv., **425**:467 (1994).

Cassoni, P., Sapino, A., Papotti, M., Bussolati, G. Int. J. Cancer, **66**:817 (1996).

Cassoni, P., Sapino, A., Fortunati, N., Munaron, L., Chini, B., and Bussolati, G. Int. J. Cancer, 72:340- 1997.

Cheng, M., Watson, P.H., Paterson, J.A., Seidah, N., Cretien, M., and Shui, R.P. Int.J. Cancer, 966-97 (1997).

Choi, K., Carter, D.A., Biswas, S., Lightman, S.L., Ho, M., and Murphy, D. Cancer Res., **54**:6434 (19

Clark, E.A., Bruggge, J.S. Science, **268:**233 (1995).

Cobb, M.H. and Goldsmith, E.J. J. Biol. Chem., 270:14843-14846 (1995).

Colditz, G.A. Cancer Supplement, **71:**1480 (1993).

Dubois J.M., Rouzaire-Dubois, B. Prog. Biophys. Mol. Biol., **59**:1 (1993).

*Fay, M.J., Friedmann, A.S., Yu, X., North, W.G. Cancer Letters, **82:**167 (1994).

* Fay, M.J., Du, J., Yu, X., and North, W.G. Peptides 17:477-481 (1996)..

* Fay M.J., Mathew, R.S., Donnelly, E.M., Memoli, V.A., and North, W.G. Immunohistochemical evalu of vasopressin gene expression in benign breast disease: Preliminary Observations. Endocrine Pathology, Ir Press (1997).

*Friedmann, A.S., Malott, K.A., Memoli, V.A., Pai, S.I., Yu, X., and North, W.G., Br. J. Cancer, **69** 2 (1994).

*Gallagher, J.D., Fay, M.J., North, W.G., and McCann, F.V., Cell. Signal., **8** 279 (1996).

Gross, A.J., Steinberg, S.M., Reilly, J.G., Bliss, D.P., Brennan, J., Tramle, P., Simmons, A., Phelps, F Mulshine, J.L., Ihde, D.C., and Johnson, B.E. Cancer Res., **53:**67 (1993).

Grynkiewick, G. Poenie, M., and Tsien, R.Y. J. Biol. Chem. **260:**3440 (1995).

Guillon, G., Kirk, C.J., and Balestre, M. Biochem. J., **240:**189 (1986).

Gupta, A., Sasarula, S., Rao, P.V. J. Assoc. Physicians India, **34**:441 (1986).

Hirasawa, A., Shibata, K., Kotosai, K., Tsujimoto, G. Biochem. Biophys. Rs. Commun., **203**:72 (1994

Hong, M., Moody, T.W. Peptides, **12:**1315 (1991).

Howard, A.C., Laing, R.W., and Hussain, F.N. Eur. J. Cancer, **29A**:2339 (1993).

Ito, Y., Kobayashi, T., Kimura, T., Matsuura, N., Wakasugi, E., Takeda, T., Shimano, T., Kubota, Y., Nobunaga, T., Makino, Y., Azuma, C., Saji, F. Endocrinol., **137**:773 (1996).

Janmey, P.A. Annu. Rev. Physiol., **56:**169 (1994).

Kirk, C.J., Guillon, G., Balestre, M., and Jard, S. Biochem. J., **240**:197 (1986).

Lopez-Ilasaca M., Crespo, P., Guiseppe-Pellici, P., Silvio-Gutkind, J., and Wetzker, R. Science, 275:394- (1997).

Monaco, M.E., Lippman, M.E., Knazek, R., and Kidwell, W.R. Cancer Res. **38**:4101 (1978).

Monaco, M.E., Kidwell, W.R., and Lippman, M.E. Biochem J., **188**:437 (1980).

*North, W.G. J. Clin. Endocrinol. Metab. **73**:1316 (1991).

*North, W.G., and Yu, X.M. Peptides, **14:**303 (1993).

*North, W.G., Pai, S., Friedmann, A., Yu, X., Fay, M., and Memoli, V. Breast Cancer Res. Treatment **34**:229 (1995).

*North, W.G., Fay, M.J., Longo, K., and Du, J. Peptides, 18: 985-993 (1997).

*North, W.G., Fay, M.J., Longo, K., and Du, J. Expression of all vasopressin receptor subtype)s by smal tumors implies a multifaceted role for this peptide. Cancer Res., Submitted, Nov. (1997).

Owens, L.V., Xu, L., Craven, R.J., Dent, G.A., Weiner, T.M., Kornberg, L., Liu, E.T., and Cance, W.(Cancer Res., **55:**2752 (1995).

Rosenbaum, L.C., Neuwelt, E.A., Van Tol, H.H.M., Loh, Y.P., Verbalis, J.G., Helstrom, I., Helstrom, K.E., and Nilaver, G. Proc. Natl. Acad. Sci. USA, **87:**9928 (1990).

Sausville, E., Carney, D., and Battey, J. J. Biol. Chem., **260:**10236 (1985).

Sethi, T. and Rozengurt, E. Cancer Res., **51:**3621 (1991).

Simon, H., Gao, Y., Franki, N., Hays, R.M. Am. J. Physiol. **265:**c757 (1993).

Sinnett-Smith, J., Zachary, I., Valverde, and Rozengurt, E. J. Biol Chem., **268:**14261 (1993).

Sugimoto, T., Saito, M., Mochizuki, S., Watanabe, Y., Hashimoto, S., Kawashima, H. J. Biol. Chem., **269**:27088 (1994).

Taylor, H., Ang, V.T.Y., Jenkins, J.S., Silverlight, J.J., Coombes, R.C., and Laqmani, Y.A. Cancer Re **50**:7882 (1990)

Thibonnier, M., Auzan, C., Madhun, Z., Wilkins, P., Berti-Mattera, L., Clausers, E. J. Biol. Chem., **269**:3304 (1994).

Woods, D.J. and Monaco, M.E., Mol. Endocrinol., **2**:350 (1988).

Zachary, I., Sinnett-Smith, J., and Rozengurt, E. J. Biol. Chem., **266:**24126 (1991).

Zachary, I., Sinnett-Smith, J., and Rozengurt, E. J. Biol. Chem., **267:**19031 (1992).

Zachary, I., Sinnett-Smith, J., Turner, C., and Rozengurt, E. J. Biol. Chem., **268:**22060 (1993).

(9) APPENDICES

The following items are located in the Appendices:

1. **North, W.G.**, Fay, M.J., and Du, J. Vasopressin and breast cancer, gene expression and trafficking. Summer neuropeptide conference (Key West, FL), June 21-26, 1997 (abstract).

2. **North, W.G.** and Du, J. Production and processing of vasopressin gene-related proteins by neuroendocrine tumors. Proc. Soc. Neuro., 23:63.4A, 1997.

3. K.A. Longo, **W.G. North**, J. Du, and M.J. Fay. Evidence for the expression of a novel vasopressin-activated calcium mobilizing receptor (HVACM) in human breast cancer and lung cancer. 1997 World Congress of Neurohypophysial Hormones (Montreal, Canada) August 8 - 12, 1997.

4. **North, W.G.**, Fay, M.J., Longo, K., and Du, J. Vasopressin gene related products in the management of breast cancer. The Department of Defense Era of Hope Breast Cancer Research Program Meeting (Washington, D.C.) October 31 - November 4, 1997.

5. Fay, M., Du, J., Longo, K., and **North, W.** The role of vasopressin and oxytocin hormones in breast cancer. The Department of Defense Era of Hope Breast Cancer Research Program Meeting (Washington, D.C.) October 31 - November 4, 1997.

6. A copy of our genebank submission for homo sapiens vasopressin-activated calcium mobilizing putative receptor protein (VACM-1) mRNA and protein, genebank accession number AF017061.

7. A copy of our genebank submissions for small-cell tumor vasopressin receptor subtypes Via, Vib, V_2 normal, and V_2 abnormal. Accession numbers are AF030625, AF030512, AF030626, AF032388.

Vasopressin and Breast Cancer: Gene expression and Trafficking.

William G. North, Michael J. Fay, and Jinlin Du, Dartmouth Medical School, Lebanon, N.H. 03756 USA.

We earlier discovered that the vasopressin gene expression occurs in probably all breast cancers, that this expression apparently arises as part of the carcinogenesis process in the mammary gland, and that 40 KDa and 20 KDa vasopressin-related proteins are generated as components of the plasma membrane in breast tumor cells. We have named the membrane proteins GRSA (*g*lycopeptide-*r*elated cell *s*urface *a*ntigens)..We have now examined aspects of vasopressin gene expression and the processing of gene-associated products in MCF-7 and ZR-75, using RT-PCR, cloning, DNA sequencing, sucrose-gradient fractionation, Western analysis, and flow cytometry. Results obtained have led us to the following conclusions:

- GRSA surface markers originate through the expression of both normal and abnormal vasopressin genes. This is because RT-PCR products of normal and increased size, as well as with normal and abnormal sequences, were obtained;
- trafficking of GRSA proteins to the cell surface is controlled by factors additional to structural elements within these proteins. This is because both abnormal 40 KDa proteins as well as seemingly normal 20 KDa provasopressin are packaged into neurosecretory vesicles;
- abnormal posttranslational processing of vasopressin-related proteins by tumor cells is not due to their inability to express intravesicular processing enzymes. This is because we were able to demonstrate that functional forms of prohormone convertase 2 (PC2) and carboxypeptidase E (CPE) are probably produced by these cells;
- GRSA proteins contain vasopressin and neurophysin structures, as well as the glycopeptide moiety of provasopressin. This is because antibodies to vasopressin, human vasopressin-associated neurophysin (VP-HNP) and vasopressin-associated glycopeptide (VAG), all react with both 40 KDa and 20 KDa protein forms, and;
- GRSA proteins can be potentially used in new immunotherapeutic treatments of breast cancer. This is because the proteins, as components of viable cells in vitro, react with specific antibodies.

63.4

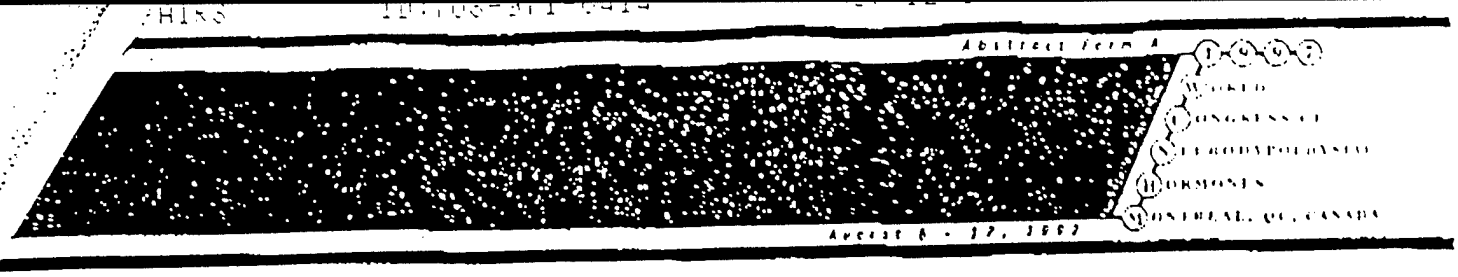
PRODUCTION AND PROCESSING OF VASOPRESSIN GENE-RELATED PROTEINS BY NEUROENDOCRINE TUMORS. W.G. North*, J. Du. Dept. of Physiol., Dartmouth Med. Sch., Lebanon, NH 03756.

We have discovered that vasopressin (VP) gene-related proteins are most probably universal lineage markers for not only small-cell carcinoma of the lung (SCCL), but also breast cancer. Unlike their production by neurons, most (>90%) of these proteins are not packaged into secretory vesicles by these tumor cells, but instead are trafficked to the plasma membrane where they uniquely form surface antigens (NRSA). RT-PCR, cloning, sequencing, immunocytochemistry, Western analysis, and flow cytometry, have allowed us to reach the following conclusions about these tumor proteins:

- NRSA originates from of both normal and abnormal VP genes;
- VP gene expression is a likely feature of the carcinogenic process that generates tumors such as SCCL and breast cancer;
- errors take place in transcription that probably lead to tumor-specific abnormal posttranslational processing;
- NRSA arises through both normal and abnormal posttranscriptional processing;
- trafficking of NRSA to the cell surface is controlled by factors additional to structural elements within the proteins translated;
- abnormal processing of proteins by tumors is not due to their inability to express intravesicular processing enzymes;
- changes in tumor differentiation (or drug resistance) does not affect the nature nor the degree of expression of NRSA.
- membrane models for NRSA require VP, neurophysin, and glycopeptide elements to be extracellular.

Proc. Society for Neuroscience, Volume 23, 1997

Proc. Soc. Neuro, 23:63.4A, 1997



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Abstract title Evidence for the expression of a novel vasopressin-activated calcium mobilizing receptor (HVACM) in human breast cancer and lung cancer cell lines

Authors including presenter (Please underline presenter's name)	Institutions
1 Kenneth A. Longo	1 Dartmouth Medical School
2 William G. North, Ph.D.	2 Dartmouth Medical school
3 Jinlin Du, M.D.	3 Dartmouth Medical School
4 Michael J. Fay, Ph.D.	4 Dartmouth Medical School

Please submit additional authors on a separate sheet of paper

Please type text of abstract ONLY (not title) within this frame

The purpose of this study was to determine if a human homologue of the rabbit vasopressin-activated calcium mobilizing (VACM-1) receptor is expressed in human cancer cells. Vasopressin (AVP) may be involved in human breast cancer and lung cancer pathophysiology, as an autocrine/paracrine factor. AVP can act through four classes of receptors: V2, V1a, V1b, and the recently cloned VACM-1, a structurally unique member of this group that contains a single transmembrane domain. (Recently, a highly homologous cDNA, termed HVACM, was cloned from human placental mRNA.) AVP induced an increase in intracellular free calcium in the breast cancer cell lines MCF-7, T47-D, and ZR-75, and in the lung cancer cell line NCI H-146. Total RNA from these cell lines and normal human tissues (kidney and lung), was used for reverse transcription polymerase chain reaction (RT-PCR) and Northern blot analysis. RT-PCR, using two primer sets designed against the rabbit VACM-1 sequence, amplified bands of the predicted sizes of 674 bp and 193 bp in all cell lines and tissues tested. Direct sequencing of PCR products obtained from H-146 revealed a high degree of identity to the rabbit VACM-1 cDNA (90%) and the human HVACM cDNA (99.5%). Northern blot analysis revealed three distinct bands (3.5, 5 and 6.5 kilobases) in the cancer cell lines. In summary, we have demonstrated the presence of mRNA for a novel AVP receptor in human cancer cell lines and normal human tissues.

VASOPRESSIN GENE-RELATED PRODUCTS IN THE MANAGEMENT OF BREAST CANCER.

William G. North, Ph.D., Michael J. Fay, Ph.D., Kenneth Longo, B.S., and Jinlin Du, M.D.

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There is currently no known universal marker system for breast cancer that can be utilized in tests for early detection, for tumor localization, and for targeted treatment. Most approaches in the management of this disease depend on mammography for detection, and combination chemotherapy and radiation for treatment. We discovered that all breast cancers we examined expressed the vasopressin gene, and set out to determine if this expression represented a universal marker system for the disease. We also have commenced examining the nature of the gene-related products generated by this expression, their role in tumor growth, and their potential usefulness in developing new methods for early detection and for rational treatments. Our approach has involved employing immunohistochemistry and a battery of our antibodies directed against different regions of the provasopressin molecule, methods of protein isolation and characterization, flow cytometry, reverse transcription followed by amplification through polymerase chain reaction (RT-PCR), DNA sequencing, sucrose-gradient fractionation, and radioimmunoassay. In our studies we have utilized surgical and biopsy specimens of breast cancer, normal breast tissue, breast fibrocystic disease, and breast carcinoma in situ, and employed five breast cancer cell lines in culture.

Results obtained using immunohistochemistry have revealed that vasopressin gene-related products are very likely universal markers of early carcinogenesis in breast tissues. This is because all of 19 breast tumors examined gave diffuse positive immunostaining for different components of the provasopressin molecule, while no staining was obtained with normal breast tissues. No cases of polycystic disease examined, including typical and atypical hyperplasia, gave positive staining and this showed tumor immunoreactivity does not simply represent tissue proliferation. All cases of carcinoma in situ gave diffuse positive staining with antibodies against vasopressin-associated human glycopeptide (VAG) suggesting

Keywords: Vasopressin Gene and Carcinogenesis, Glycopeptide-Related Surface Antigen, Targeting, Vasopressin Receptors, Autocrine Growth Factor

This work was supported in part by the U.S. Army Medical Research and Material Command under DAMD 17-94-j-4288.

DOD—
Era of Hope, Proceedings, Volume II, 1997
The Renaissance Hotel Washington, D.C.
October 31 – November 4, 1997

vasopressin gene expression is also common to this form of preinvasive breast cancer. Results obtained from protein analysis and sucrose-gradient fractionation studies, on breast cancer and the MCF-7 and ZR-75-1 cell lines, indicate vasopressin gene expression in breast cancer gives rise to unique major protein products of 40 KDa and 20 KDa that become components of the plasma membrane, and are largely (>90%) processed outside of secretory granules. We have named these proteins collectively GRSA (glycopeptide- related surface antigen) because for viable MCF-7 cells in culture they were found to react with our antibodies to VAG. Ongoing RT-PCR studies on MCF-7, T47D, ZR-75-1 cell lines, utilizing ten primers designed to produce cross-over products for the whole reading frame of vasopressin (VP) mRNA, have so-far allowed us to deduce that GRSA proteins are the products of at least two VP mRNAs, one of normal size and presumably generated from a normal gene, the other(s) containing an additional 600 bases upstream from Exon B and generated either from a normal gene through alternative splicing that includes a portion of intron 1 or from an abnormal gene with an insertion in Exon A. In our studies we have additionally found no evidence for cross-over between vasopressin and oxytocin genes in breast cancer. Although cellular trafficking of GRSA proteins is largely outside of secretory vesicles, we have determined breast cancer cells are capable of expressing proteolytic enzymes required in normal intravesicular processing. Primer pairs for amplification of cDNA fragments of prohormone convertases (PC) 1/3 and 2, and carboxypeptidase E (CPE were used in RT-PCR performed on RNA from cell lines MCF-7 and ZR-75-1. For CPE primers, products of the predicted size were obtained from both cell lines, and DNA sequencing gave a sequence identical to that published for functional CPE of anterior pituitary. Similarly, a product of predicted size and normal structure could be amplified using PC 2 primers from MCF-7 cells, but not from ZR-75-1 cells. Neither cell line seemed to express mRNA for PC 1/3. While most VP gene expression culminates in GRSA protein production, some of it appears to produce vasopressin and VAG as secretory products. This is because, using our RIAs, we were able to show these products elevated in the plasma of 5 of 7 patients with breast cancer. RIAs for VP and VAG might therefore find a use in methods for detecting tumors and monitoring treatments. Vasopressin (VP) appears to be an autocrine growth factor for breast cancer. In this respect, we were have been able to demonstrate for T47D and ZR-75-1 cells, using Indo-1AM fluorescence and flow cytometry that the peptide can increase intracellular free-Ca^{2+} in a dose-dependent manner. We were also able to show through Western analysis that VP can activate mitogen-activated protein (MAP) kinase in these cells. Although these effects both appear to be through a vasopressin V_1 receptor mechanism, RT-PCR and DNA sequencing has been used by us to show that breast cancer cells are capable of expressing all four vasopressin receptor subtypes (V_{1a}, V_{1b}, V_2, and human VACM), as well as oxytocin receptors. BT 549, MCF-7, MDA-MB-231, T47D, and ZR-75-1 cells have featured in these receptor studies.

Our studies have therefore led us to the following conclusions: 1) the vasopressin gene is a universal marker of carcinogenesis in breast tissue; 2) vasopressin gene expression in breast cancer uniquely leads to the formation of surface GRSA proteins that are potential targets for immunotherapy; 3) breast tumors are neuroendocrine and most cause plasma elevations of vasopressin gene-related products that can be potentially used for detection and monitoring treatments; 4) vasopressin is an autocrine growth factor for breast cancer; and 5) expression of multiple VP receptors subtypes implies vasopressin plays a multifaceted role in tumor growth and survival. All of these conclusions speak to the future importance of vasopressin gene-related products for developing new and sensitive methods of detecting breast cancer and monitoring treatments, and new and successful immunotherapuetic interventions.

THE ROLE OF VASOPRESSIN AND OXYTOCIN HORMONES IN BREAST CANCER

Michael Fay, Ph.D., Jinlin Du, MD, Kenneth Longo, and William North, Ph.D.

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This laboratory has demonstrated that fixed breast cancer biopsy specimens exhibit positive immunoreactivity for vasopressin and oxytocin gene-related products using the technique of immunohistochemistry and antibodies directed against different regions of the vasopressin and oxytocin prohormones. In addition, both in vitro and in vivo research indicate that neuropeptides, like vasopressin and oxytocin, modulate breast cancer cell growth. Taken together these results suggest that vasopressin and oxytocin may serve as autocrine and/or paracrine growth modulators for breast cancer cells. However, the receptors and signal transduction pathways through which vasopressin and oxytocin act to influence breast cancer cell growth remain unknown. The purpose of this research is to determine if breast cancer cells express vasopressin and oxytocin receptors, and to evaluate vasopressin- and oxytocin-induced signal transduction in breast cancer cells.

To evaluate which vasopressin and oxytocin receptor subtypes are expressed by breast cancer cells the technique of reverse-transcription polymerase chain reaction (RT-PCR) was used with primer pairs specific for the oxytocin receptor, the V1a vasopressin receptor, the V1b vasopressin receptor, the V2 vasopressin receptor, and the vasopressin-activated calcium mobilizing receptor (VACM). The VACM and V1b receptor PCR products were confirmed by direct DNA sequencing. To study vasopressin and oxytocin induced changes in intracellular-free calcium, breast cancer cells were loaded with indo-1 AM, and neuropeptide-induced changes in intracellular free calcium monitored over a four minute period using a Becton Dickinson Facstar Plus flow cytometer [excitation 356 nm, emissions 405 nm (calcium bound indo), and 485 nm (free indo)]. To determine if vasopressin causes activation of the mitogen activated protein kinase cascade (MAP kinase), MCF-7 breast cancer cells were stimulated with vasopressin, and activated (phosphorylated) MAP Kinase evaluated by western blot analysis.

Keywords: Breast Cancer Cells, Vasopressin and Oxytocin, Vasopressin and Oxytocin Receptors, Signal Transduction.

This work was supported by the U.S. Army Medical Research and Material Command under DAMD 17-94-j-4131.

Using the technique of RT-PCR evidence was obtained for the expression of mRNA(s) for a number of vasopressin and oxytocin receptor subtypes in cultured breast cancer cell lines. Using two primer pairs based on the sequence of the VACM receptor, PCR products of the predicted sizes of 674 bp and 193 bp were amplified from MCF-7, T47D, and ZR-75 breast cancer cell lines. Using a primer pair based on the oxytocin receptor, a PCR product of the predicted size of 391 bp was amplified from BT549, MCF-7, MDA-MB-231, T47D, and ZR-75 breast cancer cell lines. From the ZR-75, BT549, and MCF-7 cell lines a PCR product of the predicted size of 862 bp was amplified using primers for the V2 vasopressin receptor. In addition, using the V2 receptor primers, a PCR product which is approximately 100 bp larger than expected was amplified from these three cell lines. It is believed that this PCR product represents an incompletely spliced mRNA species containing the second intron. Using Primer pairs that amplify a 239 bp PCR product for the V1b vasopressin receptor, a product of the predicted size was amplified from the MCF7 breast cancer cell line. Preliminary PCR results using a primer pair based on the V1a vasopressin receptor indicate that a PCR product of the predicted size of 408 bp was amplified from the T47D breast cancer cell line. The identity of the VACM and V1b PCR products has been verified by direct DNA sequencing of the PCR products. Northern blot analysis for VACM using RNA from the ZR-75, MCF-7, and T47D cell lines indicates RNA species of ~ 3.5, 5, and 6.5 Kb. Using indo-1 AM loaded ZR-75 and T47D breast cancer cells neuropeptide induced changes in intracellular free calcium was monitored using flow cytometric analysis. Vasopressin (0, 10 nM, 100 nM, and 1,000 nM) was administered after approximately 20 seconds of baseline. In both cell lines vasopressin at the 100 nM and 1,000 nM doses induced a rise in intracellular-free calcium as indicated by an increase in the 405nm/485nm ratio. At all the doses studied oxytocin (10 nM, 100 nM, 1,000 nM) did not cause a noticeable rise in intracellular-free calcium in the ZR-75 and T47D cell lines. Treatment of MCF-7 breast cancer cells with 100 nM and 1,000 nM vasopressin resulted in a dose-dependent increase in tyrosine phosphorylated MAP kinase as determined by Western blot analysis.

Both in vivo and in vitro results indicate that neuropeptides like vasopressin can serve as growth modulating agents for breast cancer. Research performed in this laboratory indicates that neuropeptides, like vasopressin and oxytocin, are produced by breast cancer cells. Collectively these results suggest that neuropeptide hormones may serve as autocrine/paracrine factors for breast cancer. The results obtained in these studies provide further support for a role of vasopressin and oxytocin as paracrine/autocrine factors for breast cancer since mRNA(s) for a number of receptors for these hormones are expressed in cultured breast cancer cells. Vasopressin treatment causes a rise in intracellular free calcium in two cultured breast cancer cell lines, suggesting that the hormone might be activating VACM, V1a, or V1b receptor subtypes. Experimental results obtained with the MCF-7 breast cancer cell line suggest that the influence of vasopressin on breast cancer cell growth observed in vivo and in vitro may be due to activation of the MAP kinase cascade. These results further support a role for neuropeptide hormones like vasopressin and oxytocin in breast cancer pathophysiology. Identifying hormones involved in breast cancer cell growth, the hormone receptors through which these peptides act, and the cellular changes associated with receptor activation is crucial to identifying novel strategies for the treatment of breast cancer.

HVACM RECEPTOR

LOCUS AF017061 2461 bp mRNA PRI 16-SEP-1997
DEFINITION Homo sapiens vasopressin-activated calcium mobilizing putative receptor protein (VACM-1) mRNA, complete cds.
ACCESSION AF017061
NID g2394273
KEYWORDS .
SOURCE human.
ORGANISM Homo sapiens
Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 2461)
AUTHORS Longo,K.A., Du,J. Fay,M.J., and North,W.G.
TITLE Direct Submission
JOURNAL Submitted (02-AUG-1997) Department of Physiology, Dartmouth Medical School, 1 Medical Center Drive, Lebanon, NH 03755, USA
FEATURES Location/Qualifiers
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gene 1..2461
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CDS 1..2346
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the above report in format.

LOCUS XXXXX 1298 bp mRNA PRI 21-OCT-1997
DEFINITION Homo sapiens small-cell carcinoma of the lung vasopressin receptor subtype 1a mRNA, complete cds.
ACCESSION AF030625
KEYWORDS .
SOURCE human.
ORGANISM Homo sapiens
Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 1298)
AUTHORS Thibonnier,M., Auzan,C., Madhun,Z., Wilkins,P., Berti-Mattera,L. and Clauser,E.
TITLE Molecular cloning, sequencing, and functional expression of a cDNA encoding the human V1a vasopressin receptor
JOURNAL J. Biol. Chem. 269, 3304-3310 (1994)
REFERENCE 2 (bases 1 to 1298)
AUTHORS North,W.G., Fay,M.J., Longo,K.A. and Du,J.
TITLE Functional Vasopressin V1 Type Receptors are present in Variant as well as Classical forms of Small-Cell Carcinoma
JOURNAL Peptides 18, 985-993 (1997)
REFERENCE 3 (bases 1 to 1298)
AUTHORS Du,J., Fay,M.J., Longo,K.A. and North,W.G.
TITLE Direct Submission
JOURNAL Submitted (21-OCT-1997) Physiology, Dartmouth Medical School, 1 Medical Center Drive, Lebanon, NH 03756, USA
COMMENT Hirasawa, A. Biochemical and Biophysical Research Communications, 203,72-79,1994

Thibonnier, M. Genomics, 31, 327-334, 1996.
FEATURES Location/Qualifiers
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LOCUS XXXXX 1450 bp mRNA PRI 20-OCT-1997
DEFINITION Homo sapiens small cell lung cancer vasopressin receptor subtype 1b mRNA, complete cds.
ACCESSION AF030512
KEYWORDS .
SOURCE human.
ORGANISM Homo sapiens
Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 1450)
AUTHORS Sugimoto,T., Saito,M. , Mochizuki,S., Watanabe,Y., Hashimoto,S. and Kawashima,H.
TITLE Molecular cloning and functional expression of a cDNA encoding the human V1b vasopressin recceptor
JOURNAL J. Biol. Chem. 269, 27088-27092 (1994)
REFERENCE 2 (bases 1 to 1450)
AUTHORS Du,J., Fay,M.J., Longo,K.A. and North,W.G.
TITLE Human vasopressin receptor subtype 1b in small cell carcinoma of the lung
JOURNAL Unpublished
REFERENCE 3 (bases 1 to 1450)
AUTHORS Du,J., Fay,M.J., Longo,K.A. and North,W.G.
TITLE Direct Submission
JOURNAL Submitted (20-OCT-1997) Physiology, Dartmouth Medical School, 1 Medical Center Drive, Lebanon, NH 03756, USA
COMMENT de Keyzer, Y. FEBS Lett. 356, 215-220, 1994.
FEATURES Location/Qualifiers
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CDS 124..1398
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LOCUS XXXXX 1201 bp mRNA PRI 21-OCT-1997
DEFINITION Homo sapiens vasopressin V2 receptor mRNA, complete cds.
ACCESSION AF030626
KEYWORDS .
SOURCE human.
ORGANISM Homo sapiens
Eukaryotae; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria;
Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 1201)
AUTHORS Birnbaumer,M., Seibold,A., Gilbert,S., Ishido,M., Barberis,C.,
Antaramian,A., Brabet,P. and Rosenthal,W.
TITLE Molecular cloning of the receptor for human antidiuretic hormone
JOURNAL Nature 357, 333-335 (1992)
REFERENCE 2 (bases 1 to 1201)
AUTHORS Fay,M.J., Du,J., Yu,X. and North,W.G.
TITLE Evidence for Expression of Vasopressin V2 receptor mRNA in Human
Lung
JOURNAL Peptides 17, 477-481 (1996)
REFERENCE 3 (bases 1 to 1201)
AUTHORS Du,J., Fay,M.J., Yu,X. and North,W.G.
TITLE Direct Submission
JOURNAL Submitted (21-OCT-1997) Physiology, Dartmouth Medical School, 1
Medical Center Drive, Lebanon, NH 03756, USA
COMMENT Seibold, A. Am. J. Hum. Gent. 51,1078-1083, 1992.
FEATURES Location/Qualifiers
source 1..1201
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721 gggagattca tgccagtctg gtgccagggc catcagagag gcctgggggg cgccgcaggg
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1141 catcgtgagg agctgttggg tgtcttgcct ctagaggctt tgagaagctc agctgccttc
1201 c
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References (papers and abstracts)

1. **North, W.G.**, Pai, S., Friedmann, A., Yu, X., Fay, M., and Memoli, V. Vasopressin gene related products are markers of human breast cancer. Breast Cancer Research and Treatment, 1995; 34:229-235.
2. Gallagher, J.D., Fay, M.J., **North, W.G.**, and McCann, F.V. Ionic signals in T47D human breast cancer cells. Cellular Signaling, 1996; 8(4):279-284.
3. Fay, M.J., Mathew, R.S., Donnelly, E.M., Memoli, V.A., and **North, W.G.** Immunohistochemical evaluation of vasopressin gene expression in fibrocystic breast disease. Endocrine Pathology (submitted), paper in press.
4. Fay, M.J., Yu, X., Memoli, V., and **North, W.G.** Expression of the vasopressin gene by human breast cancer. Mol. Biol. Cell, 1994; 5:2730 (abstract).
5. Fay, M.J., Yu, X., Memoli, V., and **North, W.G.** Expression of the vasopressin gene by human breast cancer. Gordon Conference, Mammary Gland Biology (Colby Sawyer College, New London, NH), June 18-23, 1995 (abstract).
6. Longo, K.A., Fay, M.J., Du, J., and **North, W.G.** Evidence for the expression of a novel vasopressin-activated calcium mobilizing receptor (VACM-1) in human breast cancer and lung cancer. Mol. Biol. Cell, 1996; 7:3761 (abstract).
7. **North, W.G.**, Fay, M.J., and Du, J. Vasopressin and breast cancer, gene expression and trafficking. Summer neuropeptide conference (Key West, FL), June 21-26, 1997 (abstract).
8. Longo, K.A., **North, W.G.**, Du, J., and Fay, M.J. Evidence for the expression of a novel vasopressin-activated calcium mobilizing receptor (HVACM) in human breast cancer and lung cancer cell lines. 1997 World congress of neurohypophysial hormones (Montreal, Canada) August 8-12, 1997 (abstract).
9. **North, W.G.** and Du, J. Production and processing of vasopressin gene-related proteins by neuroendocrine tumors. Proc. Soc. Neuro., 23:63.4A, 1997.

This work was supported by the U.S. Army Medical Research and Material Command under DAMD 17-94-J-4288.