

Review

Cardiac natriuretic peptides: hormones with anticancer effects that localize to nucleus, cytoplasm, endothelium, and fibroblasts of human cancers

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Summary. Four cardiac peptide hormones, i.e., vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) synthesized by the same gene decrease within 24 hours up to 97% the number of human breast, colon, pancreatic, and prostate adenocarcinoma cells as well as human small-cell and squamous carcinomas of the lung cells. These peptide hormones completely inhibit the growth of human pancreatic adenocarcinomas growing in athymic mice. Immunocytochemical investigations have revealed that LANP, vessel dilator, kaliuretic peptide and ANP localize to the nucleus and cytoplasm of human pancreatic adenocarcinomas, which is consistent with their ability to decrease DNA synthesis in the nucleus of this cancer mediated by the intracellular messenger cyclic GMP. These peptide hormones also localize to the endothelium of capillaries and fibroblasts within these cancers. These are the first growth-inhibiting peptide hormones ever demonstrated to localize to the nucleus. Their ability to decrease the activation of growth promoting substances such as Extracellular Receptor Kinase (ERK)-1/2 and Nuclear Factor Kappa Beta (NFκB) suggests that in addition to inhibiting DNA synthesis their ability to decrease the activation of growth promoting substances helps to mediate their ability to inhibit the growth of human cancers.

Key words: Cancer, Nucleus, DNA synthesis, Natriuretic peptides

Introduction

Cardiac natriuretic peptides consist of a family of peptide hormones that are synthesized by three different genes (Rosensweig and Seidman, 1991; Ogawa et al., 1995; Flynn, 1996; Gardner et al., 1997; Vuolteenaho et al., 1997) and then stored as three different prohormones [i.e., 126 amino acid (a.a.) atrial natriuretic peptide (ANP), 108 a.a. brain natriuretic peptide (BNP), and 103 a.a. C-type natriuretic peptide (CNP) prohormones] (Brenner et al., 1990; Vesely, 2002, 2003). In healthy adults, the ANP prohormone's main site of synthesis is the atrial myocyte, but it is also synthesized in a variety of other tissues (Gardner et al., 1986; Vesely et al., 1992). The sites of synthesis of the natriuretic peptides in the approximate order in which they contribute to the synthesis as well as their amino acid sequences has recently been reviewed (Vesely, 2003) and will not be reviewed here.

Peptide hormones originating from the ANP prohormone

Within the 126 a.a. ANP prohormone are four peptide hormones (Fig. 1), with blood pressure-lowering, natriuretic, diuretic, and/or kaliuretic (i.e., potassium-excreting) properties in both animals (Mack et al., 1984; Vesely et al., 1987; Martin et al., 1990; Gunning et al., 1992; Benjamin and Peterson, 1995; Habibullah et al., 1995; Dietz et al., 1995, 2001; Zicdel, 1995; Villarreal et al., 1999) and humans (Vesely et al., 1994a,b, 1998, 1999; Nasser et al., 2001). They, thus, are important for blood pressure regulation and maintenance of plasma volume (Vesely, 2003). These peptide hormones, numbered by their a.a. sequences beginning at the NH₂-terminal end of the ANP prohormone, consist of the first 30 a.a. of the prohormone [i.e., proANP 1-30; long acting natriuretic peptide (LANP), a.a. 31-67 [i.e., proANP 31-67; vessel dilator], a.a. 79-98 [proANP 79-98; kaliuretic peptide], and a.a. 99-126 (ANP) (Fig. 1).

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Each of these four peptide hormones circulates in healthy humans, with LANP and vessel dilator concentrations in plasma being 17 to 22-fold higher than ANP, 33-48-fold higher than BNP and 124-177-fold greater than CNP (Vesely et al., 1989; Winters et al., 1989; Ackerman et al., 1997; Hunter et al., 1998; DePalo et al., 2000; Franz et al., 2000, 2001). More than one peptide hormone originating from the same prohormone is common with respect to the synthesis of hormones (Vesely, 1992). Adrenocorticotrophic hormone (ACTH), for example, is derived from prohormone that contains four known peptide hormones (Vesely, 1992). The BNP and CNP genes, on the other hand, appear to each synthesize only one peptide hormone within their respective prohormones, i.e., BNP and CNP (Lang et al., 1992; Ogawa et al., 1995; Barr et al., 1996; Flynn, 1996; Gardner et al., 1997; Lainchbury et al., 1997). The natriuretic effects of LANP, kaliuretic peptide, and vessel dilator have different mechanism(s) of action from ANP, in that they inhibit renal $\text{Na}^+\text{-K}^+\text{-ATPase}$ secondarily to their ability to enhance the synthesis of prostaglandin E_2 which ANP does not do (Gunning et al., 1992; Chiou and Vesely, 1995). The effects of ANP, BNP, and CNP in the kidney are mediated by cGMP (Brenner et al., 1990; Gunning and Brenner, 1992; Ruskoaho, 1992).

Immunocytochemical localization of cardiac natriuretic peptides in the kidney

The kidney is a prime target organ (along with vasculature) of the physiological effects of the cardiac natriuretic peptides (Brenner et al., 1990; Levin et al., 1998; Clark et al., 2000; Vesely, 2001). Immunohistochemical studies have localized ANP, vessel dilator, and LANP to the sub-brush border of the pars convoluta and pars recta of the proximal tubules of animal (Ramirez et al., 1992) and human (Saba et al., 1993) kidneys (Fig. 2). Immunofluorescent studies reveal that each of these peptides has a strong inclination for the perinuclear region in both the proximal and distal tubules and the alternate cells of the collecting ducts (Fig. 2) (Ramirez et al., 1992; Saba et al., 1993). The ANP prohormone is synthesized within kidney (Poulos et al., 1996; Shin et al., 1997; Totsune et al., 1998; Gower et al., 2003). The amount of ANP prohormone present in the kidney, however, is only one one-nineteenth of that produced in the atria of the heart. The ANP prohormone gene is present and can be expressed in the kidney (Poulos et al., 1996; Shin et al., 1997; Totsune et al., 1998; Gower et al., 2003). The gene is upregulated within the kidney in early renal failure in diabetic animals (Gower et al., 2003) and in the remnant kidney of rats with 5/6 reduced renal mass (Totsune et al., 1998).

Cardiac natriuretic peptides as anticancer agents

Cardiac natriuretic peptides from the ANP prohormone, i.e., long acting natriuretic peptide

(LANP), vessel dilator, kaliuretic peptide and atrial natriuretic peptide have significant anticancer effects of eliminating up to 97% of human breast, colon, pancreatic and prostate adenocarcinomas as well as human small-cell and squamous carcinomas of the lung cells *in vitro* within 24 hours with no proliferation of the remaining cancer cells in three days after this decrease in cancer cell number (Vesely et al., 2003, 2004, 2005a,b,c, 2006). With vessel dilator, for example, there was a 60%, 72%, 92% and 97% decrease in human prostate cancer cell number at its 1 μM , 10 μM , 100 μM and 1 mM concentrations (Vesely et al., 2005a).

Dose-response curves have revealed a significant ($P < 0.05$) decrease in human prostate cancer number with each tenfold increase in the concentration from 1 μM to 1000 μM (i.e., 1 mM) of these four peptide hormones (Vesely et al., 2005a). There was a 97.4%, 87%, 88% and 89% ($P < 0.001$) for each) decrease in prostate cancer

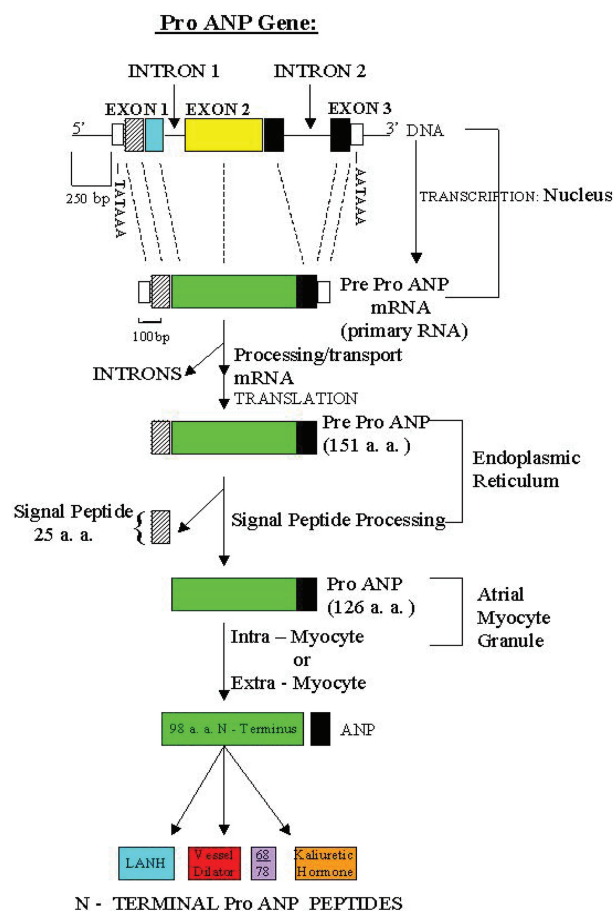


Fig. 1. Structure of the atrial natriuretic peptide prohormone (proANP) gene. Four peptide hormones [e.g., atrial natriuretic peptide (ANP), long acting natriuretic peptide (LANP), vessel dilator, and kaliuretic peptide] are synthesized by this gene. Each of these peptide hormones has biological effects, e.g., natriuresis and diuresis. LANP, long acting natriuretic hormone (a different nomenclature for LANP); a.a., amino acids. Reprinted from D.L. Vesely, Atrial Natriuretic Hormones, 1992 by permission (Pearson Education, Inc.).

cells secondary to vessel dilator, LANP, kaliuretic peptide and ANP, respectively, at their 1 mM concentrations within 24 hours, without any proliferation in the three days following this decrease. These same hormones decreased DNA synthesis from 68% to 89% ($P<0.001$) in the human prostate adenocarcinoma cells (Vesely et al., 2005a). When utilized with their respective antibodies their ability to decrease prostate adenocarcinoma cells or inhibit their DNA synthesis was completely blocked indicating that these anticancer effects are very specific (Vesely et al., 2005a). BNP does not have anticancer effects on any of the above human cancer cells (Vesely et al., 2003, 2005a,b,c, 2006). Western blots revealed that for the first time natriuretic peptide receptors (NPR) A- and C- were present in prostate cancer cells (Vesely et al., 2005a). These peptide hormones effects on DNA synthesis can be mimicked in human prostate cancer cells with their intracellular mediator cyclic GMP (Vesely et al., 2005a).

To determine how specific cyclic GMP's effects are in mediating the anticancer and DNA synthesis effects of these peptide hormones cyclic GMP antibody was utilized in a series of experiments in human colon cancer

cells (Gower et al., 2005). There was a 89 to 97% decrease ($P<0.001$) for each) in human colon adenocarcinoma cells within 24 hours with 1 mM of the above four peptide hormones. These same hormones decreased DNA synthesis 65% to 83% in the colon cancer cells ($P<0.001$). Cyclic GMP antibody inhibited 75% to 80% of these peptides' ability to decrease colon adenocarcinoma cell number and inhibited 92 to 96% of their DNA synthesis effects and 97% of cyclic GMP's effects. Thus, the cyclic GMP antibody blocked essentially all of cyclic GMP's and the cardiac natriuretic peptides' effects on DNA synthesis in the colon cancer cells suggesting that the cardiac hormones effects on DNA synthesis are specifically mediated by cyclic GMP. The cyclic GMP antibody's ability to block 75-80% of these cardiac hormones' ability to decrease human colon adenocarcinoma cell number would suggest that these peptide's mechanism(s) of action as anticancer agents is mediated partially but not completely by cyclic GMP. This information is compatible with the information that one of these peptides', i.e., kaliuretic peptide's ability to decrease activation of extracellular receptor kinase (ERK) 1/2, a

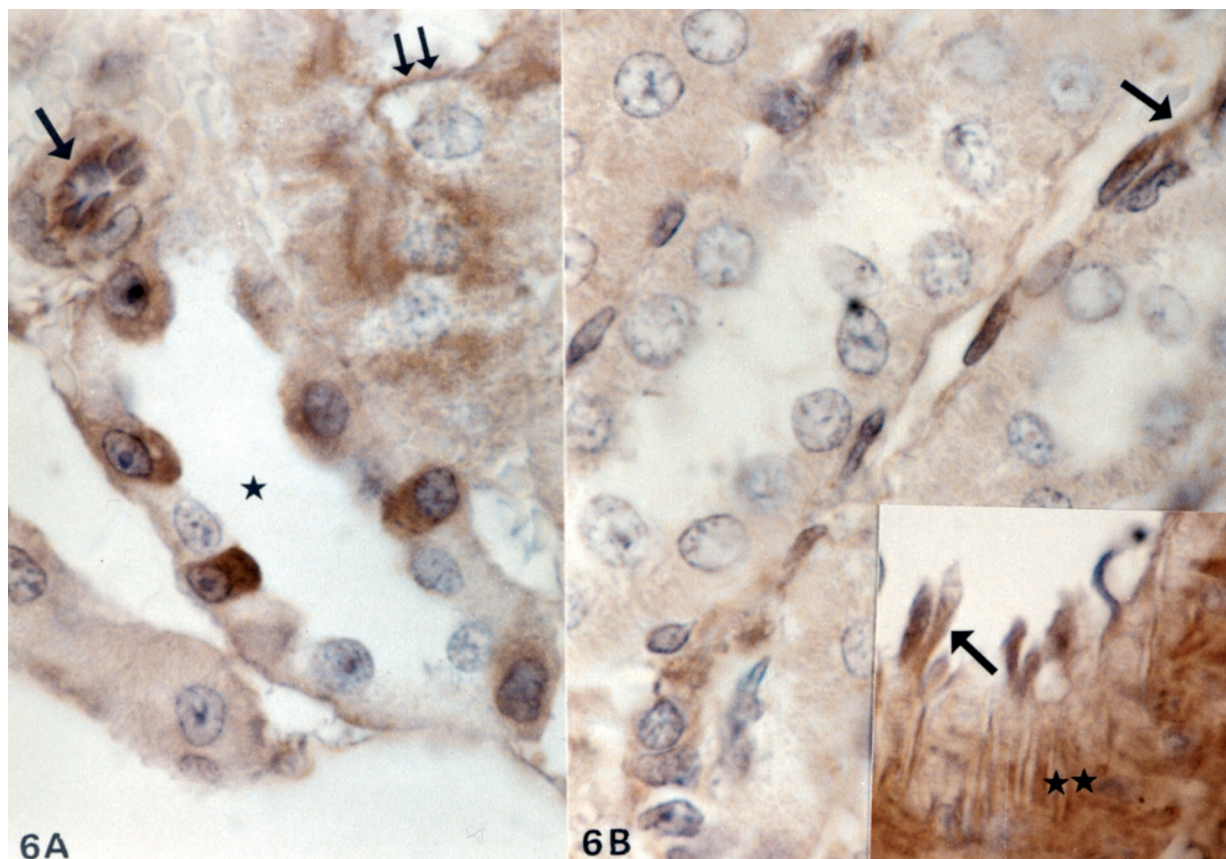


Fig. 2. Immunoperoxidase localization of ANP to endothelium (long arrows) of the interstitial arteriole, artery and peritubular capillary (A and B). The media of the interstitial artery also stained positive for ANP (B, insert **). In the B insert, one can clearly discern the ANP immunoperoxidase staining of endothelial cells (arrow). ANP immunoperoxidase was again seen in the convoluted tubules (double arrow) and in alternate cells of collecting ducts (A, *). (Magnification: 940x). Reprinted from Ramirez et al. *Kidney International* 41: 334-341, 1992 with permission of Blackwell Publishing, Ltd.

cancer growth promoting peptide which translates from the extracellular membrane to the nucleus of the cell to promote growth (Mohapatra et al., 2004). Kaliuretic peptide and ANP also significantly decrease of the activation Nuclear Factor Kappa Beta (NF κ B), an intracellular mediator of growth (Mohapatra et al., 2004). Thus, one additional mechanism of these peptides' antigrowth effects is their ability to decrease the activation of growth promoting peptides (Mohapatra et al., 2004) as well as directly inhibiting DNA synthesis within the nucleus (Saba et al., 2005).

Cardiac natriuretic peptide stop growth and decrease volume of human pancreatic adenocarcinoma growth in athymic mice

Vessel dilator (139 ng min⁻¹ kg⁻¹ of body weight) infused subcutaneously for 14 days completely stopped the growth of human pancreatic adenocarcinomas in athymic mice (n=14) with a decrease in their tumor volume, while the tumor volume increased 69-fold (P<0.001) in the placebo (n=30)-treated mice (Vesely et al., 2004). When these peptide hormones (each at 1.4 μ g min⁻¹ kg⁻¹ of body weight) were infused for four weeks, vessel dilator, long acting natriuretic peptide and kaliuretic peptide decreased tumor volume after one week by 49%, 28%, and 11%, respectively, with a one-fold increase in the tumor volume with ANP (Vesely et al., 2004). Cyclic GMP (2.4 μ g min⁻¹ kg⁻¹ of body weight) inhibited after one week the growth of this cancer 95% (Vesely et al., 2004).

It is important to note that human pancreatic adenocarcinomas have the lowest five-year survival of all common cancers (Pitchumoni, 1998; Wolff et al., 2000). The five-year survival of persons with pancreatic adenocarcinomas is 1% (Pitchumoni, 1998; Wolff et al., 2000). With surgery plus current chemotherapy the median survival is four months (Pitchumoni, 1998; Wolff et al., 2000). This aggressive human pancreatic adenocarcinoma, whose growth was stopped and volume decreased by the above peptide hormones, when not treated with these peptide hormones had its volume increased 172-fold at three weeks and was 300-fold increased in four weeks after the tumor became palpable (Vesely et al., 2004). After two months, the volume of the adenocarcinomas was 1306-fold greater than when the cancers first became palpable (Vesely et al., 2004).

In the above study of human pancreatic adenocarcinomas the peptide hormones were infused subcutaneously for four weeks without fresh hormone(s) added during the four weeks (Vesely et al., 2004). When vessel dilator and LANP are given for a month via subcutaneous infusion pumps the majority of human breast cancers disappear after three weeks when fresh peptide hormone in pumps is changed weekly in athymic mice (Vesely et al., unpublished observation). When examined by necropsy eight weeks after the infusion began there was no sign of the human breast cancers, which were present before the infusion began, in any

tissue or organ of the athymic mice (Vesely et al., unpublished observation).

Localization of cardiac natriuretic peptides within the human pancreatic adenocarcinomas

Immunocytochemical evaluation after removal of the human pancreatic adenocarcinomas revealed that vessel dilator, LANP, kaliuretic peptide and ANP each localized to nucleus and cytoplasm of the cancer cells (Fig. 3; Saba et al., 2005). These peptide hormones also localized to the capillaries growing into these human cancers and to the fibroblasts within the cancers (Saba et al., 2005). Immunoperoxidase staining of vessel dilator was very strong (+++) in the cytoplasm of the human adenocarcinoma cells (Fig. 3A) compared to control adenocarcinoma cells (Insert, Fig. 3D), which did not receive a vessel dilator infusion *in vivo* (Saba et al., 2005). Essentially all of the cytoplasm and perinuclear areas of the pancreatic adenocarcinoma cells had strong staining with vessel dilator (Fig. 3A). The nucleus of the adenocarcinoma cells had weaker (+) but discernible vessel dilator immunoperoxidase staining (Insert, Fig. 3A). Vessel dilator also localized (+++) to the endothelium of the small capillaries invading the human pancreatic adenocarcinoma. On histological (H&E) staining there were not any large blood vessels (arteries, etc) within these tumors but numerous small capillaries invading this tumor could be visualized (Fig. 4). In the H & E evaluation one can observe mitosis occurring within the cancer cells with their nuclei clearly discernable (Fig. 4). Each of the tumors had necrotic centers in both the treated and untreated adenocarcinomas. Vessel dilator also localized to the fibroblasts within the adenocarcinomas where the cytoplasm but not the nuclei of the fibroblasts stained positive for vessel dilator (Fig. 3A).

Long acting natriuretic peptide (LANP) had a slightly stronger (++++) immunoperoxidase staining of the cytoplasm (Saba et al., 2005), but similar intensity (+++) of immunoperoxidase staining of the perinuclear area and nucleus (+) of the human pancreatic adenocarcinomas compared to vessel dilator (Fig. 3B). Strong (++++) LANP immunoperoxidase staining was noted in the endothelium in the small capillaries within the adenocarcinomas (Fig. 3B). LANP staining of the fibroblasts within this tumor was more intense (++++) than with vessel dilator (Fig. 3B).

Kaliuretic peptide immunoperoxidase staining in the adenocarcinomas infused with kaliuretic peptide had a decreased intensity compared to the other peptide hormones (Table 1 and Fig. 3C). Kaliuretic peptide, however, localized to the same structures within the human pancreatic adenocarcinomas (Fig. 3C). Thus, kaliuretic peptide immunoperoxidase staining (+) localized to the nucleus of the cancer cells similar to that observed with vessel dilator and LANP (Fig. 3C). Staining of the cytoplasm (++) of the human pancreatic adenocarcinoma cells with kaliuretic peptide was less

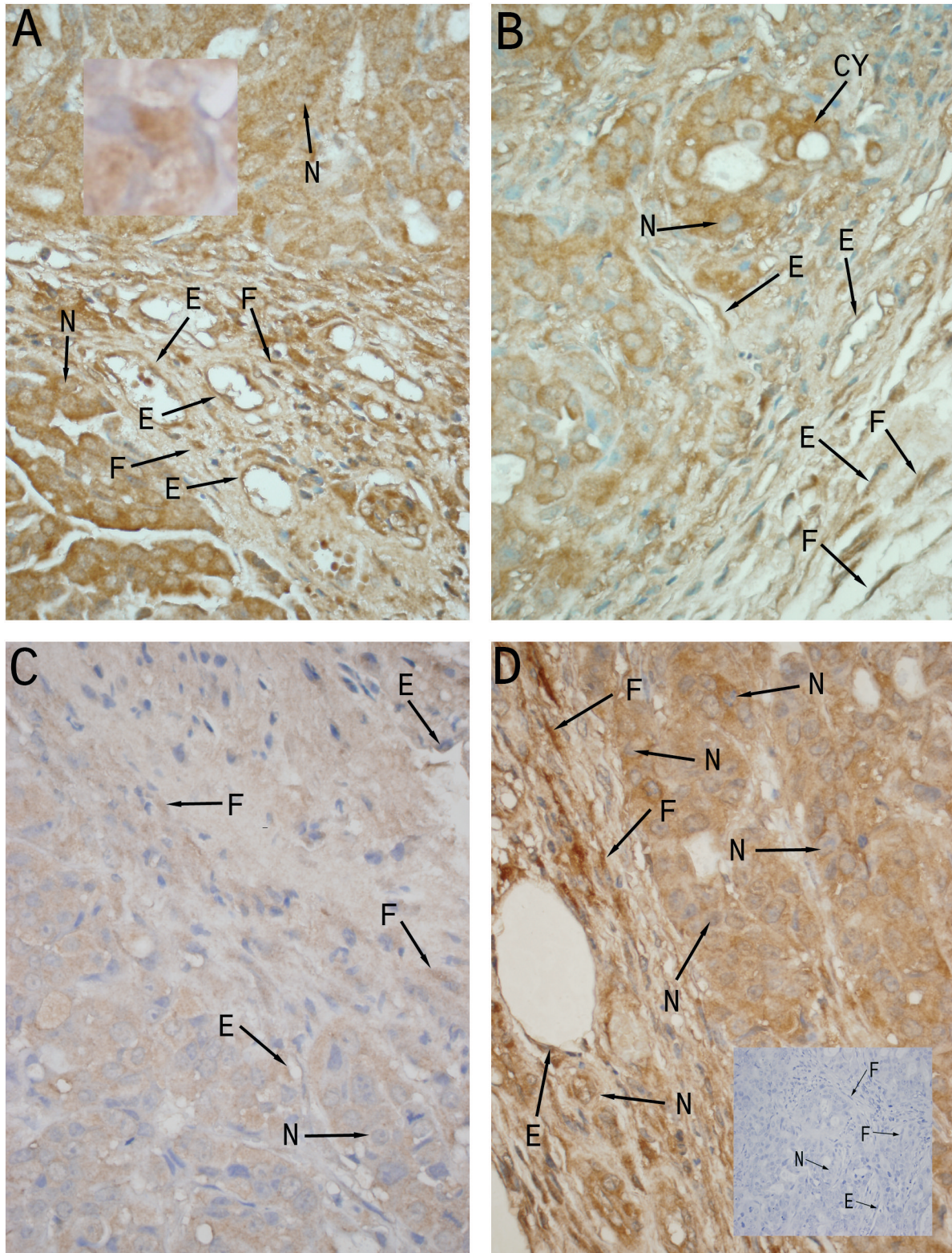


Fig. 3. Immunoperoxidase localization of vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) within human pancreatic adenocarcinomas with each of these peptide hormones strongly localizing to cytoplasm (Cy), nucleus (N), endothelium (E), and fibroblasts (F). The light blue stain in the fibroblasts is the nuclei of the fibroblasts. (A) Vessel dilator treated, (B) LANP treated, (C) kaliuretic peptide treated, and (D) ANP treated. Primary antibody of each peptide was diluted 1:800. Original magnification x 60. The inset in (A) is an isolated nuclei illustrating that vessel dilator has immunoperoxidase staining within the nucleus. The inset in (D) is a negative control using the human pancreatic adenocarcinoma with substitution of the primary antibodies with normal rabbit serum. Reprinted from Saba S.R. et al. *J. Histochem. Cytochem.* 53: 989-995, 2005 with permission of the Histochemical Society.

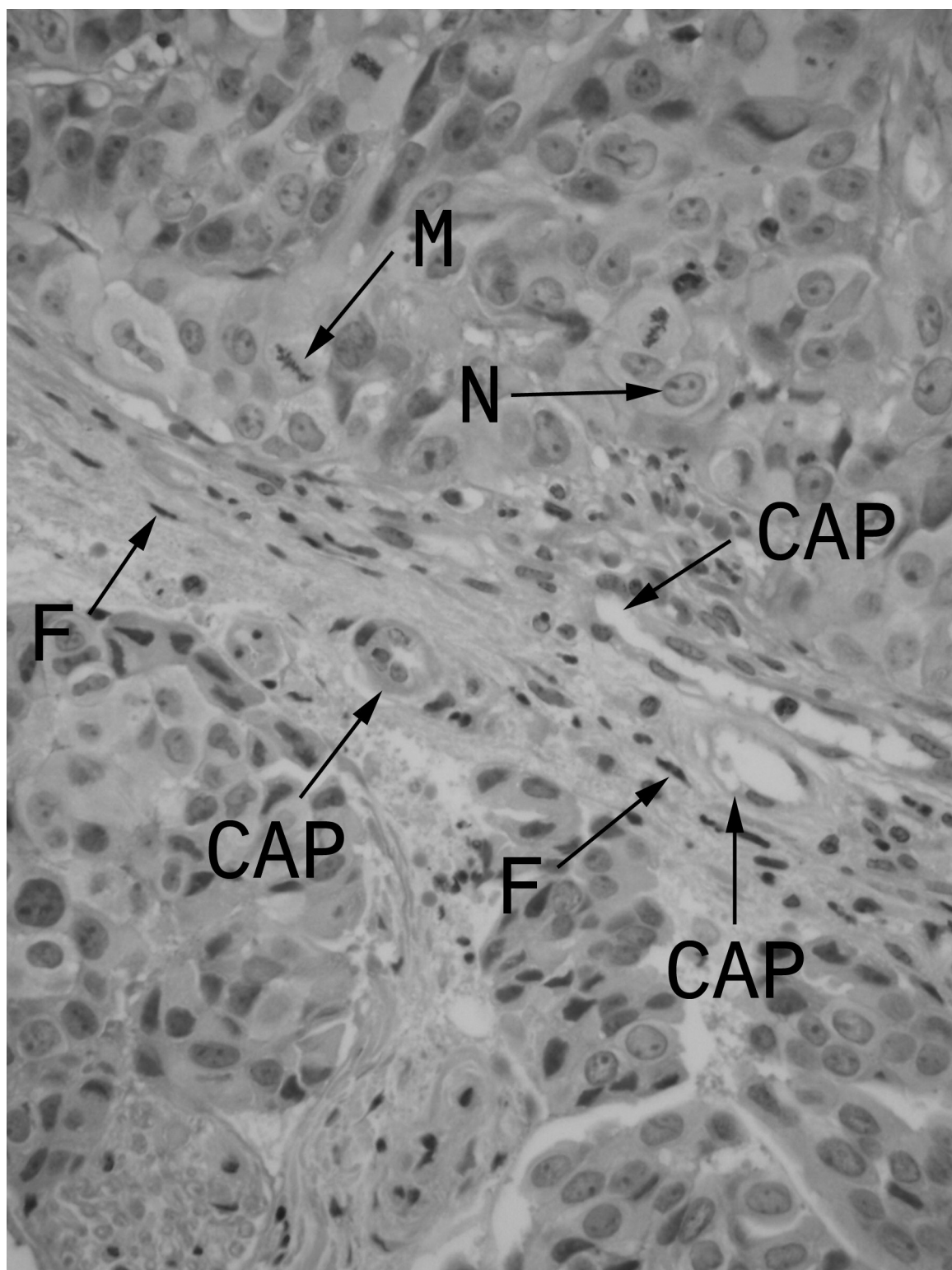


Fig. 4. Histology (hematoxylin and eosin) of human pancreatic adenocarcinoma illustrating capillaries (but not large blood vessels) growing into adenocarcinoma and numerous mitosis occurring. CAP: capillaries, N: nucleus of human pancreatic adenocarcinoma cell, M: mitosis within adenocarcinoma cell, and F: fibroblasts with human pancreatic adenocarcinoma. It is important to note that there are several cancer cells undergoing mitosis in this very rapidly growing cancer. Original magnification x 60. Reprinted from Saba S.R. et al. *J. Histochem. Cytochem.* 53, 989-995, 2005 with permission of Histochemical Society.

than that observed with vessel dilator but there was definite localization of kaliuretic peptide to the cytoplasm (Saba et al., 2005). Kaliuretic peptide also localized to the endothelium of the small capillaries and to fibroblasts (Fig. 3C) with an intensity similar to that of its localization to cytoplasm (Saba et al., 2005).

ANP had very strong immunoperoxidase (++++) localization to the cytoplasm of the human pancreatic cells (Fig. 3D). ANP had slightly stronger immunoperoxidase staining of the nucleus (++) than each of the other peptide hormones (Table 1). ANP also had a very strong (+++) localization to the endothelium of the capillaries invading this cancer (Saba et al., 2005). Strong (++++ ANP immunoperoxidase staining was also present in the fibroblasts in this tumor (Fig. 3D). The immunohistochemical data are summarized in Table 1.

There was not any immunoperoxidase staining with vessel dilator, LANP, kaliuretic peptide or ANP in the human adenocarcinomas when their respective primary antisera were either substituted with normal rabbit serum (Fig. 3D) or when the primary antibody was preincubated with an excess of vessel dilator, LANP, kaliuretic peptide, and/or ANP in their respective immunoperoxidase assays for 24 hours at 37°C (Saba et al., 2005). Each of the four peptide hormones in the above investigation (Saba et al., 2005) had localization in the nucleus of the cancer cells. This information correlates with the knowledge that each of these peptide hormones strongly inhibit DNA synthesis (83% to 91% decrease) within the human pancreatic adenocarcinomas of the present investigation (Vesely et al., 2003) as the nucleus is the site of DNA synthesis. Further correlation of the findings of the localization of these peptide hormones in the nucleus of the human pancreatic adenocarcinomas by Saba et al., 2005 are flow cytometry studies demonstrating that each of these peptide hormones, except ANP, decrease the number of adenocarcinoma cells in synthetic phase of the cell cycle (i.e., where DNA synthesis occurs) (Vesely et al., 2005c). ANP decreases the number of adenocarcinoma cells in the mitotic phase of the cell cycle (Vesely et al., 2005c).

Vessel dilator, LANP, kaliuretic peptide, and ANP

Table 1. Immunohistochemical localization of vessel dilator, long acting natriuretic peptide (LANP), kaliuretic peptide, and atrial natriuretic peptide (ANP) in human pancreatic adenocarcinomas.

	Vessel dilator	LANP	Kaliuretic peptide	ANP
Cytoplasm	+++	++++	++	++++
Nucleus	+	+	+	++
Endothelium	+++	++++	++	++++
Fibroblasts	+++	++++	++	++++

Immunoperoxidase staining graded 0 to +++, with ++++ being the strongest staining observed. Reprinted from Saba S.R. et al. J. Histochem. Cytochem. (2005) 53, 989-995 with permission of Histochemical Society.

had a very strong localization to the cytoplasm of these cancer cells. We had hypothesized that if these peptide hormones were reaching the nucleus to inhibit DNA synthesis, they should also be abundantly present in the cytoplasm after binding to the cell surface receptors on plasma membranes of the cancer cells. These peptides are known to bind to specific receptors in the plasma membranes of normal and cancer cells (Vesely et al., 1987, 1990, 1992). After ANP binds to its receptor, the receptor internalizes and ANP was then thought to be degraded, with the receptors recycling to the plasma membrane (Hirata et al., 1985; Napier et al., 1986; Hughes et al., 1987; Morel and Heisler, 1988). Part of the cytoplasmic demonstration of these peptide hormones within the cancer cells may be the cardiac natriuretic peptides attached to their receptors that are being internalized. However, the intense cytochemical localization throughout the cytoplasm and the new knowledge of the present investigation that these peptides also localize strongly to the perinuclear area and are in the nucleus suggests that these peptides are not all being degraded in the cytoplasm as previously thought, but rather are traveling through the cytoplasm to reach the nucleus to directly inhibit DNA synthesis.

There is evidence that other peptides are transported from the external plasma membrane to the nucleus (Burack and Shaw, 2000). One example of this is Extracellular Receptor Kinase (ERK), which is transported to the nucleus to cause proliferation (i.e., a growth promoting protein) (Burack and Shaw, 2000). Proteins such as ERK are thought to move from the plasma membrane to the nucleus via attaching to a scaffolding protein, which moves them to the nucleus (Burack and Shaw, 2000). One could envision a similar type of scaffolding proteins to transport the four growth-inhibiting proteins of the present investigation, but this protein(s) has not been defined at present. The activation and movement of ERK to the nucleus can be inhibited by one of the cardiac natriuretic peptides (i.e., kaliuretic peptide which is the only one of the natriuretic peptides tested for this property at present) (Mohapatra et al., 2004).

It is important to note that all of the previously reported peptide hormones that localize to the nucleus (i.e., insulin, epidermal growth factor, nerve growth factor, platelet derived growth factor, luteinizing hormone releasing hormone, and human chronic gonadotrophin) (Burwen and Jones, 1987) as well as ERK (Burack and Shaw, 2000) have been growth-promoting hormones. The investigation of Saba et al., 2005 is the first demonstration that we are aware of that growth-inhibiting peptide hormones localize to the nucleus, where they can directly interact to inhibit the effects of the growth-stimulating hormones as well as act via their demonstrated direct decrease of DNA synthesis in the nucleus (Vesely et al., 2003, 2005a,b,c, 2006).

LANP, vessel dilator, ANP and kaliuretic peptide also localize to the fibroblasts within adenocarcinomas (Saba et al., 2005). It could not be determined with

certainly whether these peptide hormones were being synthesized by the fibroblasts or whether they localized to the fibroblasts after their infusion. ANP prohormone mRNA is present in fibroblasts, which indicates that vessel dilator, LANP, kaliuretic peptide, and ANP, which are derived from the ANP prohormone, are synthesized by fibroblasts (Kwano et al., 2000). It is of interest that these peptide hormones did not localize to the nucleus of the fibroblasts, but only to the nucleus of the cancer cells.

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