EB2005 SYMPOSIUM INTRODUCTION

Diagnosis and Treatment Utilizing Natriuretic Peptides

One of the missions of the American Federation for Medical Research (AFMR) is to facilitate translational research. With that objective, AFMR is proud to continue to sponsor the "Bench to Bedside Symposia" at the annual Experimental Biology (EB) meeting. More details on how to apply for holding a symposium for future EB meetings are provided at <www.afmr.org>.

The symposium "Diagnosis and Treatment Utilizing Natriuretic Peptides," sponsored by the AFMR, was held at the 2005 EB meeting in San Diego, California, on April 4, 2005. This symposium was cosponsored by Quest Diagnostics, Inc. The session's content is summarized in the four articles that follow, each authored by one of the speakers at the symposium. Dr. Adolfo J. deBold (Ottawa Heart Institute, Ottawa, ON), who discovered the first of the natriuretic peptides (ie, atrial natriuretic factor, also termed atrial natriuretic peptide), reviewed what regulates natriuretic peptide production by the heart. Dr. John Burnett Jr (Mayo Clinic, Rochester, MN) reviewed natriuretic peptides as treatment modalities of congestive heart failure. Dr. Walter H. Hörl (University of Vienna, Vienna, Austria) reviewed natriuretic peptides as treatment modalities of acute and chronic kidney disease and the effects of hemodialysis on the circulating levels of natriuretic peptides. Lastly, Dr. David Vesely (University of South Florida Cardiac Hormone Center, Tampa, FL) presented data on the basic science and potential clinical application of the newest property of some of the natriuretic peptides, that is, their potential as anticancer agents.

The following articles discuss the molecular biology, animal experiments, and human investigations that have led to one of the natriuretic peptides to be used clinically to treat congestive heart failure. Further, their potential to move from the bench and animal experiments to the bedside to treat renal failure and cancer is outlined for the reader.

David L. Vesely, MD, PhD, Symposium Chair
Abdulla K. Salahudeen, MD, AFMR Vice-President for Meetings and Symposia

EB2005 SYMPOSIUM

Atrial Natriuretic Peptides: Anticancer Agents

David L. Vesely

ABSTRACT

Atrial natriuretic peptides (ANPs) consist of a family of six peptide hormones that are synthesized by three different genes and then stored as three different prohormones. Within the 126–amino acid ANP prohormone are four peptide hormones: long-acting natriuretic peptide (LANP), vessel dilator, kaliuretic peptide, and ANP, whose main known biologic properties are blood pressure regulation and maintenance of plasma volume. The newest discovered property of these peptide hormones is their anticancer effects. Vessel dilator, LANP, kaliuretic peptide, and ANP decrease the number of human pancreatic adenocarcinoma cells in culture by 65%, 47%, 37%, and 34%, respectively, within 24 hours at their 1 μM concentrations. Similar results have been found with breast adenocarcinomas, squamous cell lung cancer, and small cell lung cancer cells, each associated with an 83% or greater inhibition of deoxyribonucleic acid (DNA) synthesis by these four peptide hormones. Brain natriuretic peptide has no effects even when increased 100-fold (ie, 100 μM). C-type natriuretic peptide has no effects when increased 10-fold, but when increased 100-fold, it decreases 39% of the cancer cells. At this higher 100 μM concentration, vessel dilator kills 92% of the cancer cells within 24 hours. The four peptide hormones synthesized by the ANP gene given subcutaneously via osmotic pumps in athymic mice with human

From the Departments of Internal Medicine, Physiology, and Biophysics, University of South Florida Cardiac Hormone Center, and James A. Haley Veterans' Administration Medical Center, Tampa, FL.

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Address correspondence to: Dr. David L. Vesely, Cardiac Hormone Center, University of South Florida for Health Sciences, 13000 Bruce B. Downs Blvd, Tampa, FL 33612; e-mail: david.vesely@med.va.gov.

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pancreatic adenocarcinomas completely stop the growth of these adenocarcinomas at 1 week. Vessel dilator, LANP, and kaliuretic peptide within 1 week decrease the volume by 49%, 28%, and 11% of the human pancreatic adenocarcinomas, which, with current anticancer treatment, have a mean survival of only 4 months.
Key Words: natriuretic peptides, cancer, cyclic guanosine monophosphate, DNA synthesis

Atrial natriuretic peptides (ANPs) consist of a family of peptide hormones that are synthesized by three different genes and then stored as three different prohormones: 126–amino acid ANP, 108–amino acid brain natriuretic peptide (BNP), and 103–amino acid C-type natriuretic peptide (CNP) prohormones.[1–3] Within the 126–amino acid ANP prohormone are four peptide hormones (Figure 1), whose main known biologic properties are blood pressure regulation and maintenance of plasma volume in animals[4–10] and humans.[11–13] These peptide hormones, numbered by their amino acid sequences beginning at the N-terminal end of the ANP prohormone, consist of the first 30 amino acids of the prohormone, that is, long-acting natriuretic peptide (LANP), amino acids 31 to 67 (vessel dilator), amino acids 79 to 98 (kaliuretic peptide), and amino acids 99 to 126 (ANP).[3,14] The BNP and CNP genes, on the other hand, appear to each synthesize only one peptide hormone within their respective prohormones, that is, BNP and CNP.[2,15,16] Each of these peptide hormones

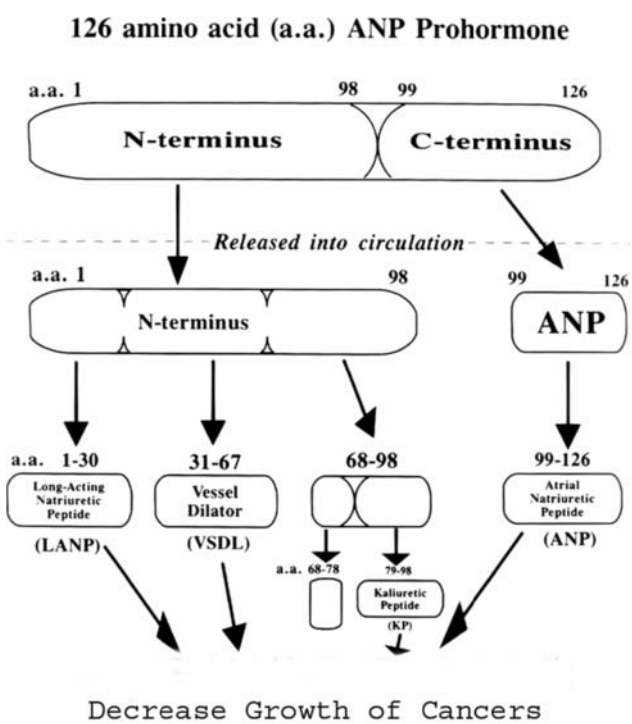

FIGURE 1 Atrial natriuretic peptide (ANP) gene synthesizes a 126–amino acid prohormone that contains four peptide hormones consisting of amino acids 1 to 30 (ie, long-acting natriuretic peptide), 31 to 67 (vessel dilator), 79 to 98 (kaliuretic peptide), and 99 to 126 (ANP).[2–14] Reproduced with permission from Vesely DL et al.[41]

circulates in healthy humans, with vessel dilator and LANP's concentrations in plasma being 17- to 24-fold higher than ANP, BNP, and CNP.[17–23]

ANP has growth regulatory properties.[24–27] In vascular smooth muscle, ANP inhibits cell proliferation (hyperplasia) and smooth muscle cell growth (hypertrophy).[24–27] ANP has growth regulatory properties in a variety of other tissues, including brain, bone, myocytes, red blood cell precursors, and endothelial cells.[28–35] In the kidney, ANP causes antimitogenic and antiproliferative effects in glomerular mesangial cells.[28–30,32]

ATRIAL NATRIURETIC PEPTIDES DECREASE THE NUMBER AND DNA SYNTHESIS OF HUMAN PANCREATIC ADENOCARCINOMAS

The first cancer studied both in vitro and in vivo was human pancreatic adenocarcinomas, which have the lowest 5-year survival rate of all common cancers.[36,37] The 5-year survival rate of persons with adenocarcinoma of the pancreas is 1%.[36,37] The median survival is 4 months.[36,37] Current cancer chemotherapy and surgery prolong survival by a few months, but the above-mentioned survival rates are for persons treated with surgery and/or current cancer chemotherapeutic agents.[36,37]

Vessel dilator, LANP, kaliuretic peptide, and ANP (each at 1 μM) decrease the number of human pancreatic adenocarcinoma cells in culture by 65% ($p < .001$), 47% ($p < .01$), and 37% and 34% (both at $p < .05$), respectively, within 24 hours.[38] This decrease was sustained without any proliferation of the adenocarcinoma cells occurring in the 3 days following this decrease in number.[38] Thus, when exposed to vessel dilator, LANP, kaliuretic peptide, and ANP for 48 hours, the inhibition of the number of cancer cells compared with controls was 68% ($p < .001$), 43%, 40%, and 33% ($p < .05$ for these three peptides), respectively.[38] At both 72 and 96 hours, the decrease in the number of adenocarcinoma cells secondary to vessel dilator was 70% ($p < .001$).[38] LANP for 72 and 96 hours resulted in the number of adenocarcinoma cells being reduced 47% and 48% ($p < .01$ for both), respectively.[38] At 72 and 96 hours, the number of cancer cells with kaliuretic peptide present was decreased by 39% and 42% compared with untreated control cells ($p < .05$ for each).[38] The number of adenocarcinoma cells at 72 and 96 hours was decreased secondary to ANP by 37% and 35% ($p < .05$ for both).[38] At least part of the mechanism of the decrease in the cancer cell number and antiproliferative effects of these peptide hormones was a 83% or greater inhibition of deoxyribonucleic acid (DNA) synthesis but not owing to enhanced apoptosis, that is, programmed cell death.[38] Thus, vessel dilator, LANP, kaliuretic peptide, and ANP, each at their 1 μM concentrations, inhibited DNA synthesis when incubated with adenocarcinoma cells for 24 hours by 91%, 84%, 86%, and 83%, respectively ($p < .001$ for each) (Figure 2). One of the known mediators[39,40] of mechanisms of action of these

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peptide hormones, that is, cyclic guanosine monophosphate (cGMP), inhibited DNA synthesis in these adenocarcinoma cells by 51%. Dose-response curves revealed that 8-bromo-cGMP, the cell-permeable analogue of cGMP, decreased DNA synthesis in these cancer cells 46%, 42%, 39%, and 34% (all $p < .05$) at its 3 mM, 1 mM, 100 µM, and 1 µM concentrations, respectively.[38] Even at 1 nM (ie, 10^{-9} M) of 8-bromo-cGMP, there was a 25% decrease in DNA synthesis in the adenocarcinoma cells ($p < .05$).[38] At 100 pM of 8-bromo-cGMP, its effects on DNA synthesis in these adenocarcinoma cells became not significant (14% decrease).

ANPS STOP THE GROWTH OF HUMAN PANCREATIC ADENOCARCINOMAS IN VIVO

In vivo, the effects of these peptide hormones as anticancer agents were even more impressive. Vessel dilator (139 ng $min^{-1}kg^{-1}$ of body weight) infused subcutaneously for 14 days via osmotic pumps completely stopped the growth of human pancreatic adenocarcinomas in athymic mice ($n = 14$) with a decrease in their tumor volume, even when the tumor volume was large[41] (ie, 60-fold increase in size over basal palpable tumor before peptide infusion was begun, to mimic what occurs in humans; ie, the pancreatic adenocarcinomas in humans are usually large before they are discovered[36]). The tumor volume increased 69-fold in this 2-week period ($p < .001$) when measured with electronic Vernier calipers in the placebo-treated mice ($n = 30$).[41] Dose-response studies revealed that at concentrations as low as 1.7 ng/min^{-1} 20^{-1} g mouse, vessel dilator could completely stop the growth of the human pancreatic adenocarcinomas, but at this concentration, there was no decrease in the volume of the tumor by vessel dilator.[41] The tumor volume of the untreated human pancreatic adenocarcinoma increased 172-fold in 3 weeks and was almost 300-fold increased 4 weeks after the tumors first became palpable.[41] After 2 months, the volume of this untreated aggressive adenocarcinoma was 1,306-fold greater than when the tumors first became palpable.[41] When these peptide hormones at 10-fold higher concentrations (ie, at 1.4 µg/min^{-1} kg^{-1} body weight) were infused for 4 weeks, in addition to completely stopping the growth of this aggressive adenocarcinoma, vessel dilator, LANP, and kaliuretic peptide decreased the tumor volume of human pancreatic adenocarcinomas after 1 week by 49%, 28%, and 11%, respectively (Figure 3), with a 1- and 20-fold increase in the tumor volume in ANP- and placebo-treated mice.[41] cGMP (0.05 µg/min^{-1} 20^{-1} g mouse body weight) inhibited after 1 week the growth of this cancer by 95%.[41] There was no evidence of cytotoxicity in any of the normal tissues during the infusion of these peptide hormones.[41]

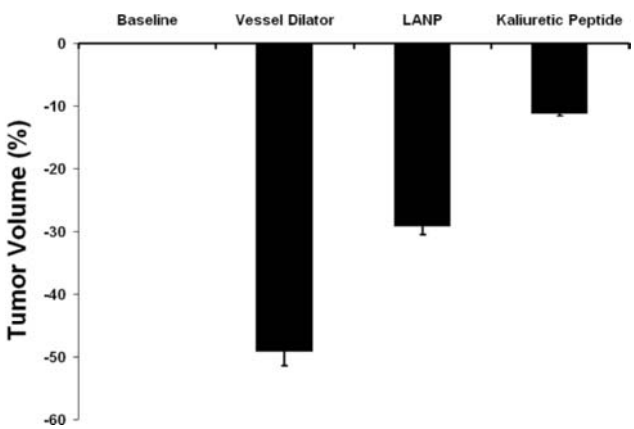

FIGURE 2 Inhibition of deoxyribonucleic acid (DNA) synthesis by vessel dilator, long-acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) in pancreatic adenocarcinoma cells. This inhibition of DNA synthesis is illustrated as the percentage of DNA synthesis occurring with the respective peptide hormones, each at 1 µM, versus the amount of DNA synthesis without the addition of any of these peptide hormones. The amount of inhibition of DNA synthesis by each of these peptide hormones was significant at $p < .001$ when evaluated by repeated-measures analysis of variance. Reproduced with permission from Vesely BA et al.[38]

LOCALIZATION OF ANPS WITHIN HUMAN PANCREATIC ADENOCARCINOMAS

Immunocytochemical evaluation after removal of the human pancreatic adenocarcinomas revealed that vessel dilator, LANP, kaliuretic peptide, and ANP, each localized to the nucleus and cytoplasm of the cancer cells and to the endothelium of the capillaries growing into these tumors.[42] This investigation is the first demonstration of any anti–growth peptide hormone localizing to the nucleus, where these peptide hormones could directly inhibit DNA synthesis.[42] It is, thus, of interest that all four of these peptide hormones, which inhibit DNA synthesis, localized to the nucleus. Growth-promoting peptides,

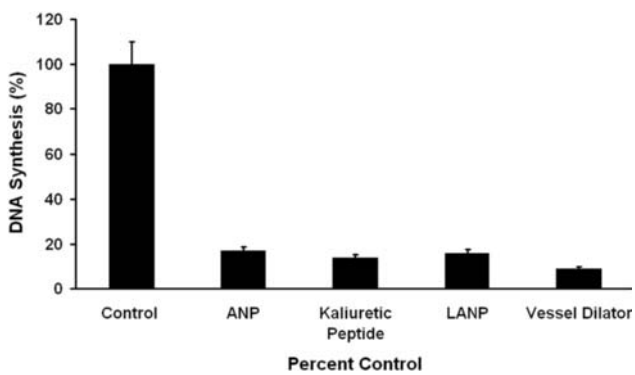

FIGURE 3 Vessel dilator, long-acting natriuretic peptide (LANP), and kaliuretic peptide decrease tumor volume during the first week of treatment with 1.4 µg min^{-1} kg^{-1} body weight. Compared with their respective baseline tumor volumes, the decrease in tumor volume was significant at $p < .01$ for vessel dilator and $p < .05$ for LANP and kaliuretic peptide when evaluated by analysis of variance with repeated-measures design with Fisher's least significant difference as a post hoc test ($n = 5$ in each group). Reproduced with permission from Vesely DL et al.[41] ANP = atrial natriuretic peptide; DNA = deoxyribonucleic acid.

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such as extracellular receptor kinase (ERK)-1, have been shown to move from the plasma membrane to the nucleus to cause proliferation, and, recently, we showed that a slightly modified kaliuretic peptide can substantially decrease the activation of ERK-1 and -2.[43] These peptide hormones may, thus, inhibit the growth of cancer cells not only by directly inhibiting DNA synthesis in the nucleus but by also decreasing the activation of growth-promoting substances, such as ERK-1 and -2, which promote the growth of cancer cells.[43]

DECREASE IN NUMBER AND DNA SYNTHESIS OF HUMAN BREAST ADENOCARCINOMA CELLS BY FOUR PEPTIDE HORMONES SYNTHESIZED BY THE ANP GENE

Within 24 hours, vessel dilator, LANP, kaliuretic peptide, ANP, and 8-bromo-cGMP (each at 1 μM) decreased the number of human breast adenocarcinoma cells 60%, 31%, 27%, 40%, and 31%, respectively.[44] There was no proliferation in the 3 days following this decrease in breast adenocarcinoma cell number. These same four hormones decreased DNA synthesis by 69 to 85% in the human breast adenocarcinoma cells ($p < .001$).[44] BNP and CNP, each at the same concentration, did not significantly decrease the number of breast adenocarcinoma cells (0% and 2%, respectively).[44] Each of the peptide hormones except BNP caused an increase in cellular debris when examined by flow cytometry, indicating that cellular necrosis was occurring secondary to these peptide hormones.[44]

CELL-CYCLE ARREST OF ADENOCARCINOMA CELLS WITH NATRIURETIC PEPTIDES

Cell-cycle progression was directly affected by several of the natriuretic peptides. The majority of the natriuretic peptides had their strongest modification of cell-cycle progression in the synthetic (S) phase of the cell cycle.[44] Vessel dilator, LANP, kaliuretic peptide, and 8-bromo-cGMP (each at 1 μM) decreased the number of breast cancer cells in the S phase of the cell cycle by 62%, 33%, 50%, and 39%, respectively (all $p < .05$).[44] ANP caused a 40% decrease in the G_2–M proliferative phase of the cell cycle.[44] There was an accumulation of cells in the resting G_0–G_1 phase secondary to LANP, vessel dilator, kaliuretic peptide, and ANP.[44] Vessel dilator, which caused the largest decrease in cells in the S phase, had the largest accumulation of cells in the G_0–G_1 phase.[44] BNP had no effect on the S phase or any other portion of the cell cycle.[44]

NATRIURETIC PEPTIDE RECEPTORS A AND C ARE PRESENT IN BREAST ADENOCARCINOMA CELLS

When the breast adenocarcinoma cells were evaluated by Western blots, natriuretic peptide receptors A and C (NPR-A and NPR-C) were demonstrated to be present.[44] It is of interest that the breast adenocarcinoma cells have developed NPR-A and NPR-C to mediate ANP's effects via membrane-bound guanylate cyclase (NPR-A)– and non–membrane-bound guanylate cyclase (NPR-C)–mediated mechanisms, respectively.[45] ANP's signaling via the NPR-C is thought to involve a cascade of Ca^{2+} influx, activation of endothelial nitric oxide synthase with resulting formation of nitric oxide activating cytosolic guanylate cyclase, which, in turn, increases the concentration of cGMP.[45] The presence of these receptors helps explain that ANP, but not BNP and CNP, has effects at its 1 μM concentration because ANP binds to both receptors with a stronger affinity than BNP or CNP; thus, a lower concentration of ANP is needed to have an effect.[46] When the concentrations of CNP and BNP are increased 100-fold, in dose-response curves,[47] CNP, but not BNP, has an effect on the number of cancer cells. This is consistent with CNP's binding to the NPR-C with a stronger affinity than BNP but not as strong as ANP; that is, binding to NPRs is ANP > CNP > BNP.[46]

GENERALIZED ANTICANCER EFFECTS OF NATRIURETIC PEPTIDES

In addition to the above-mentioned adenocarcinomas, these four peptide hormones synthesized by the ANP gene decrease the cancer cell number and DNA synthesis in all cancers examined thus far. Included in these cancers are the most common cancers, including small cell lung cancer[47] and squamous lung cancer cells,[48] and uncommon cancers, such as primary malignant tumors of the heart (David L. Vesely et al, unpublished observations, 2004). ANP, but not the other peptide hormones, has been investigated on hepatoblastoma cells and was found to decrease their number in culture.[49] When the concentration of vessel dilator is increased in dose-response curves 100-fold to 100 μM, it decreases the number of cancer cells by 92% within 24 hours,[47] suggesting that at this concentration it has very potent anticancer effects.

NATRIURETIC PEPTIDES DO NOT HAVE SIDE EFFECTS OF CURRENT ANTICANCER AGENTS

The four cardiovascular peptide hormones do not cause nausea, vomiting, alopecia, or myleosuppression, which are common with current cancer chemotherapy, or the more severe toxicity of permanent ovarian dysfunction and leukemia and/or secondary tumors, which occurs with currently used anticancer agents.[36,37] The lack of these side effects and their beneficial effects of decreasing the number of cancer cells suggest that the cardiovascular hormones synthesized by the ANP prohormone gene may have use in the future as anticancer agents.

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