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Elimination of Up to 80% of Human Pancreatic Adenocarcinomas in Athymic Mice by Cardiac Hormones

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Abstract. *Background: Four cardiac hormones have anticancer effects in vitro: i) atrial natriuretic peptide (ANP), ii) vessel dilator, iii) long acting natriuretic peptide (LANP), and iv) kaliuretic peptide. Materials and Methods: These cardiac hormones were infused subcutaneously for 28 days with weekly fresh hormones at 3 nM min^{-1} kg^{-1} body weight in athymic mice bearing human pancreatic adenocarcinomas. Results: ANP, vessel dilator, LANP and kaliuretic peptide eliminated 80%, 33%, 20% and 14% of the pancreatic adenocarcinomas. Even in the treated animals which did not have a total cure, their tumor volume decreased to less than 10% (and with vessel dilator to 2%) of that of the untreated animals. The natriuretic peptide receptor (NPR)-A receptor was decreased 33% to 55% in the metastatic lesions compared to the primary pancreatic adenocarcinoma. Conclusion: Four cardiac hormones eliminated up to 80% of human pancreatic adenocarcinomas in athymic mice.*

Human pancreatic adenocarcinomas have the lowest median survival of all common cancers (1, 2). The median survival is 4 months (1, 2). Surgery and current cancer chemotherapy prolong survival by a few months but the above-mentioned median survival rate of four months is for patients treated with surgery and/or currently available cancer chemotherapeutic agents (1, 2).

A family of peptide hormones are synthesized within the heart and stored in the atrial myocyte as prohormones for rapid release in response to stimuli (3, 4). One gene in the heart of this peptide hormone family synthesizes a 126-amino acid (a.a.) prohormone which contains four cardiac peptide hormones consisting of a.a. 1-30 (long acting natriuretic peptide, LANP), a.a. 31-67 (vessel dilator, VDL), a.a. 79-98 (kaliuretic peptide, KP) and a.a. 99-126 of this 126 a.a. prohormone (atrial natriuretic peptide, ANP) (3-5) (Figure 1). These peptide hormones have significant anticancer effects on human pancreatic, breast, colon, kidney and prostate adenocarcinomas (6-10), as well as small-cell and squamous cell lung carcinoma cells *in vitro* (11, 12). The present *in vivo* investigation was designed to determine whether these peptide hormones can eliminate human pancreatic adenocarcinomas in athymic mice when infused subcutaneously for 28 days with the peptide hormone being refreshed weekly.

Abbreviations: ANP, atrial natriuretic peptide; LANP, long acting natriuretic peptide; KP, kaliuretic peptide; VDL, vessel dilator.

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Materials and Methods

Human pancreatic adenocarcinoma cells. A cell line [American Type Culture Association [ATCC, (Manassas, VA, USA) number CRL-2119] of human pancreatic adenocarcinoma cells (HPAC) was purchased from the ATCC. This pancreatic adenocarcinoma cell line was derived in 1994 from a nude mouse xenograft of a primary tumor removed from the head of the pancreas of a 64-year-old Caucasian female patient in our laboratory (13).

Culture of pancreatic adenocarcinoma cells for tumor formation in vivo. Propagation of these cells was in Dulbecco's modified Eagle's medium supplemented with FBS 10%, at a temperature of 37˚C, as recommended by the ATCC. Cells were dispensed into new flasks with subculturing every 6-8 days. The growth medium was changed every three days. Only single cell suspension with a viability of >90% was used for the injections. Cell pellets were resuspended in saline prior to injections.

Animals.

Human pancreatic adenocarcinomas in the athymic mice. Homozygous (nu/nu) athymic nude mice from the National Cancer Institute (NCI) were used for these studies. Twenty-gram mice were given subcutaneous injections of $1x10^6$ of human pancreatic

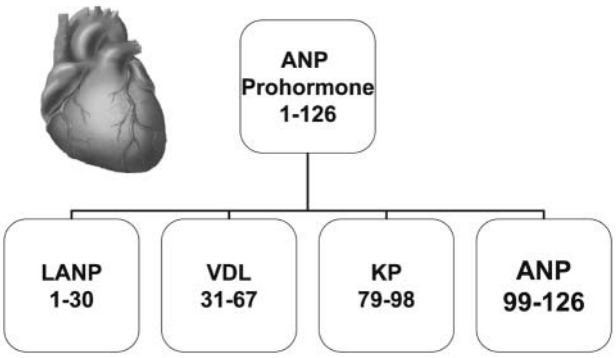

Figure 1. *The atrial natriuretic peptide gene in the heart synthesizes a 126 amino acid (a.a.) prohormone with which proteolytic processing results in the formation of four cardiac hormones. These four cardiac hormones, long acting natriuretic peptide (LANP) consist of the first 30 amino acids of the 126 a.a. prohormone, vessel dilator, a.a. 31-67 of the prohormone, kaliuretic peptide, a.a. 79-98 of this prohormone and atrial natriuretic peptide (ANP), consisting of a.a. 99-126 of the 126 a.a. prohormone. This figure is reproduced with permission from Anticancer Res 26: 3217-3222, 2006.*

adenocarcinoma cells in 250 μl of phosphate-buffered saline, pH 7.4 on the left side of the back of the mice with ketamine 80 mg/kg and xylazine 10 mg/kg body weight intraperitonealy as anesthesia. Tumor growth was followed by electronic digital Vernier caliper measurements every day with tumor volume recorded daily (14). Tumor volume was calculated by the formula $V=(a \times b^2)/2$ where a = largest superficial diameter and b = the smallest superficial diameter (14).

Research protocol. The injected adenocarcinoma cells coalesced into well-defined tumors of at least 1 mm x 1 mm (volume = 0.5 mm^3) in approximately 21 days. Osmotic pumps (Alzet Model 1007D, Duret Corporation, Cupertino, CA, USA) containing either 0.9% saline (control infusion) or one of the respective hormones in 0.9% saline, as previously described from our laboratory (15), were implanted subcutaneously under anesthesia in the upper back of the athymic mice after three independent investigators having confirmed that a tumor was present. All of the peptide hormones for these experiments were synthesized by Phoenix Pharmaceuticals Inc., Belmont, CA, USA. The Alzet Model 1007D osmotic pump for mice delivers all of its contents (100 μl) over 7 days at a rate of 0.5 μl/h and then stops pumping. A 1 mM concentration of these cardiac hormones *in vitro* eliminates up to 97% of cancer cells *in vitro* within 24 hours (8, 9). When a 1 mM concentration in the osmotic pump is infused over seven days the concentration per kg body weight/minute is 3 nM/kg/body weight/min or 99 nM/30 gram mouse/minute. This research protocol was approved by the Institutional Animal Care and Use Committee of the University of South Florida and the James A. Haley Veterans Medical Center and followed the "Guiding Principles for Research Involving Animals and Human Beings".

Natriuretic peptide A- and C-receptors in primary versus metastatic pancreatic adenocarcinomas. When some of the human pancreatic adenocarcinomas become metastatic in the present investigation, we hypothesized that the metastatic lesions may have mutated losing their NPR-A and/or C-receptors in order that they could no longer respond to ANP. NPR-A and C-receptors were evaluated by Western blotting as previously described from our laboratory (7-12) with the following modifications for evaluation of solid tumors versus evaluation of individual cancer cells.

Western blot analysis of primary and malignant human pancreatic adenocarcinomas. Briefly, 50-100 mg pieces of human pancreatic adenocarcinomas were homogenized and then 50 μg of protein from the pellet and supernatant fractions were loaded onto separate lanes of a Criterion Precast 7.5% Tris-HCl gel (Bio-Rad; Hercules, CA, USA). The proteins were separated by SDS-PAGE (25 volts for 8 hours) and then electrically transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences Corporation, Piscataway, NJ, USA) for 75 minutes at 100 Volts in Towbin buffer followed by Ponceau S staining to verify transfer, as previously described from our laboratory (7-12). The membranes were incubated overnight at 4˚C with gentle rocking and with a 1:4000 dilution of R1214 polyclonal antibody directed against the COOH terminus of the NPR-A receptor (generously provided by Dr. David L. Garbers, University of Texas Southwestern, Dallas, TX) or with a 1:1000 dilution (prepared in 5% BSA 1X TBS) of Omori polyclonal antibody to the NPR-C receptor (kindly provided by Dr. Kenji Omori, Osaka, Japan). The blot was washed four times with 1X TBS and then incubated for 1 hour at room temperature with moderate shaking in a solution of 5% nonfat dry milk goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA, USA) at a dilution of 1:7000 for NPR-A or 1:3000 for NPR-C. The immunoreactive bands were then detected by using the Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) according to instructions by the manufacture. Precision plus protein dual color standards were used to identify bands corresponding to the NPR-A and NPR-C receptors.

Densitometric analysis of immunogenic bands. Densitometry of immunogenic bands was performed using the ImageQuant software version 5.2 (Molecular Dynamics, Sunnyvale, CA, USA). The abundance of NPR-A and NPR-C protein in the metastatic tumors was determined relative to that of primary tumor.

Statistical analysis. Univariate statistics (percent of treated mice or tumor volume) were calculated to describe the ability of the cardiac hormones to reduce or eliminate the growth of human pancreatic adenocarcinomas. To test whether the observed differences between groups over time were statistically significant we employed linear mixed (growth curve) models as described by Verbeke (16). The dependent variable for the analysis were mean tumor volume. The independent variables for the analyses were group membership, which had five levels (4 experimental and 1 control groups), time, modeled as a continuous random variable, and group by time interaction term. This group by time interaction term compares the slope of means of the dependent variable by group across the follow-up period. In our first model we compared results from the experimental groups with the control group. We followed this analysis with a second model, excluding the control group, to investigate whether the mean tumor volume was different over time for the experimental groups. All models employed restricted maximum likelihood estimations (REML). Fit statistics were compared for models using several covariance structures and the

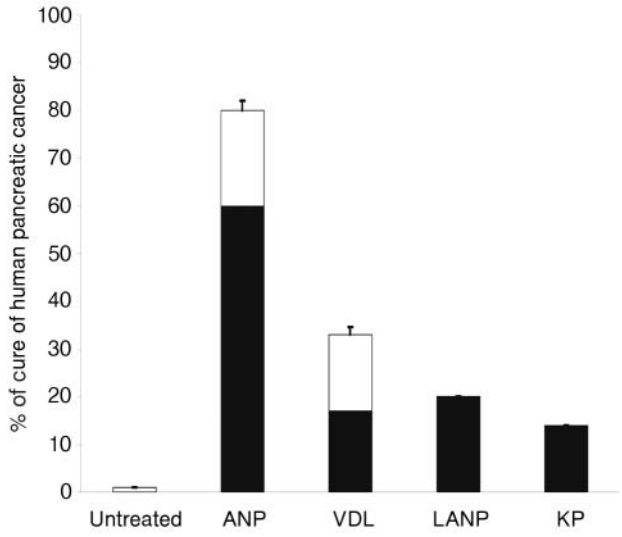

Figure 2. *Complete elimination of human pancreatic adenocarcinomas with cardiac hormones. The total bar graph for each peptide hormones indicates the percent of complete elimination of the primary human pancreatic adenocarcinomas. The white bar inside the larger bar reveals the percent treated mice who had the primary lesion never recur but developed a second lesion i.e., metastatic lesion. The ANP-treated mouse with a metastatic lesion responded with the addition of a vessel dilator. Atrial natriuretic peptide = ANP, long acting natriuretic peptide = LANP, vessel dilator = VDL, and kaliuretic peptide = KP. The complete elimination of pancreatic cancer with ANP and vessel considered dilator were considered significant at $p<0.001$, while LANP and kaliuretic peptide were significant at $p<0.05$ compared to all the treated pancreatic adenocarcinomas in their groups when evaluated by ANOVA with repeated measures design for within group comparisons with fisher's LSD as a post-hoc test.*

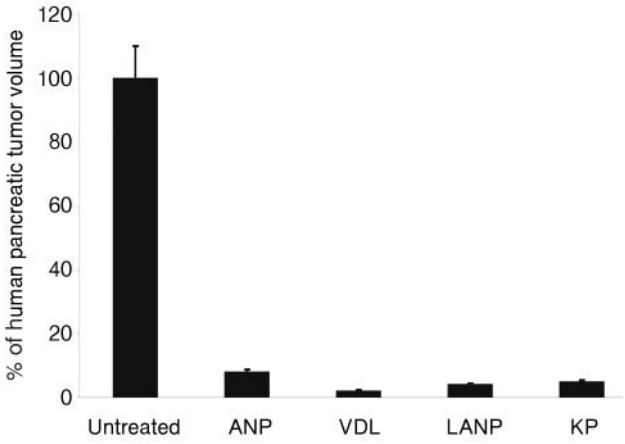

Figure 3. *Treatment with cardiac hormones decrease the volume of all human pancreatic adenocarcinomas by 92 to 98% compared to untreated human pancreatic adenocarcinomas. The decrease in tumor volume of the human pancreatic adenocarcinomas by vessel dilator (VDL, 98% decrease in volume), long acting natriuretic (LANP, 96% decrease), kaliuretic peptide (KP, 95% decrease) and atrial natriuretic peptide (ANP, 92% decrease compared to untreated pancreatic cancers' volume) were each was considered significant at $p<0.001$ after comparing the tumor volume of the untreated pancreatic adenocarcinomas evaluated by repeated measures of ANOVA.*

structure with the best fit (first-order autoregressive) was retained. All statistics analyses were conducted using SAS version 9.1. In all cases, $p<0.05$ was considered the criterion for statistical significance.

Results

The four cardiac hormones as a group eliminated the human pancreatic adenocarcinomas growing in 35% of the athymic mice. Thus, each of the four cardiac peptide hormones had the ability to completely eliminate some of the human pancreatic adenocarcinomas growing *in vivo*. In the present investigation, ANP had the most consistent anticancer effects, eliminating the primary human pancreatic adenocarcinomas in 80% of the treated mice (Figure 2). Vessel dilator caused the human pancreatic adenocarcinomas to disappear in 33% of the athymic mice while long acting natriuretic peptide (LANP) and kaliuretic peptide caused the human pancreatic adenocarcinomas to be eliminated in 20% and 14% of the mice, respectively, (Figure 2). There was not a single incidence of the adenocarcinomas recurring in the primary site when the cancer was eliminated by any one of these cardiac hormones.

Although the rest of the athymic mice harboring human pancreatic adenocarcinomas did not have a total cure, all of the animals treated with cardiac hormones had tumor reduction to less than 10% of the tumor volume of the untreated animals (Figure 3). Excluding the animals which had a complete response with elimination of the human pancreatic cancers, *i.e.*, as these animals which were obviously better than the untreated animals and would thereby decrease the overall mean tumor size of the treated groups, the increase in tumor size at the end of the treatment period for the groups which did not have a complete response was 1.6-fold, 1.2-fold, 2.1-fold and 2.9-fold for vessel dilator, kaliuretic peptide, LANP and ANP, respectively, compared to 300-fold increase in tumor volume in the untreated mice (n=30). Results of the linear mixed model analysis of these data found that the slopes of the mean tumor volume in the experimental groups over the follow-up period were statistically significantly lower than that of the control mice ($p<0.0001$). Results of the second linear model, including only the experimental groups, found that the mean slope of tumor volume was statistically significantly lower for the ANP group than the other three experimental groups ($p<0.001$).

When the treated animals were euthanized the volume of the tumors had increased 8-fold in the ANP animal which did not have a complete response, 2-fold in the vessel dilator group of incomplete responders, 5-fold in the kaliuretic peptide incomplete responder group and 4.4-fold

Table I. *Cardiac hormones stop the growth and/or eliminate human pancreatic adenocarcinomas in vivo.*

Volume (mm^3) of tumor	Treatments/(Weeks)												
	0	1	2	3	4	5	6	7	8	9	10	11	12
ANP													
1.	9.3	0	0	0	0	0	0	0	0	0	0	0	0
2.	30.1	5.5	0	0	0	0	0	0	49.8*	11.2	0	0	0
3.	14.2	0	5.3	0	0	0	0	0	0	0	0	0	0
4.	1.2	0	0	12.5	0	0	0	0	0	0	0	0	0
5.	17.9	14.3	35.3	29	51.9	123.5	135.6	147.2	152.6	98.8	62.1	–	–
Mean	15	4	20	15	10	25	27	29	41	22	12	0	0
Vessel dilator													
1.	0.3	3.1	1.3	6	0	0	0	0	0	0	0	0	0
2.	16.7	17.9	0	0	0	0	0	38.1#	37.8	75.3	113	208	121
3.	2.7	3.9	16.4	25.1	14.7	23.2	40.3	18.8	–	–	–	–	–
4.	217	51.3	53.8	59.1	100.4	141.9	183.2	311	297	–	–	–	–
5.	14.5	34	19.8	37.5	45.1	73.8	79	178	58.9	–	–	–	–
6.	2.9	13	49.5	174.7	226.7	172.2	317.2	305	–	–	–	–	–
Mean	42	21	24	50	65	69	103	142	99	38	57	104	61
Kaliuretic peptide													
1.	2.5	1	1	0	0	0	0	0	0	0	0	0	0
2.	12.7	8.8	4.3	6.5	15.9	23.7	49.1	94	9.6	–	–	–	–
3.	165	9.7	11.1	20.6	27.4	42.4	118.7	136.3	–	–	–	–	–
4.	21	0	5.5	24.2	25.7	39.5	29.7	112	28	32.4	–	–	–
5.	17	48	74	68	100	182	143	204	135	–	–	–	
6.	5.2	4.2	0	0	0	11.4	104.1	228.3	200.7	–	–	–	
7.	7.5	61.8	47.5	44.2	101.6	178.8	318.7	388.8	–	–	–	–	
Mean	33	18	20	23	39	68	109	166	78	0	0	0	
LANP													
1.	14.6	0	0	5.4	0	0	0	0	0	0	0	0	
2.	121.7	46.7	64.4	34	87	119	130.2	106	–	–	–	–	
3.	15.3	19.6	13.7	36	44	89	104.8	147.5	–	–	–	–	
4.	21.8	8.5	57.8	123.6	154.9	231.5	219.8	214.2	–	–	–	–	
5.	2.8	5.2	31.1	73.9	60.6	157.9	205.7	241.8	–	–	–	–	
Mean	88	16	33.4	54.6	69.3	119.5	132.1	141.9	0	0	0	0	
Untreated (n=30)													
Mean	2	35	124	309	539	1003	1307	1783	2351				

ANP: atrial natriuretic peptide; LANP: long acting natriuretic peptide; –: mouse euthanized and autopsy performed; Weeks: beginning with start of infusion for four weeks followed by eight weeks post-infusion. "0" weeks represent the baseline size of the tumor prior to infusion of the respective peptide hormones. * 4-week ANP infusion begun when metastatic lesion appeared. #4-week infusion of vessel dilator begun when this metastatic lesion appeared.

in the LANP treated group of incomplete responders. During the same period of time the human pancreatic adenocarcinomas which were untreated (n=30), increased almost 1000-fold. There was, thus, a dramatic slowing of the growth and decrease in tumor volume of all the human pancreatic adenocarcinomas treated with these peptide hormones, with the volume of these tumors being less than 10% of the tumor volume of the animals not treated with these cardiac hormones (Figure 3). The volume of the pancreatic adenocarcinomas in the vessel dilator treated animals was only 2% of the tumor volume of the untreated mice (Figure 3). There was not a single-treated animal which had a tumor volume that was 10% or more of the tumor volume of the untreated age-matched animals when followed for exactly the same period of time.

One animal treated with ANP (#2 in Table I) seven weeks after elimination of the primary human pancreatic cancer developed a new lesion on the opposite side of the body. This metastatic lesion provided an opportunity to test the hypothesis that treating this metastatic lesion with a different cardiac hormone which works through a different receptor(s) from ANP (18-20) could eliminate the metastatic lesion.

Vessel dilator treatment decreased the volume of this lesion in half in one week. After two weeks of vessel dilator treatment this metastatic lesion completely disappeared and never recurred (Table I). In a further evaluation of this hypothesis, one animal treated with vessel dilator (vessel dilator animal #2 is Table I) had a large human pancreatic adenocarcinoma on its upper back completely disappear after two weeks of treatment and the pancreatic adenocarcinoma never returned in the primary site. This animal, however, developed a metastatic pancreatic cancer lesion on the opposite side of the body in the flank region six weeks after the adenocarcinoma in the primary site had been eliminated. This new lesion was then treated with ANP to determine if a peptide whose effects are medicated through a different receptor than vessel dilator might eliminate this lesion. The second lesion stopped growing at first with ANP treatment, but then began to grow and was not eliminated.

In the majority of mice with this aggressive human pancreatic cancer, which increased 1300-fold in volume in two months when untreated (n=30), there was no evidence of metastasis at necropsy of the animals treated with these cardiac hormones. A kaliuretic peptide-treated animal #7 (Table I) did have metastasis to the abdominal wall and into the liver in addition to its primary human pancreatic adenocarcinoma that was not eliminated in its upper back. It was hypothesized that the metastatic lesions had lost one or more of their natriuretic peptide receptors (NPR)-A and -C, which allowed the metastatic lesions to grow.

NPR-A and C-receptors in primary and metastatic pancreatic cancers. The reasoning for the hypothesis to study the NPR receptors in the primary and metastatic cancer tissues is based upon the knowledge that the more aggressive breast cancers are the ones that have lost their estrogen/progesterone receptors and with respect to the human pancreatic cancers, the more aggressive human pancreatic adenocarcinomas may have lost their receptors for ANP in the metastatic lesions to allow them not to respond to these peptide hormones. When the primary lesion and metastatic lesions in the abdominal wall and liver were examined by Western blots, the NPR-A and -C receptors were demonstrated to be present in the primary lesion but the NPR-A or active receptor was markedly reduced in the metastatic abdominal (33% less) and liver (less than 55% compared to primary lesion) (Figure 4).

Side-effects. There was no evidence of any side-effects with the peptide hormones *in vivo*. The animals were monitored daily for any evidence of side-effects and none of the animals had seizures, hypotension, nausea, vomiting or any signs of pain or distress which in mice may be evidenced by immobility and silence, withdrawal, reduced grooming, hunched up posture, or reduced food and water intake. None of these occurred in the treated animals, which were lively and social. Physiological variables, such as reduced depth of respiration, increased heart rate or reduced hydration status did not occur with the cardiac hormone treatments. In the animals which lived a normal lifespan after treatment there were signs of aging with their skin becoming wrinkled and they lost weight compared to their weight at mid-life. At autopsy there was no evidence of any cancer in the animals which lived a normal lifespan after treatment.

Discussion

This is the first investigation demonstrating that cardiac hormones can completely eliminate any cancer growing *in vivo*. Human pancreatic adenocarcinomas were chosen for this investigation as they have the lowest mean survival (*i.e.*, only four months) of all common cancers and this total survival of four months is after being treated with surgery, radiotherapy, and plus current chemotherapy (1, 2). In 2006 there were an estimated 17,150 new cases and 16,090 deaths in men and 16,580 new cases and 16,210 deaths in women with pancreatic cancer in the United States (17). Combined, there were an estimated 33,700 new cases and 32,300 deaths from pancreatic cancer in the United States with present treatments in 2006 (17) justifying the need for new treatment(s) for human pancreatic cancer.

In the present investigation, approximately 1/3 of the human pancreatic adenocarcinomas were completely cured when all treatment groups were combined. The ANP and vessel dilator groups had 80% and 33% of the pancreatic adenocarcinomas completely disappearing when examined at autopsy. After nine months follow-up there was no recurrence or metastasis of the human pancreatic cancers in the majority of the animals which became cancer-free (Figure 2). None of the primary human adenocarcinomas that were eliminated recurred in the primary site in any of the four peptide hormone treated groups. To put this nine-month cancer-free post-treatment in perspective, the lifespan of athymic mice is approximately one year (source: Harlan, Indianapolis, IN, USA). The mice in the present investigation were evaluated for the complete lifespan of healthy cancer-free athymic mice. Thus, they were one month old when they arrived from the NCI, took one month to develop the human adenocarcinomas, were treated for one month and had a nine-month post-treatment follow-up. The mice did not die of pancreatic cancer but rather of old age with no cancer present on autopsy in the animals that had the human pancreatic carcinomas eliminated by these cardiac hormones.

Western blots of the NPR-A and -C receptor in the present investigation revealed that the metastatic lesions had less NPR-A receptor as a possible cause as how the metastatic lesions grow, *i.e.*, they had lost a significant amount of the receptor(s) that allows the cancer to respond to ANP. This

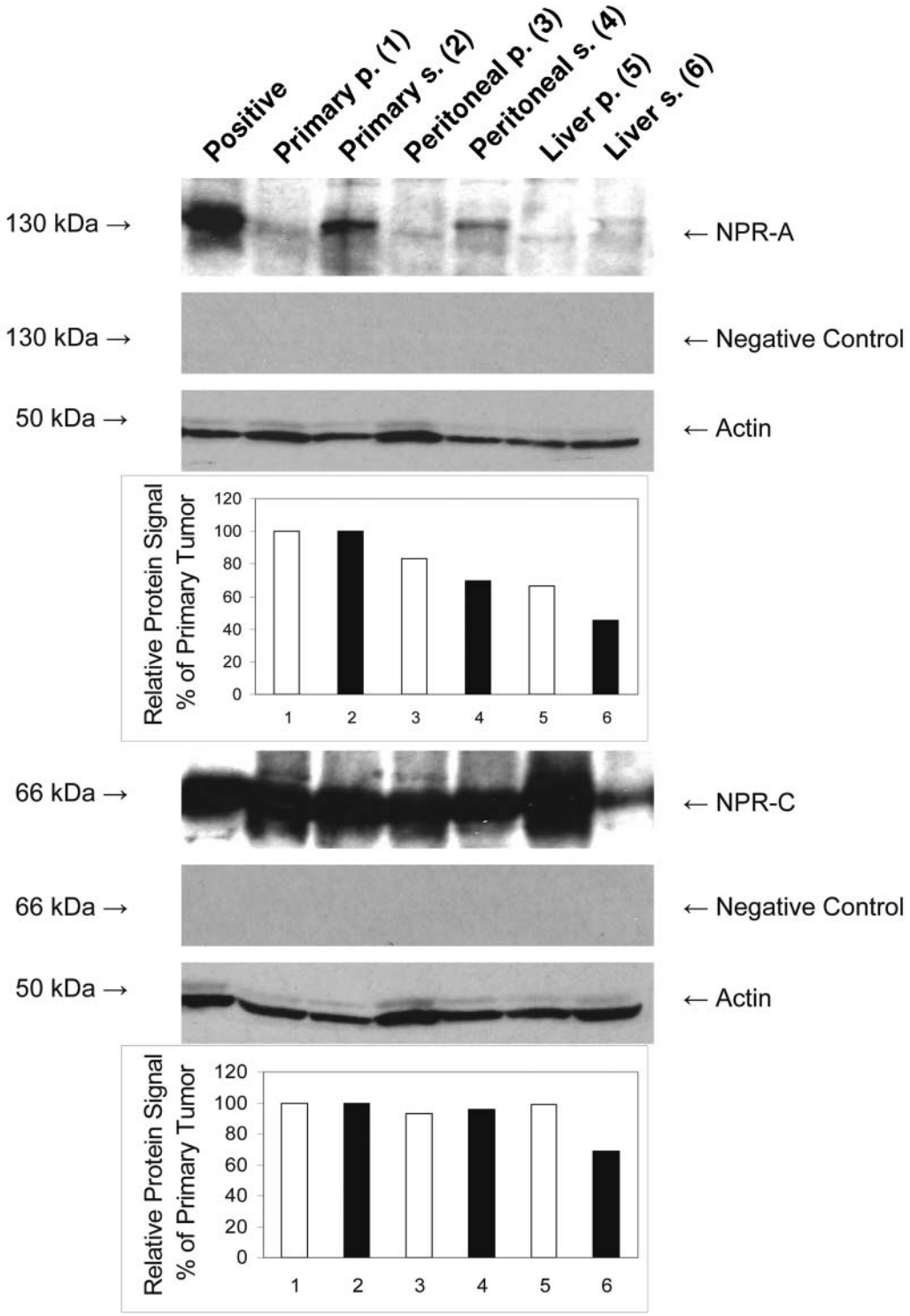

Figure 4. *Western blot analysis of NPR-A (top panel) and NPR-C (lower panel) of human pancreatic adenocarcinoma primary tumor, metastatic peritoneal wall lesion, and liver metastatic lesion from kaliuretic peptide-treated athymic mouse. Western blot analysis (50 μg protein/lane) was performed on the membranous (pellet, P, □) and supernatant (soluble, S, ■) fractions. (The receptors are usually in membranous fractions in individual cells but when a tumor is solubilized, as in the present investigation, the receptors are found in both the membranous and soluble fractions.) Relative to the primary tumor, the total amount of NPR-A was less ($p<0.01$) for the peritoneal wall lesion (~17% for pellet and ~33% for supernatant decrease in A-receptor) and much less ($p<0.001$) for the liver lesion (33% for pellet and ~55% decrease in NPR-A for supernatant) when analyzed by repeated measures of analysis of variance (ANOVA). Band intensities were assessed by densitometry and the results were expressed as percentages of primary tumor values.*

information suggests that measuring NPR-A receptor on human pancreatic cancers surgically removed might help us differentiate which tumors will grow more aggressively and which ones will respond to ANP as an adjunct therapy similar to currently utilized estrogen/progesterone receptor evaluation in breast cancer.

The loss of the NPR-A (or active) receptor helps to explain why the metastatic lesion treated with ANP was not eliminated while the other metastatic lesion treated with vessel dilator was eliminated. ANP works *via* the NPR-A receptor while vessel dilator has a receptor distinct from the NPR-A receptor (18-20). Loss of the NPR-A receptor by the metastatic lesion would cause metastatic lesions not to respond to ANP but loss of this receptor would still allow the metastatic lesion to respond to vessel dilator, which, as above, has its own distinct receptor (18-20).

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