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Four Cardiac Hormones Eliminate Up to 82% of Human Medullary Thyroid Carcinoma Cells Within 24 Hours

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Four cardiac hormones, i.e., atrial natriuretic peptide, vessel dilator, long-acting natriuretic peptide, and kaliuretic peptide, which have anticancer effects, were evaluated for the first time on any endocrine cancer to determine if they have anticancer effects in an endocrine cancer. These four cardiac hormones were evaluated for their anticancer, DNA synthesis, and receptor status in human medullary thyroid cancer cells. There was a significant ($p < 0.001$) decrease in human medullary thyroid cancer cells with each 10-fold increase from 1 to 100 μ*M* of the four cardiac hormones. There was an 81%, 68%, 71%, and 66% elimination within 24 h of medullary thyroid cancer cells secondary to vessel dilator, kaliuretic peptide, atrial natriuretic peptide, and long-acting natriuretic peptide, respectively ($p < 0.0001$). Three days after treatment with these peptide hormones, there was no proliferation of the medullary thyroid cancer cells. These cardiac hormones decreased DNA synthesis in the medullary thyroid cells from 65% to 84% ($p < 0.0001$). Western blots revealed natriuretic peptide receptors-A and -C were present in human medullary thyroid cancer cells. These results indicate the four cardiac hormones have potent anticancer effects by eliminating up to 82% of human medullary thyroid carcinoma cells within 24 h of treatment.

Key Words: Medullary thyroid cancer; cardiac hormones; DNA synthesis; receptors.

Introduction

The most common endocrine cancer is thyroid cancer *(1)*. Medullary thyroid carcinoma has a worse prognosis than either papillary or follicular thyroid cancer *(1)*. The Surveillance, Epidemiology and End Results (SEER) evaluation of 15,698 patients revealed that the 10-yr age and gender-corrected survival rates were 98% for papillary, 92% for follicular, but only 80% for medullary thyroid carcinoma *(1)*. In 2005 there were an estimated 25,690 new cases of thyroid cancer with 1490 thyroid cancer deaths *(2)*. Sporadic medullary thyroid carcinoma (MTC) accounts for 80% of all cases of MTC with the remainder having inherited tumor syndromes such as multiple endocrine neoplasia types 2A or 2B or familial medullary thyroid carcinoma *(3)*. Metastatic cervical adenopathy is noted in approx 50% of patients with sporadic MTC at initial presentation *(4)*, and lymph node metastases increase to 80% at time of diagnosis if the MTC is larger than 1 cm in diameter *(5,6)*. Radiotherapy is frequently used to treat MTC because of this high incidence of metastatic lesions, but there is no evidence it has any effect on survival *(7)*. Targeted radiotherapies using radiolabeled somatostatin analogs *(8,9)* radiolabeled monoclonal antibodies directed against CEA *(10)*, and radiolabeled MIBG *(11)* have been used with limited success *(3)*. There is, thus, need for a new adjunct treatment(s) of medullary thyroid carcinomas.

Cardiac natriuretic hormones are a family of peptide hormones that have significant anticancer effects on some cancer cells and tumors *(12–19)*, but these peptide hormones have never been investigated for their anticancer effects on any endocrine cancer. Of this family of peptide hormones, one gene in the heart synthesizes a 126 amino acid (aa) prohormone which with proteolytic processing results in four peptide hormones consisting of (1) the first 30 aa of this prohormone (i.e., long-acting natriuretic peptide, LANP), (2) vessel dilator (VDL, aa 31–67), (3) kaliuretic peptide (KP, aa 79–98), and (4) atrial natriuretic peptide (ANP, aa 99–126) of the 126 aa prohormone (Fig. 1). The rationale for studying these cardiac hormones on thyroid cancer cells is that ANP receptors have been shown by competitive binding techniques to be present on thyroid cells *(20,21)* to mediate their potential anticancer effects. Our hypothesis is that these cardiac hormones will significantly decrease (i.e., eliminate) medullary thyroid cancer cells in culture through receptors on these thyroid cancer cells and that these anticancer effects will be mediated in part by a strong inhibition of

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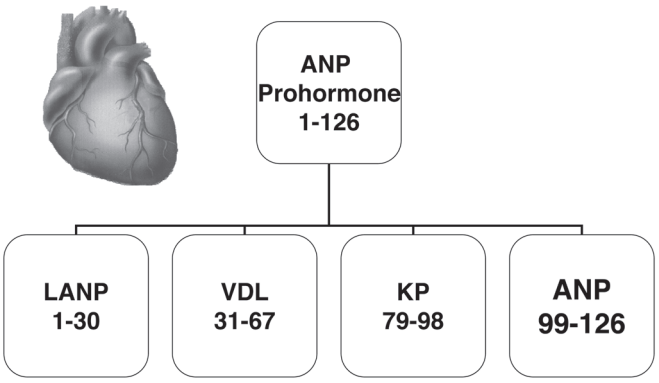

Fig. 1. The *atrial natriuretic peptide* gene in the heart synthesizes a 126-amino-acid (aa) prohormone with which proteolytic processing results in the formation of four cardiac hormones. These four cardiac hormones, i.e., (1) long-acting natriuretic peptide (LANP) consists of the first 30 amino acids of the 126 aa prohormone, (2) vessel dilator (VDL), aa 31–67 of the prohormone, (3) kaliuretic peptide (KP), aa 79–98 of this prohormone, and (4) atrial natriuretic peptide (ANP), consisting of aa 99–126 of the 126 aa prohormone.

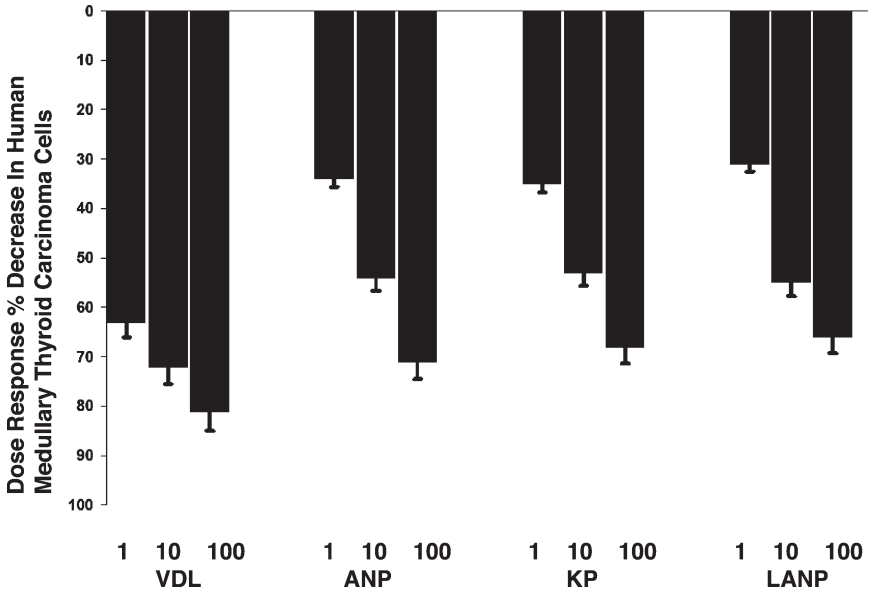

Fig. 2. Dose–response of kaliuretic peptide (KP), atrial natriuretic peptide (ANP), vessel dilator (VDL), and long-acting natriuretic peptide's (LANP) anticancer effects on human medullary thyroid carcinoma (MTC) cells. As each increasing micromolar concentration from 1 to 100 of these four peptide hormones, there was a significant ($p < 0.05$) decrease in medullary thyroid cancer cells within 24 h when evaluated by repeated measures of ANOVA. Vessel dilator caused the same decrease as the other peptide hormones at 10-fold lower concentrations, as observed in this figure ($n = 60$ for each group).

DNA synthesis in human medullary cancer cells. This inhibition of DNA synthesis is the final step in these cardiac hormones' mechanism(s) of action, which includes their ability to inhibit up to 97% of the phosphorylation of extracellular-signal regulated kinase (ERK) 1,2, i.e., cancer growth promoting kinases that translate from the extracellular membrane to the nucleus to promote growth *(22,23)*. This hypothesis is based on these cardiac hormones' effects on non-endocrine cancer cells *(12–18)*. The four peptide hormones synthesized by the cardiac gene were evaluated for their effects on the thyroid cancer with the worst prognosis of the three most common thyroid cancers, i.e., MTC *(1)*. The present investigation was designed to determine if four cardiac hormones can decrease human medullary thyroid carcinoma cell number, inhibit DNA synthesis in human MTC cells, and determine if receptors were present on the cancer cells to mediate atrial natriuretic peptide's effects.

Results

Decreased Number of Human Medullary Thyroid Carcinoma Cells by Four Peptide Hormones Synthesized by the Cardiac Atrial Natriuretic Peptide *Gene*

The number of human medullary thyroid carcinoma (MTC) cells in culture for 24 h decreased 81% (down to 20 ± 2 cancer cells from 109 ± 3 cells), 72%, and 63% secondary to vessel dilator at its 100 μ*M*, 10 μ*M*, and 1 μ*M* concentrations, respectively ($p < 0.0001$ for each) (Fig. 2). Dose–response curves revealed that LANP in culture for 24 h decreased the number of MTC cells 66% (decreased to 37 ±

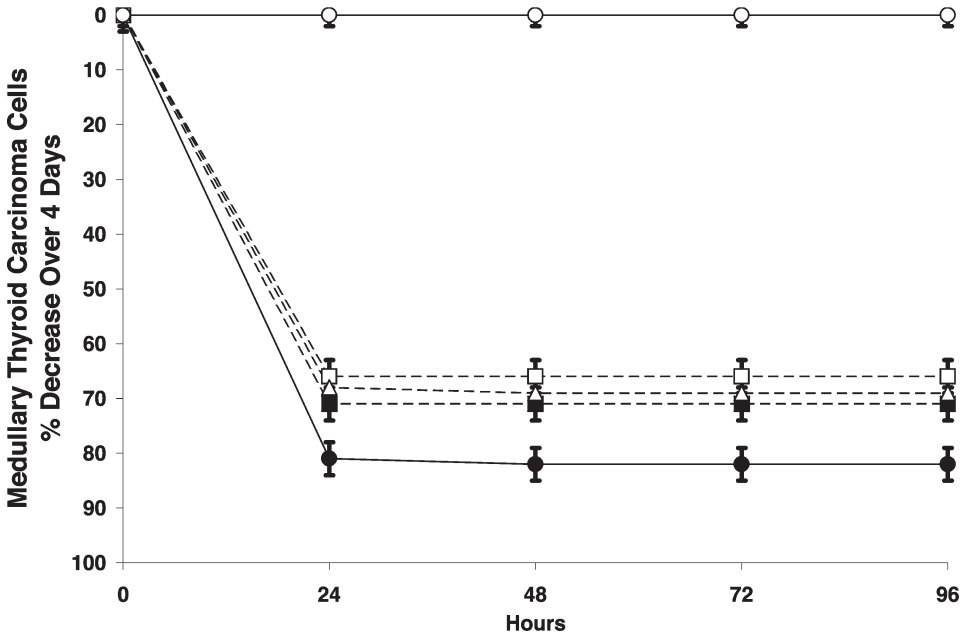

Fig. 3. Time course in decrease of human medullary thyroid cancer cell number with 100 μ*M* concentration of atrial natriuretic peptide (■), kaliuretic peptide (△), vessel dilator (●), and long acting natriuretic peptide (□), at 24, 48, 72, and 96 h were significant at $p < 0.001$ compared to placebo-treated serum-free control (○) medullary thyroid carcinoma cells when evaluated by repeated analysis of variance ($n = 60$ for each group).

2 cancer cells), 50%, and 31% at its 100 μ*M*, 10 μ*M*, and 1 μ*M* concentrations, respectively ($p < 0.001$ for each) (Fig. 2). Exposure of the human MTC cells to kaliuretic peptide resulted in a 68% (35 ± 2 MTC cells), 53%, and 36% decrease at its 1 μ*M*, 10 μ*M*, and 100 μ*M* concentrations, respectively ($p < 0.001$ for each) (Fig. 2). The addition of ANP decreased the number of MTC cells in 24 h by 71%, 54%, and 33% at its 100 μ*M*, 10 μ*M*, and 1 μ*M* concentrations. Thus, with respect to their ability to inhibit the growth of human medullary thyroid cancer when these cells were exposed to identical 100 μ*M* concentrations of these peptide hormones for 24 h was vessel dilator > ANP > kaliuretic peptide > LANP. When the number of medullary thyroid carcinoma cells was examined immediately after incubation with the respective peptide hormones, there was no decrease in the number of MTC cells. In the wells with a decreased number of MTC cells secondary to the cardiac hormones, there was evidence of cellular debris. The serum-free controls at 24, 48, 72, and 96 h were viable without any evidence of necrosis in the serum-free controls.

Decreased Medullary Thyroid Carcinoma Proliferation for 3 d after Initial 24 h Exposure of These Peptide Hormones

When the medullary thyroid cancer cells were followed for 3 d after treatment with vessel dilator, LANP, kaliuretic peptide, and ANP, there was complete inhibition of proliferation of MTC cells at 48, 72, and 96 h after the decrease in the number of the MTC cells at 24 h by the peptide hormones from the cardiac ANP prohormone gene (Fig. 3). Thus, when exposed to vessel dilator for 24 h but without exposure to vessel dilator for the next 24 h, the decrease in number of human MTC cells at 48 h was 82%, 72%, and 63% at 100 μ*M*, 10 μ*M*, and 1 μ*M* of vessel dilator (non-significant difference from the amount of decrease at 24 h). Likewise at 48 h the decrease in MTC cells secondary to kaliuretic peptide was nearly identical to that observed at 24 h with a 69%, 53%, and 36% decrease at 48 h with 100 μ*M*, 10 μ*M*, and 1 μ*M* of kaliuretic peptide (non-significant difference from 24 h). At 48 h, after an exposure to LANP for only 24 h, there was a 66%, 50%, and 31% decrease in MTC cell number at its 100 μ*M*, 10 μ*M*, and 1 μ*M* concentrations. Exposure to ANP for 24 h but without exposure ANP for the next 24 h resulted in no proliferation of MTC cells as there was a 71%, 54%, and 33% decrease in MTC cells with 100 μ*M*, 10 μ*M*, and 1 μ*M* of ANP (non-significant difference comparing these different concentrations 24 h and 48 h). Two days after exposure to the respective peptide hormones (i.e., 72 h) as illustrated in Fig. 3 for 100 μ*M* concentration, there was no proliferation in the remaining MTC cells. Thus, with vessel dilator there was an 82%, 72%, and 63% decrease, while with kaliuretic peptide there was a 69%, 53%, and 35% decrease at 72 h in human medullary thyroid carcinoma cells at their 100 μ*M*, 10 μ*M*, and 1 μ*M* concentrations, respectively. LANP caused a 66%, 50%, and 31% decrease while with ANP there was a 71%, 54%, and 33% decrease in MTC cancer numbers at their 100 μ*M*, 10 μ*M*, and 1 μ*M* concentrations at 72 h.

Three days after exposure to the respective peptide hormones, there was no proliferation of the medullary thyroid cancer cells (Fig. 3). Thus, the MTC cells that had not been exposed to vessel dilator for 3 d had an 82%, 71%, and 63%

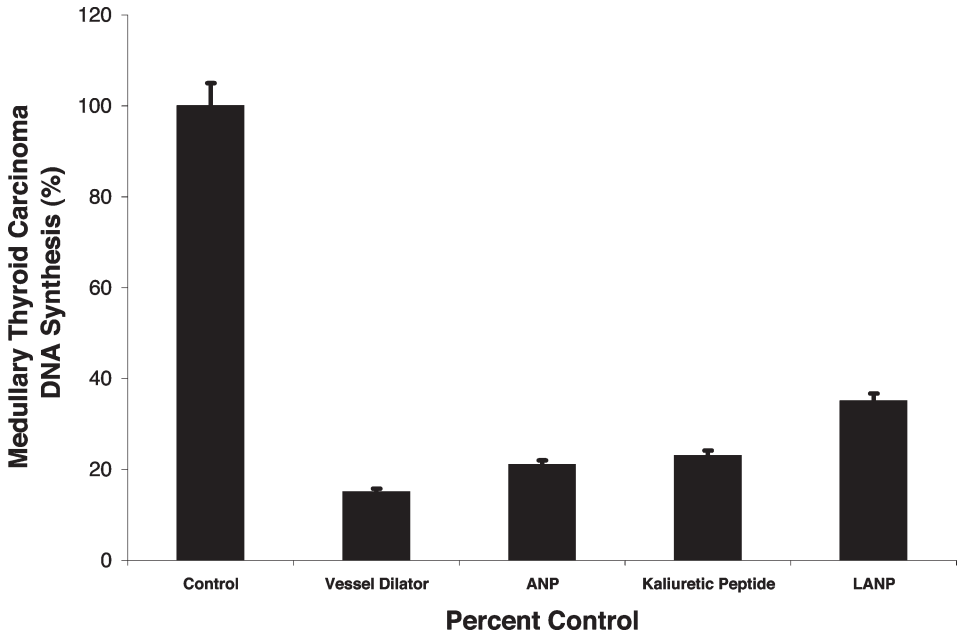

Fig. 4. Decrease in DNA synthesis by atrial natriuretic peptide (ANP), kaliuretic peptide, vessel dilator, and long-acting natriuretic peptide (LANP). The 65–85% decrease in DNA synthesis in the medullary thyroid cancer cells secondary to the four cardiac hormones (each at 1 μ*M*) was significant ($p < 0.001$) compared to control (i.e., untreated) cells when evaluated by repeated measures of analysis of variance (ANOVA) ($n = 30$ for each group).

decrease in cancer cell number compared to control and with kaliuretic peptide there was a 69%, 53%, and 35% decrease. The decrease secondary to LANP and ANP at 96 h was 66%, 50%, and 31% and 71%, 54%, and 33% at their 100 μ*M*, 10 μ*M*, and 1 μ*M* concentrations, respectively. This is a relatively slow-growing cancer in culture as evidenced by the control number of MTC cells increasing 11%, 21%, and 29% at 48, 72, and 96 h compared to 24 h.

Inhibition of DNA Synthesis in Human Medullary Thyroid Carcinoma Cells by LANP, Vessel Dilator, ANP, and Kaliuretic Peptide

To help determine the mechanism of medullary thyroid carcinoma cells decrease in number and cellular proliferation by the above four hormones, the present study investigated if their effects were owing to an inhibition of DNA synthesis. Vessel dilator, LANP, kaliuretic peptide, and ANP each at their 1 μ*M* concentrations inhibited DNA synthesis when incubated with the human medullary thyroid carcinoma cells for 24 h by 85%, 65%, 77%, and 79%, respectively ($p < 0.001$ for each) (Fig. 4).

NPR-A and -C Receptors Are Present in Human Medullary Thyroid Carcinoma Cells

Medullary thyroid carcinoma cells have never been evaluated to determine whether they have NPR-A and/or -C receptors. When the human medullary thyroid carcinoma cells were evaluated by Western blots, the NPR-A and -C receptors were demonstrated to be present (Fig. 5).

Discussion

This investigation is the first evidence of anticancer effects of four peptide hormones synthesized within the heart on any endocrine tumor. Medullary thyroid cancer (MTC) was chosen for this investigation as thyroid cancer is the most common of the endocrine cancers and, of the three most common forms of thyroid cancer, MTC has the worst 10-yr survival *(3)*. Medullary thyroid cancer is of further interest as this neuroendocrine cancer is metastatic in 80% when first diagnosed if the MTC is larger than 1 cm *(5,6)* and is metastatic in approx 50% of all sporadic MTC *(4)*. This knowledge indicates that there is a strong need for adjunct anticancer therapy in this endocrine cancer. Radiotherapy is utilized for MTC but there is no evidence it has any effect on survival *(7)*. Targeted radiotherapy utilizing radiolabeled somatostatin analogs *(8,9)* and radiolabeled MIBG *(11)* have limited success in treating medullary thyroid cancer *(3)*. There is, likewise, scant evidence that Octreotide®, a somatostatin analog currently used in clinical practice, reduces medullary thyroid carcinoma tumor mass or improves its survival rate *(24,25)*. Thus, the four cardiovascular hormones that eliminate up to 82% of the medullary thyroid carcinoma cells in 24 h may be useful adjunct(s) to surgery to treat medullary thyroid carcinomas.

Vessel dilator was the most potent of these peptide hormones in decreasing the number of human medullary thyroid carcinoma cells at each of the respective concentrations of the peptide hormones (Fig. 2). In the dose–response curves

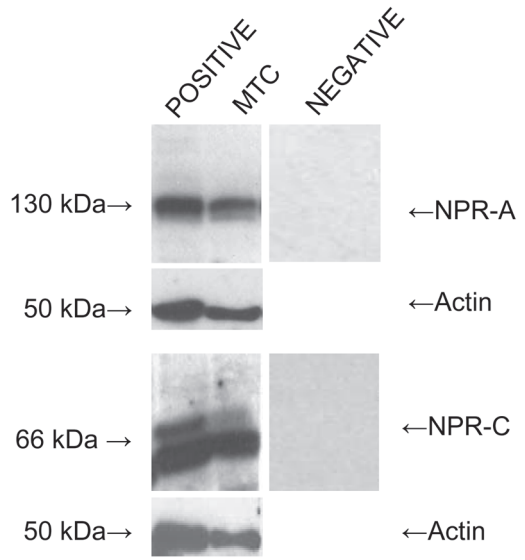

Fig. 5. Natriuretic peptide receptors (NPR)-A, and -C are present in human medullary thyroid cancer (MTC) cells. Western blot analysis with a 1:4000 dilution of R1214 polyclonal antibody directed against the COOH terminus of the natriuretic peptide A receptor (kindly provided by Dr. David L. Garbers, University of Texas Southwestern, Dallas, TX) and a 1:1000 dilution of Omori antibody to the NPR-C receptor (generously provided by Dr. Kenji Omori, Osaka, Japan). The left side blots are the positive controls for each of the receptors. The NPR-A receptor in human MTC cells is in the upper panel at 130 kDa. The lower panel blot demonstrates the NPR-C receptor at 66 kDa in the human MTC cells. The negative controls are in the right panel of this figure. Albumin (bovine serum albumin, BSA) (70 kDa) was used in addition to BIO RAD Precision Plus Protein Dual Color standards to identify the bands corresponding to the NPR-A and -C receptors, respectively. Re-probing with actin was used as a loading control. The MTC cells for receptor analysis were scraped from 100 mm dishes in ice-cold mammalian protein extraction reagent (M-PER; Pierce; Rockford, IL) containing Halt™ phosphatase inhibitor (Pierce) and Halt™ protease inhibitor (Pierce).

of the present investigation, when vessel dilator concentration was increased 10-fold and 100-fold (i.e., 10 μ*M* and 100 μ*M*), vessel dilator decreased the number of human medullary thyroid carcinoma cells by 72% and 81%, respectively, within 24 h compared to 63% decrease at its 1 μ*M* concentration (Fig. 2). Vessel dilator also eliminated other cancer cells in vitro the most *(12–18)*. This information plus the knowledge that vessel dilator decreases the tumor volume of human pancreatic adenocarcinomas the most in vivo *(19)* suggests that vessel dilator has the most significant anticancer properties of the four peptide hormones with anticancer effects in the present investigation. At each 10-fold increase in concentration of the respective peptide hormones in the present investigation (Fig. 2), vessel dilator's anticancer effects on human medullary thyroid cancer cells were more significant than the other four peptides ($p < 0.05$). This was especially apparent at their respective 1 μ*M* concentrations where vessel dilator decreased the number of MTC cells by more than double the amount of decrease secondary to the other peptide hormones (Fig. 2).

The remaining three peptide hormones synthesized by the cardiac ANP gene, however, had significant effects on decreasing the number of human medullary thyroid cancer cells. When the concentration of kaliuretic peptide, ANP, and LANP were increased to 100 μ*M*, they caused a very significant 66–71% decrease ($p < 0.001$) in the number of MTC cells within 24 h. There appears to be a difference in these peptide hormones ability to decrease cancer cell number depending on the type of cancer. For example, kaliuretic peptide's (1 μ*M*) ability to decrease the number of human MTC cells (36% decrease) is more than most other cancers with a 30% decrease in prostate adenocarcinoma *(13)* and small-cell lung cancer cells *(16)* but its effects in the present investigation are similar to its effect on human pancreatic adenocarcinoma cells (37% decrease) *(12)*. It is important to note that after 24 h of incubation with the four peptide hormones that cellular debris was present, suggesting that cellular necrosis was occurring. There was no necrosis in the serum-free cells that were viable at 24, 48, 72, and 96 h. Three days after no further exposure to these peptide hormones, the human medullary thyroid carcinoma cells did not to proliferate suggesting that these peptide hormones may need to be given only every 5 d or longer to stop proliferation of the remaining MTC cells after their initial elimination of up to 82% of the medullary thyroid cancer cells. This 1-d treatment had similar results to giving these peptide hormones continuously to human cancer cells for 4 d, where there is also no proliferation of cancer cells *(12–18)*. These peptide hormones' anticancer effects are at concentrations above concentrations at which they normally circulate in the human body. The circulating concentrations at healthy humans of LANP, vessel dilator, kaliuretic peptide, and ANP are 1528 ± 158 pg/mL, 1595 ± 157 pg/mL, 213 ± 42 pg/mL, and 63 ± 2 pg/mL, respectively *(26)*. The anticancer effects of these peptide hormones are, thus, at pharmacological rather than physiological concentrations.

Similar doses to those utilized in the present investigation have been used to treat human pancreatic adenocarcinomas in athymic mice with no side effects observed in these in vivo experiments *(19)*. With respect to the possibility to treat human medullary thyroid cancers with these peptide hormones as an adjunct therapy, it is important to note that similar doses to those utilized in the present investigation have been given to 100 humans, i.e., 50 healthy humans *(27)* and 50 persons with congestive heart failure *(28)*, and there were no side effects and, in particular, no hemodynamic side effects with vessel dilator, kaliuretic peptide, or long-acting natriuretic peptide *(27,28)*. Atrial natriuretic peptide caused hypotension in one healthy person and in one person with congestive heart failure *(27,28)*, which may make it less desirable than the other cardiovascular hormones for treating persons with cancer.

With respect to the mechanism and specificity of these cardiac hormones for the treatment of cancer(s), their mechanism of action after binding to their respective receptors involves their ability to inhibit up to 97% of extracellular signal regulated kinase (MAP kinase) important for the growth of cancers *(22,23)*. Membrane-associated Ras activates the Raf-mek 1/2–ERK 1/2 kinase cascade, and this cascade is specifically blocked by the four cardiac hormones of the present investigation *(22,23)*. Their ability to inhibit the phosphorylation of ERK 1/2 is specifically mediated by the intracellular mediator cyclic GMP as when one adds a cyclic GMP antibody these peptide hormones are unable to inhibit the phosphorylation of ERK 1/2 *(22,23)*. The final step in this kinase cascade is the stimulation of DNA synthesis within the nucleus *(22,23)*. As demonstrated in the present investigation these peptide hormones are strong inhibitors of DNA synthesis in medullary thyroid cancer as the final step in their mechanism of action. Furthermore, it is important to note these peptide hormones have been demonstrated to localize to the nucleus of cancer cells where they can inhibit DNA synthesis *(29)*. Their ability to inhibit DNA synthesis in cancer cells is also specifically mediated by cyclic GMP as demonstrated with a cyclic GMP antibody completely blocking their effects on DNA synthesis *(15)*. Each of the four peptide hormones from the cardiac ANP prohormone inhibited 65–85% of the amount of DNA synthesis in the human medullary thyroid carcinoma cells. With respect to their specificity of inhibiting DNA synthesis, we have previously demonstrated that the DNA synthesis–inhibiting properties of these peptide hormones synthesized by the cardiac *ANP* gene are directly due to the peptide hormones themselves as, when their specific antibodies are incubated with the peptide hormones, the antibodies completely block these peptide hormones' ability to decrease cancer cell number and DNA synthesis *(13)*. The antibodies by themselves did not block DNA synthesis *(13)*. Further evidence that these peptide hormones' effects are specific to these peptide hormones and not to peptides in general is that brain natriuretic peptide (BNP), a peptide hormone also made in the heart with identical amino acids in an identical ring structure with ANP but different amino acids outside the ring structure, has no anticancer effects when utilized in 100-fold higher concentration than those utilized in the present investigation with medullary thyroid cancer cells *(14,18)*.

With an estimated 1490 thyroid cancer deaths in 2005 with surgery, current cancer chemotherapy, Octreotide®, and radiation *(2)* and the knowledge that up to 80% of medullary thyroid carcinomas have metastasized at the time of diagnosis *(4–6)*, there is definite need to develop new approaches for the therapy of medullary thyroid cancer. The present investigation details not only one but four new potential therapies, i.e., four peptide hormones that kill up to 82% of human medullary thyroid cancer cells within 24 h. The four human cardiac hormones that circulate normally in the human body *(26,30–36)* have no known cytotoxic effects to normal cells *(12)*. Present use of chemotherapy commonly causes toxicity in the form of nausea, vomiting, alopecia, and myelosuppression. None of these toxicities occur with the cardiac peptide hormones *(12,27,28,35,36)*. In conclusion, this investigation demonstrates that four cardiac hormones can eliminate up to 82% of human medullary thyroid cancer cells within 24 h with no further proliferation of these cells in the 3 d following treatment. This decrease in medullary thyroid cancer cells was mediated in part by these peptide hormones' ability to decrease DNA synthesis 65–85% in these neuroendocrine cancer cells.

Materials and Methods

Materials

The peptide hormones used in this investigation were from Phoenix Pharmaceuticals, Inc., Belmont, CA.

Human Medullary Thyroid Carcinoma Cells

Human medullary thyroid carcinoma cells (ATCC number CRL-1803) were purchased from American Type Culture Collection (ATCC, Manassas, VA). This MTC cell line was deposited by S. S. Leong. This medullary thyroid carcinoma cell line from a 77-yr-old woman is also called the TT cell line and produces calcitonin, somatostatin, and carcinoembronyic antigen (CEA) *(37)*.

Culture of the Human Medullary Thyroid Carcinoma Cells

Propagation of these human MTC cells was in Ham's F12K medium with 2 m*M* L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 90%, and 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at a temperature of 37°C in a 5% CO_2 environment as recommended by the ATCC. Cells were dispensed in new flasks with subculturing two to three times per week.

Research Protocol

After the medullary thyroid carcinoma cells were subcultured for 24 h, they were then seeded to coverslips in 24-well plates (Nunclon™, Roskilde, Denmark) with 1 mL of the above media. There were 65,000 cells seeded to each coverslip. After 24 h, the well plates were washed twice with phosphate-buffered saline to remove the fetal bovine serum. Removal of serum was carried out to completely remove all variables (EGF, etc.) present in serum in order that interpretation of any data obtained would be straightforward. After 24 h of serum deprivation, media volume was reduced to 250 μL per well with, or without, i.e., serum-free, controls, the respective peptide hormones in dose–response curves with concentrations up to and including 100 μ*M* (1% of this volume). Human MTC cells were then incubated for various periods of time (24,48,72, and 96 h). The number of MTC cells were then counted with a cell counter (Thomas Scientific®, Swedesboro, NJ) evaluating 10 fields of the microscope slide at ×40 along the *X*-axis with an Olympus BH-2 microscope (Atlanta, GA). This

evaluation was repeated on six separate occasions with the number of human medullary thyroid carcinoma cells reflecting 60 observations for each group, i.e., 60 observations for controls and 60 observations for each of the four groups with respective peptide hormones. In the Results section, the number of cancer cells reported is the number of cells in each individual field. Ten fields were examined on each microscope slide. The results of the 10 fields were pooled and the average of the 10 fields is illustrated in the Results section.

Determination of DNA Synthesis

To investigate whether these peptide hormones were inhibiting DNA synthesis, bromodeoxyuridine (BrdU) incorporation *(38–41)* into the human medullary thyroid carcinoma cells was utilized as previously described from our laboratory *(12–18)*. BrdU was from BD Bioscience, San Jose, CA. After 24 h in culture with 1 μ*M* of LANP, vessel dilator, kaliuretic peptide, and ANP, respectively, or with no peptide hormone (i.e., control), BrdU in a final concentration of 10 μ*M* in the cell culture medium was added for 45 min, which is the time in which the cells are in the logarithmic phase of cell proliferation. For immunohistochemistry, a BrdU *in situ* detection kit (Becton Dickinson Immunocytochemistry Systems, San Jose, CA) was utilized. Incorporation of the BrdU stain into the nucleus was counted using a Nikon Inverted Diaphot-TMD Microscope (Tokyo, Japan). The number of stained nuclei were compared in the four peptide hormone groups with the positive control. The negative control for these studies was provided by Becton Dickinson Immunocytochemistry Systems.

ANP Receptors in Human Medullary Thyroid Carcinoma Cells

Medullary thyroid carcinomas have never been examined to determine if they have natriuretic peptide hormone receptors. When it was found that these ANPs decreased the number of human MTC cells, it was then evaluated whether medullary thyroid carcinoma cells have ANP receptors to mediate these effects. Western blots of the natriuretic peptide receptors (NPR)-A, and -C were performed as follows.

Western Blotting

Seventy-five micrograms of protein extract from MTC cells, measured by using the bicinchoninic acid (BCA) protein assay kit (Pierce; Rockford, IL), were loaded onto each lane of a discontinuous 7.5% Tris-HCl SDS-PAGE gel (Bio-Rad; Hercules, CA), separated by electrophoresis for 120 min at 100 V, and then transblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) for 75 min at 100 V in Towbin buffer. Blots were blocked for 1 h at room temperature in a 5% solution of dry milk, washed with Tris-buffered saline, and then incubated for 1 h in a 5% solution of bovine serum albumin (Fraction V; Sigma, St. Louis, MO) in Tris-buffered saline that contained a 1:4000 dilution of R1214 polyclonal antibody directed against the COOH terminus of the NPR-A receptor protein (generously provided by Dr. David L. Garbers, University of Texas Southwestern, Dallas, TX) or a 1:1000 dilution of Omori antibody to the NPR-C receptor (kindly provided by Dr. Kenji Omori, Osaka, Japan). After being washed with Tris-buffered saline, the membranes were incubated for 1 h at room temperature in a solution of dry milk with a 1:3000 dilution of horseradish peroxidase–conjugated goat anti-rabbit IgG antibody (Bio Rad). After washing with Tris-buffered saline, the bands were identified by Super Signal West Pico^{TM} chemiluminescent substrate (Pierce) and visualized in a luminescent image analyzer (model LAS-1000; Fujifilm, Tokyo, Japan). Specificity was revealed by the presence of a signal in human pancreatic cancer (PANC) (positive control) and absence of a signal with normal rabbit serum, rabbit IgG, and after preabsorption of the NPR-A antibody with NPR-A protein or preabsorption of the NPR-C antibody with NPR-C protein. Re-probing with actin (Sigma, St. Louis, MO) was used as a loading control.

Statistical Evaluation

The data obtained in this investigation are illustrated as mean ± SEM. Maximum changes in cell death (evaluated six times with 10 areas of microscope slide evaluated each time) and DNA synthesis within groups were determined by repeated measures of analysis of variance (ANOVA) evaluated with statistical module of Excel software. To be considered statistically significant, we required a probability value to be < 0.05 (95% confidence limits).

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