BREASTSENTRY™

Scientific Overview of BreastSentry

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STAGE ZERO LIFE SCIENCES

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1.Plasma proneurotensin and incidence of diabetes, cardiovascular disease, breast cancer, and mortality. JAMA. 2012; 308(14):1469-75. PubMed PMID:23047361. Melander O, Maisel AS, Almgren P, Manjer J, Belting M, Hedblad B, Engström G, Kilger U, Nilsson P, Bergmann A, Orho-Melander M.

2.Validation of plasma proneurotensin as a novel biomarker for the prediction of incident breast cancer. Cancer Epidemiol Biomarkers Prev. 2014; 23(8):1672-6. PubMed PMID: 24925674. Melander O, Belting M, Manjer J, Maisel AS, Hedblad B, Engström G, Nilsson P, Struck J, Hartmann O, Bergmann A, Orho-Melander M.

3.Stable Peptide of the Endogenous Opioid Enkephalin Precursor and Breast Cancer Risk. J Clin Oncol. 2015 Aug 20;33(24):2632-8. PubMed PMID:26169618. Melander O, Orho-Melander M, Manjer J, Svensson T, Almgren P, Nilsson PM, Engström G, Hedblad B, Borgquist S, Hartmann O, Struck J, Bergmann A, Belting M.

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BreastSentry™ Overview

BreastSentry is a sophisticated blood test that measures the levels of two bio-markers, proneurotensin (pro-NT) and proenkephalin (pro-ENK), which are highly predictive of a woman's risk for developing breast cancer. Longitudinal clinical studies have shown that **elevated levels of pro-NT** and **decreased levels of pro-ENK** are strong, independent risk factors for the development of breast cancer. [4-20]

The BreastSenty test measures the levels of pro-NT and pro-ENK biomarkers in fasting plasma to help determine a patient's risk of developing breast cancer relative to the risk in an average risk population.The test is especially indicated for use in women over the age of 40 with dense breast tissue who are less likely to find breast cancer through annual mammography alone. When used for the approximately 50% of women who have dense breasts, elevated BreastSentry risk scores can help physicians determine if further screening with 3D tomography, breast ultrasound, and/or breast MRI are necessary.

Two large Swedish general population longitudinal studies were used to validate the BreastSentry test. The Malmo Diet Cancer study (MDC) and Malmo Prevention Project (MPP), found a significant predictive relationship between individual pro-NT and pro-ENK biomarkers and the development of breast cancer. Results from the MDC study in 2012 showed a highly significant relationship between the concentration of pro-NT in the blood and the risk of developing breast cancer; the MPP study confirmed these results. It was also shown that biomarker-based risk prediction for the development of breast cancer was significantly improved by combining the measurements of both plasma pro-ENK and pro-NT.

If a patient receives an elevated BreastSentry score, physicians and patients may wish to proceed with informed decision making to assess whether to pursue further screening such as breast MRI, ultrasound, or digital breast tomosynthesis (DBT).

Assay Methodology & Validation

The two large Swedish general population longitudinal studies, Malmo Diet Cancer study (MDC) and Malmo Prevention Project (MPP), found a significant predictive relationship between individual pro-NT and pro-ENK biomarkers and the development of breast cancer. The MDC study followed women for a mean of approximately 15 years and a maximum of 18 years. The MPP study followed women for a mean of approximately five years and a maximum of eight years. Results from the MDC study in 2012 showed a highly significant relationship between the concentration of pro-NT in the blood and the risk of developing breast cancer for women for the first time; the MPP study confirmed these results. It was also shown in these studies that biomarker-based risk prediction for the development of breast cancer was significantly improved by combining the measurements of both plasma pro-ENK and pro-NT.

In the MDC prospective cohort study, fasting blood plasma was collected from the normal, healthy female Swedish population. Women with prevalent cancer and lacking fasting blood samples were excluded from the biomarker study, leaving approximately 1,929 women for investigation. The blood plasma was stored in a sample bank, and in 2012, pro-NT levels were determined using the sphingotec pro-NT assay. The pro-NT levels were compared with the information collected over approximately 18 years on the health / illness status of the women involved in the study. A total of 123 women developed breast cancer during this time of observation. The results showed that an increased pro-NT level demonstrated an almost threefold higher risk in developing breast cancer in the following 10-18 years.[4,5]

The MPP study demonstrated that there is an especially strong signal of breast cancer risk prediction at five-year follow-up.[14,15] The MPP case-control study subjects for the pro-NT and pro-ENK validation consisted of 1,569 women in total without a history of breast cancer upon initial enrollment and confirmed to be free of breast cancer upon initial examination. N=130 of these women developed breast cancer during follow-up through December 31, 2010, and a control cohort of 1,439 women did not have breast cancer through the end of follow-up.

For the MPP study, pro-NT was measured in fasting plasma samples and was related to the risk of later breast cancer development using multivariate logistic regression. The risk calculation was adjusted for known risk factors in a multivariate analysis and demonstrated to be an independent predictor of breast cancer. The determination of pro-NT delivered additional information to the risk factors already known and, as reported by Melander et. al.[5], surpasses these in their significance.

StageZero licensed the pro-NT and pro-ENK biomarkers from sphingotec and developed the BreastSentry test and algorithm. GeneNews used the banked samples from the MPP and MDC studies in developing and validating the algorithm.

Clinical Utility

The BreastSentry Test is indicated for use in average risk women. Average risk is defined as women without any of the following: a personal history of breast cancer, a confirmed or suspected genetic mutation known to increase risk of breast cancer (eg, BRCA), or a history of previous radiotherapy to the chest at a young age.

The BreastSentry test provides a tool to aid healthcare professionals in the assessment, monitoring and management of a woman's risk of developing breast cancer. The BreastSentry test can be used as an aid to address the shortcomings of mammography for detection of breast cancer in women with dense breasts. Mammography misses about 16 percent of breast cancers.[63] Depending on certain factors (such as breast density) mammography may miss nearly 30 percent of breast cancers.[64,65]

The BreastSentry test provides supplemental breast cancer risk assessment for the approximately 50% of US women who have dense breast tissue that limit the effectiveness of mammograms. Twenty-seven (27) states require that when reporting mammogram results, women should also be notified if they have dense breasts .

Women with an elevated BreastSentry risk score, especially women over the age of 40 with dense breast tissue, may benefit from additional screening such as breast ultrasound, digital breast tomosynthesis (DBT), and/or a breast MRI examination. Pro-NT and pro-ENK neuropeptides, reported individually along with the BreastSentry score, have been found to be highly predictive of breast cancer risk. Published studies suggest that lifestyle changes such as exercise, diet and reduced opioid use may result in a change in pro-NT and/or pro-ENK values over time.These changes may be associated with a reduction in breast cancer risk [11,18, 20]. Annual testing with BreastSentry can assist patients in tracking their progress with lifestyle changes and updating their future risk of breast cancer. Elevated risk scores may also lead to a change in lifestyle, diet, and improvement of other clinical indicators as recommended by a physician or healthcare professional.

The BreastSentry test provides patients with their risk of developing breast cancer in the future. Physicians may monitor patients with an elevated risk score more closely, prescribe additional screening and/or encourage their patients to make lifestyle changes to reduce their risk of breast cancer. Survival rates by Stage illustrate that the earlier a breast cancer is found, the better the chances that person will survive five years post-diagnosis. Sixty-one percent of breast cancer patients diagnosed between 2006 and 2012 were diagnosed as localized. The five-year survival rate for localized breast cancer was 98.8%, compared to a 26.3% survival rate if the cancer had metastasized.[21]

A physician, or other authorized qualified health care provider must order BreastSentry.

Clinical Interpretation & Treatment Considerations

Treatment considerations are as follows:

Pro-NT above 180pmol/L and/or pro-ENK below 44pmol/L
Discuss lifestyle changes such as diet, exercise and reduced opioid use with patient.Monitor annually.

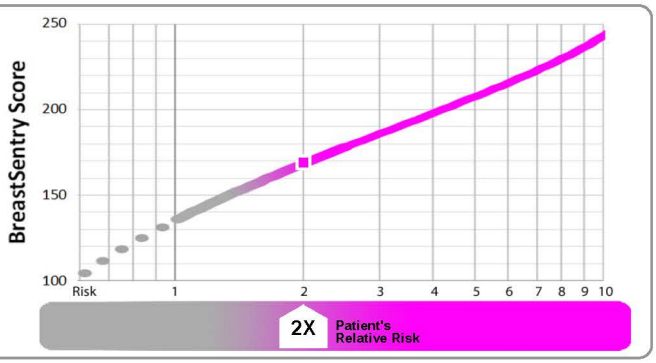
BreastSentry Average Risk Score: Patients at Average Risk should continue to follow published recommendations from the American Cancer Society and the National Cancer Institute regarding breast cancer screening as recommended by their physician. Patients may wish to supplement the mammogram with an annual BreastSentry test to determine if they have moved into the elevated risk cohort.

BreastSentry Elevated Risk Score: Patients at Elevated Risk should consider additional screening such as breast ultrasound, digital breast tomosynthesis (DBT), and/or a breast MRI examination.

Test Results and Interpretation

The patient has an **Elevated** risk score,

2X greater than a woman at average risk. Increased levels of pro-NT and decreased levels of pro-ENK are predictive of a woman's risk for development of breast cancer.



While BreastSentry reports the future probability of breast cancer, it does not specify what lifestyle, diet, exercise or other clinical causes may have the ability to change the risk of developing breast cancer.Women have the ability to lower their BreastSentry projected risk of breast cancer over time by addressing how these risk factors impact their health.

Although the exact causes of breast cancer are unknown, if the patient's BreastSentry risk curve is unacceptably elevated in the physican's clinical judgment, the physician may consider the following lifestyle and dietary modifications to help reduce a woman's risk of developing the disease:[56-62]

- Increase intake of foods that have been shown to significantly reduce inflammation and cancer risk, including fresh fruits, carotenoid-rich foods, non-starchy vegetables, raw nuts and seeds, and omega-3 fatty-acid-containing foods, such as oily fish.
- Weight loss (as appropriate)
- Regular exercise
- Decreased alcohol consumption
- Smoking cessation
- Limit dose and duration of hormone therapy
- Breast-feed—the longer you breast-feed, the greater the protective effect
- Avoid exposure to radiation and environmental pollution
- Decrease intake of foods that may increase inflammation and cancer risk, such as red/processed meat, refined grains and sugars, highly heated or oxidized oils, and trans fats

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Scientific Papers

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1.Plasma proneurotensin and incidence of diabetes, cardiovascular disease, breast cancer, and mortality. JAMA. 2012; 308(14):1469-75. PubMed PMID:23047361. Melander O, Maisel AS, Almgren P, Manjer J, Belting M, Hedblad B, Engström G, Kilger U, Nilsson P, Bergmann A, Orho-Melander M.

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Plasma Proneurotensin and Incidence of Diabetes, Cardiovascular Disease, Breast Cancer, and Mortality

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Context Neurotensin regulates both satiety and breast cancer growth in the experimental setting, but little is known about its role in the development of breast cancer or cardiometabolic disease in humans.

Objective To test if fasting plasma concentration of a stable 117-amino acid fragment from the neurotensin precursor hormone proneurotensin is associated with development of diabetes mellitus, cardiovascular disease, breast cancer, and mortality.

Design, Setting, and Participants Proneurotensin was measured in plasma from 4632 fasting participants of the population-based Malmö Diet and Cancer Study baseline examination 1991-1994. Multivariate Cox proportional hazards models were used to relate baseline proneurotensin to first events and death during long-term follow-up until January 2009, with median follow-up ranging from 13.2 to 15.7 years depending on the disease.

Main Outcome Measures Incident diabetes mellitus, cardiovascular disease, breast cancer, and mortality.

Results Overall, proneurotensin (hazard ratio [HR] per SD increment of log-transformed proneurotensin) was related to risk of incident diabetes (142 events; HR, 1.28; 95% CI, 1.09-1.50; $P=.003$), cardiovascular disease (519 events; HR, 1.17; 95% CI, 1.07-1.27; $P<.001$), and cardiovascular mortality (174 events; HR, 1.29; 95% CI, 1.12-1.49; $P=.001$) with a significant interaction between proneurotensin and sex ($P<.001$) on risk of cardiovascular disease. Exclusively in women, proneurotensin was related to incident diabetes (74 events; HR, 1.41; 95% CI, 1.12-1.77; $P=.003$), cardiovascular disease (224 events; HR, 1.33; 95% CI, 1.17-1.51; $P<.001$), breast cancer (123 events; HR, 1.44; 95% CI, 1.21-1.71; $P<.001$), total mortality (285 events; HR, 1.13; 95% CI, 1.01-1.27; $P=.03$), and cardiovascular mortality (75 events; HR, 1.50; 95% CI, 1.20-1.87; $P<.001$).

Conclusion Fasting proneurotensin was significantly associated with the development of diabetes, cardiovascular disease, breast cancer, and with total and cardiovascular mortality.

JAMA. 2012;308(14):1469-1475 www.jama.com

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BARIATRIC SURGERY OF PATIENTS with obesity has been associated with marked reduction of the incidence of diabetes, cardiovascular disease and mortality, and, in women specifically, with reduction of the incidence of cancer.[1-4] However, the causes of obesity-associated morbidity and mortality are poorly understood. Neurotensin is a 13-amino acid peptide primarily expressed in the central nervous system and gastrointestinal tract.[5-7] Neurotensin binds to 3 different receptors: neurotensin receptor 1 and 2 (Ntsr1 and Ntsr2), which are G-protein coupled receptors, and neurotensin receptor 3 (Ntsr3), which is non–G-protein coupled and also known as sortilin-1 (SORT1).[8,9] The peripheral secretion of neurotensin is stimulated by food intake, especially by fat, and is known to regulate gastrointestinal motility and

pancreatic and biliary secretion.[10] Both central (intracerebroventricular) and peripheral (intraperitoneal) injection of neurotensin acutely reduces food intake in rats, an effect mediated through Ntsr1.[11,12]

In obese as compared with normal-weight humans, postprandial plasma neurotensin concentration was reduced following a liquid fatty meal[13] and increased after gastric bypass (Roux-en-Y) treatment,[14,15] suggesting that regulation of neurotensin secretion is disturbed in human obesity. However, no study has investigated if and how neurotensin is related to measures of obesity or to obesity-associated diseases in the general population.

Neurotensin has trophic effects both on normal and neoplastic tissue and neurotensin and Ntsr1 have been suggested to be prognostic tumor biomarkers.[16,17] Neurotensin and Ntsr1 expression is common in human malignant ductal breast cancer tumors, and in mice xenografted with a malignant human breast cancer cell line, pharmacological blockade or RNA silencing of Ntsr1 reduces tumor growth.[18,19] In addition, genetic variation of 1 of the 3 receptors for neurotensin, ie, Ntsr3 (SORT1), is linked to development of coronary artery disease in humans, an effect mediated by elevated levels of low-density lipoprotein cholesterol (LDL-C).[20,21]

We hypothesized that variations of the neurotensin system may contribute to development of common diseases associated with elevated body mass index (BMI). Because mature neurotensin is unstable both in vitro and in vivo, we measured a stable 117-amino acid fragment from the *N*-terminal part of the pre-proneurotensin/neuromedin precursor hormone, referred to as proneurotensin, which is produced in stoichiometric amounts relative to the mature neurotensin.[22] Certain tissues partially produce large peptides composed of proneurotensin connected to the peptide sequences corresponding to the mature hormones (large neurotensin and large neuromedin).[23] These large hormones have been shown to weakly bind and activate Ntsr1[23]; however, it is unknown if proneurotensin has biological activity. In this study, we explored whether fasting concentration of proneurotensin is associated with future risk of diabetes mellitus, cardiovascular disease, and breast cancer, as well as with death.

METHODS

Study Population

The Malmö Diet and Cancer (MDC) study is a population-based, prospective epidemiologic cohort of 28 449 men (born 1923-1945) and women (born 1923-1950) from Malmö, Sweden, who underwent baseline examinations between 1991 and 1996.[24] From this cohort, 6103 individuals were randomly selected to participate in the MDC Cardiovascular Cohort (MDC-CC), which was designed to investigate the epidemiology of carotid artery disease between 1991 and 1994.[25] Fasting plasma samples were available for analysis of proneurotensin in 4632 participants in the MDC-CC. The 1471 excluded participants (due to lack of plasma sample) were slightly younger, but did not otherwise differ in terms of sex, smoking, diabetes, hypertension status, BMI, or plasma lipids as detailed in the eAppendix (available at http://www.jama.com).

Of the 4632 participants in whom proneurotensin was measured, those with prevalent disease prior to the baseline examination were excluded from the analyses of the main outcomes, as well as those without complete data on covariates. The baseline examination procedure has been described previously (eAppendix).[26-28] Proneurotensin was measured in stored fasting plasma specimens that were frozen to –80°C immediately at the MDC-CC baseline examination using a recent chemiluminometric sandwich immunoassay to detect a proneurotensin precursor fragment (proneurotensin 1–117) (eAppendix).[22]

Following procedures (eAppendix) previously described,[26-30] we used Swedish national and local registers to retrieve incident cases of diabetes mellitus, cardiovascular disease (myocardial infarction and stroke), breast cancer, and all-cause and cause-specific mortality during more than 12 years of follow-up. Follow-up extended to January 1, 2009, for all end points except for new-onset diabetes, for which follow-up extended to June 30, 2006.

All participants gave written informed consent and the study was approved by the ethical committee at Lund University, Lund, Sweden.

Statistics

We used multivariate Cox proportional hazards models to test the relationship between fasting plasma concentration of proneurotensin and each of the outcomes. Because we had primary hypotheses of association between proneurotensin and each of the outcomes, we did not adjust for multiple comparisons. In analyses of incident diabetes, we adjusted for age, sex, use of antihypertensive medication, systolic blood pressure, BMI, waist circumference, prevalent cardiovascular disease, current smoking, and fasting concentrations of glucose, high-density lipoprotein cholesterol (HDL-C), LDL-C, triglycerides, and insulin (diabetes risk factors).

In analyses of incident cardiovascular disease, all-cause mortality and cardiovascular mortality, we adjusted for age, sex, use of antihypertensive medication, systolic blood pressure, BMI, current smoking, diabetes mellitus, and fasting concentrations of HDL-C and LDL-C (cardiovascular disease risk factors). In analyses of incident breast cancer (women only), we adjusted for age, use of antihypertensive medication, use of hormone therapy, ever use of oral contraceptives, educational level, age at menarche, number of children, menopausal status, systolic blood pressure, BMI, diabetes mellitus, current smoking, prevalent cardiovascular disease, heredity for cancer, and fasting concentrations of HDL, LDL and insulin (breast cancer risk factors). All analyses were analyzed and checked for multicollinearity between covariates but no significant multicollinearity was found in any analyses.

Fasting plasma concentration of proneurotensin was skewed to the right and therefore transformed with the natural logarithm and thereafter normalized, and hazard ratios (HRs) were expressed per 1 (SD) increment of log-transformed proneurotensin in the Cox proportional hazards models. In addition, proneurotensin was divided into quartiles. Quartile 1 (lowest values of proneurotensin) was defined as the reference standard and quartiles 2 to 4 were compared with the reference quartile in the Cox proportional hazards models.

All analyses were performed with Stata statistical software version 11. A 2-sided *P* value of less than .05 was considered statistically significant.

RESULTS

Cross-sectional Relationship Between Cardiometabolic Risk Factors and Proneurotensin

The baseline characteristics are shown in TABLE 1 and in eTable 1 and eTable 2. Women had significantly higher proneurotensin (median [interquartile range {IQR}] than men: 109 pmol/L [79-150] vs 99 pmol/L [71-144]; $P<.001$). The relationship between proneurotensin and cardiometabolic risk factors was weak, with the strongest correlation being that with fasting insulin concentration in both sexes (eTable 3). In a linear regression model with backward elimination and a retention $P<.10$, significant independent determinants of proneurotensin were smoking and fasting concentrations of insulin, glucose, and HDL (all positive) in women, and smoking and fasting concentrations of insulin and HDL (positively related) and age and LDL (negatively related) in men (TABLE 2).

Proneurotensin and Risk of Diabetes Mellitus

Among 3704 participants free from diabetes mellitus at baseline (1484 men and 2220 women), 142 (68 men and 74 women) developed new-onset diabetes mellitus during a median (IQR) follow-up time of 13.2 years (12.6-13.7) with an event rate of 30.2 per 10 000 person-years. Each SD increase of baseline proneurotensin was associated with a multivariate-adjusted HR of 1.28 (95% CI, 1.09-1.50; $P=.003$) for the risk of new-onset diabetes in the total study population, whereas the HR was 1.41 (95% CI, 1.12-1.77; $P=.003$) in women, and not significantly elevated in men (HR, 1.21; 95% CI, 0.96-1.53; $P=.10$). There was no significant interaction between sex and proneurotensin regarding the association with new-onset diabetes ($P=.37$).

Table 1. Clinical Characteristics of the Study Population in Analyses of Incident Cardiovascular Disease, Cardiovascular Mortality, and Total Mortality

	Women (n = 2559)	Men (n = 1802)
Age, mean (SD), y	57.6 (5.9)	57.8 (6.0)
Systolic blood pressure, mean (SD), mm Hg	141 (19)	144 (19)
Diastolic blood pressure, mean (SD), mm Hg	85.7 (9.1)	89.0 (9.7)
Antihypertensive therapy, No. (%)	386 (15.1)	292 (16.2)
Diabetes mellitus, No. (%)	163 (6.4)	206 (11.4)
Fasting blood glucose, mean (SD), mmol/L	5.0 (1.2)	5.4 (1.5)
Fasting insulin concentration, (median IQR), μIU/mL	6.0 (4.0-9.0)	7.0 (5.0-10)
BMI, mean (SD)[a]	25.5 (4.2)	26.1 (3.4)
Waist circumference, mean (SD), cm	77.0 (10.2)	92.9 (9.9)
LDL-C, mean (SD), mmol/L	4.2 (1.0)	4.1 (0.89)
HDL-C, mean (SD), mmol/L	1.5 (0.37)	1.2 (0.30)
Triglycerides, mean (SD), mmol/L	1.2 (0.58)	1.4 (0.68)
Current smokers, No. (%)	653 (25.5)	497 (27.6)

Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.
SI conversion factors: To convert blood glucose to mg/dL, divide by 0.0555; insulin concentration to pmol/L, multiply by 6.945; HDL-C to mg/dL, divide by 0.0259; LDL-C to mg/dL, divide by 0.0259; triglycerides to mg/dL, divide by 0.0113.
[a] BMI calculated as weight in kilograms divided by height in meters squared.

Table 2. Independent Significant Determinants of Fasting Plasma Concentration of Proneurotensin in Women and Men From a Linear Regression Model With Backward Elimination[a]

	Women (n = 2559)		Men (n = 1802)	
Independent Proneurotensin Determinants	β Coefficient (95% CI)[b]	*P* Value	β Coefficient (95% CI)[b]	*P* Value
Insulin[c]	0.15 (0.10 to 0.20)	<.001	0.15 (0.09 to 0.20)	<.001
High-density lipoprotein	0.05 (0.00 to 0.09)	.04	0.05 (0.00 to 0.10)	.05
Smoking	0.16 (0.07 to 0.25)	<.001	0.13 (0.03 to 0.24)	.01
Glucose	0.07 (0.03 to 0.11)	.001		
Age			–0.05 (–0.10 to –0.01)	.02
Low-density lipoprotein			–0.05 (–0.10 to –0.01)	.03

[a] The following variables were entered in the linear regression model with backward elimination at $P<.10$: age, antihypertensive treatment, systolic blood pressure, diastolic blood pressure, current smoking, waist circumference, body mass index, and fasting concentrations of glucose, insulin, triglycerides, high-density lipoprotein, and low-density lipoprotein.
[b] The β is expressed as the increment of the log-transformed standardized values of proneurotensin per increment of standardized values (or presence of dichotomized risk factor) of the risk factor in question.
[c] Insulin was log-transformed before being standardized.

Among 1950 women free from impaired fasting glucose at baseline (fasting whole blood glucose, <97 mg/dL [<5.4 mmol/L]), 38 women developed diabetes during follow-up with an event rate of 15.0 per 10 000 person-years, and each SD increase of baseline proneurotensin was associated with a multivariate-adjusted HR

Table 3. Event Rates and Multivariate Adjusted Cox Proportional Hazards Models for Baseline Proneurotensin vs Incidence of Cardiovascular Disease, Breast Cancer, All-Cause Mortality, and Cardiovascular Mortality

		Quartiles					
	Overall	1	2	3	4	*P* Value[a]	*P* for trend
Cardiovascular disease[b]							
All participants, No./events, No.	4361/519	1091/118	1090/113	1092/143	1088/145		
Events/10 000 person-years	82.5	74.8	71.0	91.2	93.2		
Proneurotensin, median (range), pmol/L[c]	105 (3.3-1155)	60.2 (3.3-75.7)	89.3 (75.8-105)	123 (105-148)	190 (148-1155)		
HR (95% CI)[d]	1.17 (1.07-1.27)	1 [Reference]	1.09 (0.84-1.41)	1.39 (1.09-1.78)	1.37 (1.07-1.75)	<.001	.003
Women, No./events, No.	2559/224	640/44	641/39	639/68	639/73		
Events/10 000 person-years	59.2	46.4	40.5	72.3	78.1		
Proneurotensin, median (range), pmol/L[c]	109 (5.1-1155)	62.4 (5.1-78.6)	92.1 (78.6-109)	125 (109-150)	194 (150-1155)		
HR (95% CI)[d]	1.33 (1.17-1.51)	1 [Reference]	0.91 (0.59-1.40)	1.58 (1.08-2.32)	1.65 (1.13-2.41)	<.001	.001
Men, No./events, No.	1802/295	451/67	450/79	451/74	450/75		
Events/10 000 person-years	118	106	125	119	121		
Proneurotensin, median (range), pmol/L[c]	98.9 (3.3-1057)	58.0 (3.3-70.8)	85.9 (71.0-98.8)	118 (98.9-144)	186 (144-1057)		
HR (95% CI)[d]	1.06 (0.95-1.19)	1 [Reference]	1.27 (0.92-1.76)	1.27 (0.91-1.77)	1.22 (0.88-1.70)	.31	.27
All-cause mortality[b]							
All participants, No./events, No.	4361/603	1091/141	1090/135	1092/151	1088/176		
Events/10 000 person-years	92.2	86.6	82.1	92.2	108		
HR (95% CI)[d]	1.08 (1.00-1.17)	1 [Reference]	1.05 (0.82-1.32)	1.17 (0.93-1.48)	1.30 (1.04-1.63)	.05	.01
Women, No./events, No.	2559/285	640/62	641/65	639/67	639/91		
Events/10 000 person-years	73.3	63.9	66.5	68.8	94.1		
HR (95% CI)[d]	1.13 (1.01-1.27)	1 [Reference]	1.08 (0.76-1.52)	1.07 (0.76-1.52)	1.43 (1.03-1.97)	.03	.04
Men, No./events, No.	1802/318	451/75	450/73	451/85	450/85		
Events/10 000 person-years	120	114	110	129	128		
HR (95% CI)[d]	1.04 (0.93-1.16)	1 [Reference]	1.01 (0.73-1.40)	1.25 (0.91-1.71)	1.15 (0.84-1.58)	.47	.21
Cardiovascular mortality[b]							
All participants, No./events, No.	4361/174	1091/37	1090/31	1092/46	1088/60		
Events/10 000 person-years	26.6	22.7	18.9	28.1	36.8		
HR (95% CI)[d]	1.29 (1.12-1.49)	1 [Reference]	0.95 (0.59-1.53)	1.40 (0.91-2.17)	1.73 (1.14-2.61)	.001	.003
Women, No./events, No.	2559/75	640/13	641/13	639/20	639/29		
Events/10 000 person-years	19.3	13.4	13.3	20.6	30.0		
HR (95% CI)[d]	1.50 (1.20-1.87)	1 [Reference]	1.02 (0.47-2.21)	1.53 (0.76-3.09)	2.18 (1.13-4.20)	<.001	.008
Men, No./events, No.	1802/99	451/21	450/22	451/26	450/30		
Events/10 000 person-years	37.3	31.8	33.0	39.3	45.2		
HR (95% CI)[d]	1.16 (0.96-1.41)	1 [Reference]	1.07 (0.59-1.96)	1.36 (0.77-2.43)	1.44 (0.82-2.53)	.13	.14
Breast cancer[e]							
Women, No./events, No.	1929/123	483/20	483/25	481/32	482/46		
Events/10 000 person-years	43.2	28.0	34.7	44.8	65.7		
Proneurotensin, median (range), pmol/L[c]	108 (5.1-1132)	62.1 (5.1-77.5)	91.1 (77.6-108)	125 (108-150)	194 (150-1132)		
HR (95% CI)[d]	1.44 (1.21-1.71)	1 [Reference]	1.32 (0.73-2.38)	1.79 (1.02-3.14)	2.44 (1.44-4.15)	<.001	<.001

Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; HR, hazard ratio; LDL-C, low-density lipoprotein cholesterol.

[a] *P* values are for continuous analyses of association between proneurotensin and outcomes.

[b] Analyses of incident cardiovascular disease, all-cause mortality, and cardiovascular mortality were adjusted for age, sex, use of antihypertensive medication, systolic blood pressure, BMI, current smoking, diabetes mellitus, and fasting HDL-C and LDL-C.

[c] Because participants included and the respective proneurotensin values (fasting plasma concentration) are the same for cardiovascular disease, all-cause mortality, and cardiovascular mortality, proneurotensin values are only shown for cardiovascular disease and breast cancer.

[d] HRs (95% CIs) are expressed per 1-SD increment of log-transformed proneurotensin (in analyses of all participants, all men, and all women).

[e] Analysis of incident breast cancer in women was adjusted for age, use of antihypertensive medication, hormone therapy, ever use of oral contraceptives, educational level, age at menarche, number of children, menopausal status, systolic blood pressure, BMI, diabetes mellitus, current smoking, prevalent cardiovascular disease, heredity for cancer, and fasting concentrations of HDL-C, LDL-C, and insulin.

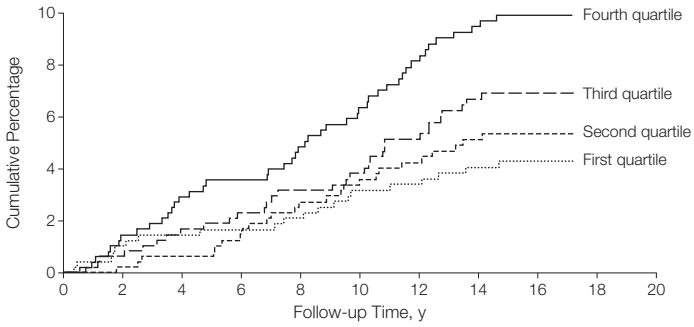
of 1.47 (1.08-2.00; $P=.01$) for incident diabetes.

Proneurotensin and Risk of Cardiovascular Disease, Cardiovascular Mortality, and All-Cause Mortality

Among 4361 participants without cardiovascular disease prior to the baseline examination (1802 men and 2559 women), 519 developed a first cardiovascular disease event during a median (IQR) follow-up time of 15.6 years (14.9-16.2), with an event rate of 82.5 per 10 000 person-years. After full adjustment, each SD increase of proneurotensin was associated with an HR of 1.17 (95% CI, 1.07-1.27) for risk of incident cardiovascular disease (TABLE 3). There was a significant interaction between proneurotensin and female sex on incidence of cardiovascular disease ($P<.001$) and sex-stratified analyses revealed that each SD increase of baseline proneurotensin was associated with a hazard ratio of 1.33 (95% CI, 1.17-1.51) and top vs bottom quartile of proneurotensin with a hazard ratio of 1.65 (95% CI, 1.13-2.41) for risk of cardiovascular disease in women, whereas there was no significant relationship among men (Table 3).

Each SD increment of proneurotensin was associated with a hazard ratio of 1.08 (95% CI, 1.00-1.17) for risk of all-cause mortality in the total population and a hazard ratio of 1.13 (95% CI, 1.01-1.27) for risk of all-cause mortality among women, with no increased risk related to proneurotensin in men (Table 3). The excess risk of death associated with proneurotensin in women appeared to be mainly accounted for by cardiovascular deaths with a hazard ratio of 1.50 (95% CI, 1.20-1.87) per SD increment of proneurotensin and 2.18 (95% CI, 1.13-4.20) in women belonging to the top as compared with the bottom quartile of proneurotensin (Table 3). There was no significant interaction between sex and proneurotensin regarding the association with all-cause ($P=.26$) and cardiovascular mortality ($P=.08$).

Figure. Cumulative Breast Cancer Event-Free Survival During Follow-up



No. at risk

First quartile	483	476	472	463	454	445	434	426	151
Second quartile	483	482	476	470	459	448	436	420	151
Third quartile	481	477	468	460	451	440	431	414	168
Fourth quartile	482	472	463	455	440	426	416	398	187

Kaplan-Meier plot shows 1 minus cumulative breast cancer event-free survival during follow-up in quartiles: first (lowest values) to fourth quartile of the baseline fasting plasma concentration of proneurotensin. Median (range) concentrations of the quartiles 1 to 4 are shown in Table 3. The numbers at risk are shown at 2-year intervals.

Proneurotensin and Breast Cancer in Women

During a median follow-up time of 15.7 years (IQR, 15.1-16.2) of 1929 women without cancer prior to the baseline examination, there were 123 incident cases of breast cancer with an event rate of 43.2 per 10 000 person-years. The cumulative incidence of breast cancer in quartiles of baseline fasting plasma concentration of proneurotensin is depicted in the FIGURE. After adjustment for breast cancer risk factors, each SD increase of proneurotensin was associated with an HR of 1.44 (95% CI, 1.21-1.71) for the risk of future breast cancer, and the top vs bottom quartiles of proneurotensin were associated with an HR of 2.44 (95% CI, 1.44-4.15) for risk of breast cancer (Table 3). In a model including a more limited set of breast cancer risk factors (age, use of hormone therapy, ever use of oral contraceptives, educational level, age at menarche, number of children, menopausal status, BMI, current smoking, and heredity for cancer), each SD increase of proneurotensin was associated with an HR of 1.42 (95% CI, 1.19-1.67; $P<.001$) for risk of breast cancer.

COMMENT

To our knowledge, this is the first epidemiological study on fasting concentration of proneurotensin, a stable *N*-terminal fragment of the precursor of the satiety hormone neurotensin, in relation to risk of future disease. We show that proneurotensin is associated with the development of diabetes mellitus, cardiovascular disease, total mortality, cardiovascular mortality, and breast cancer in women during long-term follow-up.

The relationship between proneurotensin and morbidity and mortality was only significant in women. It has been repeatedly shown that estradiol up-regulates expression of neurotensin.[18,31,32] Thus, it can be speculated that the higher proneurotensin observed in women than in men, and the fact that the association between proneurotensin and adverse outcomes was only significant in women, is partially explained by higher lifetime exposure to estrogen in women than in men. It should be noted that our study population included more women than men and that the interaction between proneurotensin and sex was only significant for the endpoint of incident cardiovascular dis-

ease. Thus, we cannot exclude that there may exist an association between proneurotensin and adverse outcomes also in men.

The elevation of proneurotensin several years before onset of disease indicates that proneurotensin is a marker of underlying disease susceptibility rather than being an expression of subclinical disease. As an observational study, our results do not prove any causation between proneurotensin and cardiometabolic disease and breast cancer. Two limitations of our study to consider when interpreting the results are that we did not correct for multiple comparisons and that we lack a replication cohort. Our results warrant replication in other prospective population-based studies and should encourage further research aimed at testing whether targeting the neurotensin system may have advantageous effects in preventing these common diseases in animal models and ultimately in humans.

As a satiety hormone, one may intuitively expect that high levels of proneurotensin would be related to less overeating and thus less obesity, diabetes, and cardiovascular disease. In contrast, high proneurotensin was persistently associated with increased risk of morbidity and mortality in women. The cause of this relationship is unclear. The key metabolic actions of neurotensin include digestion and metabolism of fat.[8,10] One can speculate that high plasma proneurotensin in the fasting state may be a result of compensatory increase in secretion of neurotensin due to resistance to the actions of neurotensin at the level of either its receptors or downstream of them, ie, neurotensin resistance.

Whatever the cause of high proneurotensin, experimental studies demonstrating increased expression of neurotensin and Nstr1 in breast tumors and reduced tumor growth after pharmacological blockade or RNA silencing of Ntsr1[18,19] lend support to a direct, mechanistic relationship between high-fasting proneurotensin and breast cancer development. Whereas the links between neurotensin and breast cancer are likely to be mediated through the Ntsr1, the major candidate receptor linking neurotensin to cardiovascular disease and diabetes is the Ntsr3 (SORT1), a protein that sorts various luminal proteins from the *trans*-Golgi. We and others identified genetic variation of the Ntsr3 as one of the strongest common susceptibility genes for coronary artery disease in the genome, an effect mediated through elevated levels of LDL-C.[20,21] In addition, Ntsr3 has been suggested to be an insulin-sensitive regulator of the key glucose transporter in muscle and adipose tissue, ie, glucose transporter 4 (GLUT4),[33] suggesting a role of the neurotensin system not only in metabolism of LDL-C and coronary artery disease but also in insulin resistance and diabetes development. In fact, fasting insulin concentration was one of the strongest correlates of proneurotensin.

Thus, it could be expected that insulin-resistant women, such as those with polycystic ovary syndrome, would drive the association with diabetes and cardiovascular disease in women. However, we had no data on polycystic ovary syndrome, however, the relationship between proneurotensin and cardiometabolic diseases was independent from both fasting insulin concentration and LDL-C. Thus, the mechanisms behind the relationship between high proneurotensin and cardiometabolic diseases remain to be identified.

In conclusion, fasting proneurotensin was significantly associated with development of diabetes, cardiovascular disease, breast cancer, and with total and cardiovascular mortality. The relationships between proneurotensin and all end points were significant in women but not in men; however, because there was only significant interaction between sex and proneurotensin for the outcome of incident cardiovascular disease, it remains to be shown whether the association between proneurotensin and adverse outcome is specific for women.

Author Contributions: Dr Melander had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Study concept and design: Melander, Bergmann, Orho-Melander.
Acquisition of data: Melander, Manjer, Hedblad, Bergmann, Orho-Melander.
Analysis and interpretation of data: Melander, Maisel, Almgren, Manjer, Belting, Hedblad, Engström, Kilger, Nilsson, Orho-Melander.
Drafting of the manuscript: Melander, Maisel, Bergmann.
Critical revision of the manuscript for important intellectual content: Melander, Maisel, Almgren, Manjer, Belting, Hedblad, Engström, Kilger, Nilsson, Bergmann, Orho-Melander.
Statistical analysis: Melander, Maisel, Almgren, Engström, Orho-Melander.
Obtained funding: Melander, Hedblad, Bergmann, Orho-Melander.
Administrative, technical, or material support: Melander, Maisel, Manjer, Hedblad, Bergmann, Orho-Melander.
Study supervision: Melander, Belting, Engström, Kilger, Bergmann.
Conflict of Interest Disclosures: All authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Dr Bergmann reports being the president of Sphingo Tec GmbH, which holds the patent rights for use of proneurotensin in prediction of diabetes, cardiovascular disease, and breast cancer; and also holding stock/stock options with SphingoTec GmbH. Dr Melander reports being listed as inventor on the same patent application. Dr Maisel reports provision of consultancy services for SphingoTec GmbH. Dr Engström reports being employed as a senior epidemiologist by AstraZeneca R&D. The other authors reported no disclosures.
Funding/Support: Funding was obtained from the European Research Council (StG-282255) (Dr Melander); the Swedish Heart and Lung Foundation, and the Swedish Research Council (Drs Melander and Orho-Melander); the Skåne University Hospital donation funds; the Medical Faculty, Lund University (Dr Melander); the governmental funding of clinical research within the national health services (Drs Melander and Orho-Melander); and the Albert Påhlsson Research Foundation, Region Skane, the King Gustaf V and Queen Victoria Foundation, and the Marianne and Marcus Wallenberg Foundation (Dr Melander).
Role of the Sponsors: The funding organizations had no role in the design and conduct of the study; the collection, management, analyses, and interpretation of the data; or the preparation or approval of the manuscript.
Online-Only Material: eAppendix, eTables 1 to 3, and eReferences are available at http://www.jama.com.

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Published OnlineFirst June 12, 2014; DOI: 10.1158/1055-9965.EPI-13-1200

Cancer Epidemiology, Biomarkers & Prevention

Research Article

Validation of Plasma Proneurotensin as a Novel Biomarker for the Prediction of Incident Breast Cancer

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Abstract

Background: High fasting plasma proneurotensin concentration was associated with the development of breast cancer in the Malmö Diet and Cancer Study (MDCS). Here, we aimed at replicating the initial finding in an independent second cohort.

Methods: The Malmö Preventive Project (MPP) is a population study and comprised 18,240 subjects when examined in 2002–2006. Of women without history of breast cancer at examination, we included all who developed breast cancer during follow-up ($n = 130$) until December 31, 2010, and a random sample of women without breast cancer until the end of follow-up ($n = 1{,}439$) for baseline plasma proneurotensin assessment (mean age, 70.0 ± 4.4 years). Proneurotensin was measured in fasting plasma samples and was related to the risk of later breast cancer development using multivariate logistic regression.

Results: Proneurotensin [odds ratio (OR) per standard deviation (SD) increment of LN-transformed proneurotensin] was significantly related to incident breast cancer [OR, 2.09; 95% confidence interval (CI), 1.79–2.44; $P < 0.001$; adjusted for age, body mass index (BMI), smoking, and hormone replacement therapy]. The effect estimate in the MPP was larger than in the discovery cohort (MDCS), with the main difference between the two cohorts being that women of the MPP study were on the average about 10 years older and follow-up time was shorter than that of the MDCS.

Conclusion: As initially found in the MDCS, fasting plasma proneurotensin was significantly associated with the development of breast cancer in the MPP study as well.

Impact: Measurement of plasma proneurotensin warrants further investigation as a blood-based marker for early breast cancer detection. *Cancer Epidemiol Biomarkers Prev; 23(8); 1672–6. ©2014 AACR.*

Introduction

Neurotensin is a 13–amino acid peptide primarily expressed in the central nervous system and gastrointestinal tract (1–3). Neurotensin binds to three different receptors: neurotensin receptor 1 and 2 (Ntsr1 and Ntsr2), which are G-protein–coupled receptors, and neurotensin receptor 3 (Ntsr3), which is non–G-protein–coupled and also known as Sortilin-1 (SORT1; refs. 4, 5). Interestingly, neurotensin has trophic effects on both normal and neoplastic tissue, and neurotensin and Ntsr1 have been suggested to be prognostic tumor biomarkers (6, 7). Neurotensin and Ntsr1 expression is common in human malignant ductal breast cancer tumors, and in mice xenografted with a malignant human breast cancer cell line, pharmacologic blockade or RNA silencing of the Ntsr1 reduces tumor growth (8, 9). The peripheral secretion of neurotensin is stimulated by food intake, especially of fat, and is known to regulate gastrointestinal motility and pancreatic and biliary secretion (10). Both central (intracerebroventricular) and peripheral (intraperitoneal) injection of neurotensin acutely reduces food intake in rats, an effect mediated through the Ntsr1 (11, 12), and has therefore been implicated in obesity as a satiety hormone.

Experimental data suggest neurotensin to have a role in breast tumor growth, and there is epidemiologic evidence that obesity increases the risk of breast cancer (6, 9, 13). Thus, we recently tested whether fasting plasma concentration of a stable 117–amino acid fragment from the N-terminal part of the prepro-neurotensin/neuromedin precursor hormone, referred to as proneurotensin, which is produced in stoichiometric amounts relative to the mature neurotensin, predicts the development of breast cancer during long-term follow-up in the Malmö Diet and Cancer Study (MDCS), a population-based prospective cohort study from Southern Sweden. We found that there was a strong and graded positive relationship between

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doi: 10.1158/1055-9965.EPI-13-1200

Published OnlineFirst June 12, 2014; DOI: 10.1158/1055-9965.EPI-13-1200

fasting plasma concentration of proneurotensin and later development of breast cancer with the top versus the bottom quartile of proneurotensin having an approximately 2.4-fold increased risk (14). Here, we attempted to replicate the finding in an independent cohort of women from the Malmö Preventive Project (MPP).

Materials and Methods

Study subjects

The study subjects are from the MPP. The MPP is a Swedish single-center prospective population-based study. Between 1974 and 1992, a total of 33,346 men and women of the homogenous ethnic background from the Malmö city area were recruited and screened for traditional risk factors of all-cause mortality and cardiovascular disease (CVD). A detailed description of baseline procedures may be found elsewhere (15, 16). In the years 2002–2006, all survivors from the original MPP cohort were invited for a reexamination. Of these, 18,240 participants ($n = 6{,}682$ women) responded to the invitation and were reexamined including blood sampling and immediate −80°C storage of EDTA plasma aliquotes. The reexamination in 2002–2006 represents the baseline time point in the current study. Of women participating in the 2002–2006 examination who had not participated in the MDCS discovery study (14) and were without history of breast cancer at this baseline examination, we included all who developed breast cancer during follow-up ($n = 130$) until December 31, 2010. As control group, we selected 1,500 women randomly among those participating in the 2002–2006 examination of the MPP who fulfilled the following criteria: (i) were without history of breast cancer at the 2002–2006 examination, (ii) remained free from breast cancer until the end of follow-up (December 31, 2010), and (iii) did not participate in the MDCS discovery study (14). Of these 1,500 women, fasting plasma samples for measurement of proneurotensin were available in 1,439. Thus, a total of 1,569 women were included using a case–control design. The baseline characteristics of the study population are shown in Table 1.

Table 1. Clinical characteristics of the study population

	Women in MPP ($n = 1{,}569$)
Age, y	70.0 ± 4.4
BMI, kg/m^2	27.1 ± 4.8
Current smokers, *n* (%)	307 (19.6)
HRT, *n* (%)	186 (11.9)

NOTE: Data are given as mean ± SD for normally distributed variables. Categorical data are presented as numbers (percentages).

Clinical examination, assays, endpoint assessment, and statistics

Body mass index (BMI) was defined as the weight in kilograms divided by the square of the height in meters. The use of hormone replacement therapy (HRT) and current cigarette smoking was assessed using a questionnaire. Proneurotensin was measured in stored fasting plasma specimens that were frozen at −80°C immediately at the MPP examination 2002–2006 using a recent chemiluminometric sandwich immunoassay to detect a proneurotensin precursor fragment (pro-NT 1–117) as described previously (17).

Breast cancer events were retrieved by record linkage with the Swedish Cancer Registry (SCR) using the unique 10-digit personal identification number. We included cancer *in situ* of the breast in our definition. Approximately 99% of all tumors diagnosed at Swedish Hospitals are registered in the SCR and 98% are morphologically verified (18, 19). Tumor site was registered according to ICD-7 and the International Classification of Diseases (ICD) version used at diagnosis.

We calculated odds ratios (OR) and 95% confidence intervals (CI) for the risk of incident breast cancer in a model adjusted for age, BMI, smoking, and use of HRT. Crude differences in proneurotensin concentration between cases of breast cancer and controls were calculated using the Mann–Whitney test. Because of a skewed distribution, proneurotensin was transformed with the natural logarithm (LN) and its OR is expressed per 1 standard deviation (SD) increase of the LN-transformed value in continuous analyses. We also analyzed quartiles of proneurotensin in relation to breast cancer risk.

Results

The mean age of the women in the study was 70 years (Table 1), and among the 130 women who developed breast cancer during follow-up, the mean time from the baseline examination until diagnosis of breast cancer was 3.1 ± 1.8 years. The median fasting plasma concentration ranged from 57.1 pmol/L in the lowest quartile of proneurotensin to 172 pmol/L in the highest (Table 2). The median (interquartile range) of proneurotensin in women who developed breast cancer as compared with those who did not was 123 (83.1–218) pmol/L versus 79.1 (63.0–110) pmol/L ($P < 0.001$). Each SD increment of LN-transformed proneurotensin was associated with a highly significant doubling of the OR for risk of incident breast cancer (Table 2). Additional adjustment for the use of antihypertensive medication, systolic blood pressure, diabetes mellitus, prevalent cardiovascular disease, and fasting concentrations of high-density lipoprotein cholesterol (HDL) and low-density lipoprotein cholesterol (LDL) resulted in an OR of 2.11 and 95% CI of 1.80–2.47, with $P < 0.001$. Thus, such additional adjustment did not lead to any attenuation of the strength of the results. There was no correlation between proneurotensin and the time from baseline to breast cancer diagnosis in women who developed breast cancer ($r = 0.04$; $P = 0.66$). Analysis of quartiles of

Published OnlineFirst June 12, 2014; DOI: 10.1158/1055-9965.EPI-13-1200

Table 2. Fasting plasma concentration of pro-neurotensin (pro-NT) in relation to future risk of breast cancer in the MPP

	All women	*P*	Quartile 1 (lowest pro-NT)	Quartile 2	Quartile 3	Quartile 4	P_{trend}
N/N events[a]	1,569/130		391/9	394/21	392/30	392/70	
pro-NT, pmol/L[b]	81.4 (34.5–1,100)		57.1 (34.5–63.6)	71.2 (63.7–81.4)	94.0 (81.5–115)	172 (116–1,100)	
OR (95% CI)[c]	2.09 (1.79–2.44)	<0.001	1.0 (ref)	2.34 (1.06–5.18)	3.46 (1.62–7.40)	9.11 (4.47–18.6)	<0.001

Abbreviation: pro-NT, fasting plasma concentration of pro-neurotensin.

[a]*N/N* events refer to number of participants/number of first breast cancer events.

[b]pro-NT is given as median (range).

[c]OR (95% CI) are expressed per 1 SD increment of LN-transformed pro-NT and in analyses of quartiles the lowest quartile of pro-NT (quartile 1) was defined as the reference category and the OR (95% CI) for each of quartiles 2, 3, and 4 were compared with the reference quartile. Analyses were adjusted for age, BMI, current smoking, and the use of HRT.

proneurotensin indicated a graded positive relationship over quartiles 1–4 and women belonging to the highest quartile of proneurotensin had a 9-fold increased risk of developing breast cancer as compared with the lowest quartile of proneurotensin.

To test whether the effect size of the proneurotensin association with incident breast cancer was similar over different age ranges within the MPP, fasting proneurotensin was related to risk of incident breast cancer within each quartile of baseline age. The effect size of each SD increment of LN-transformed proneurotensin was associated with a significantly increased risk in all four strata of baseline age in the MPP. The OR (95% CI) for risk of incident breast cancer in the first quartile of age (57–67 years; $P < 0.001$) was 1.97 (1.48–2.62), whereas it was 1.73 (1.29–2.33) for the second age quartile (age 67–70 years), 2.94 (2.06–4.21) for the third age quartile (70–73 years), and 2.31 (1.57–3.39) for the fourth age quartile (73–80 years; all $P < 0.001$).

To test whether the original discovery study cutoff levels that defined quartiles in the MDCS (14) were applicable in the MPP, we divided the current study sample into four categories defined by proneurotensin cutoff levels derived from quartile boundaries of the MDCS discovery study (≤77.5, >77.5–108, >108–150, >150 pmol/L, $Q1_{MDCS}$–$Q4_{MDCS}$; ref. 14). As compared with the referent group ($Q1_{MDCS}$, defined as proneurotensin ≤77.5 pmol/L; ref. 14), increasing concentration of proneurotensin concentration ($Q2_{MDCS}$, $Q3_{MDCS}$, and $Q4_{MDCS}$) was associated with an OR (95% CI) of 2.89 (1.66–5.01), 3.87 (2.09–7.16), and 8.15 (4.81–13.8; $P < 0.001$).

Discussion

Being a satiety hormone implicated in breast cancer pathogenesis, we recently hypothesized that neurotensin may underlie part of the epidemiologic relationship between obesity and breast cancer development. We found a strong positive and graded relationship between fasting plasma concentration of a stable fragment of the precursor hormone, proneurotensin, and incident breast cancer among women in the MDCS (14). Here, we replicate the original finding in an independent sample of women from the MPP and demonstrate an even stronger relationship than in the discovery cohort. There was a 9-fold increased risk of developing breast cancer among women belonging to the top as compared with the bottom quartile of proneurotensin.

There are differences between the discovery cohort of MDCS (14) and the current study. One of those differences, which may in part explain the higher effect sizes observed in the current study, is that the follow-up time was shorter. In the current study, the average follow-up time was 3.1 ± 1.8 years from baseline to event in women who developed breast cancer compared with a follow-up time of 15.7 years in the MDCS (14). Another difference between the discovery and replication study samples is that the women of the replication study were on the average 70 years of age, suggesting that they were all postmenopausal at baseline, as compared with the discovery cohort, which had a mean age of 58 ± 6 years, and thus also included some premenopausal women. Thus, it can be speculated that the relationship between proneurotensin and breast cancer risk is stronger in postmenopausal women than in premenopausal women. Finally, we cannot exclude the possibility of bias caused by unmeasured factors, which influence both proneurotensin and the incidence of breast cancer. One such factor could be that high proneurotensin is associated with an intensive health-seeking behavior, which in turn could make early diagnosis of breast cancer more likely. However, the fact that there was no relationship between proneurotensin and time from baseline to breast cancer diagnosis, argues against such a bias.

We noted that proneurotensin concentration was nominally lower in the older MPP women than in the MDCS. One possible explanation of this is that neurotensin production declines with age in postmenopausal women. Importantly, however, the relationship between proneurotensin and incidence of breast cancer

was independent of age and was equally strong and highly significant across increasing strata of age, suggesting that despite a slightly lower proneurotensin concentration with increasing age, its relationship with breast cancer risk was similar in all age strata studied.

Continuous analyses and quartile analyses within the discovery study of MDCS as well as within the current replication sample of the MPP clearly indicated that the relationship between proneurotensin and breast cancer risk was direct and linear. To test the robustness of the association and the linearity, we applied discovery study–derived quartile cutoff levels ($Q1_{MDCS}$–$Q4_{MDCS}$) on the current study and found that the association remained highly significant with a seemingly linear relationship with ORs comparable with quartile analysis based on the actual MPP data.

In summary, this study replicates the previous findings in an independent cohort, showing that proneurotensin strongly predicts future incidence of breast cancer among women with no history of breast cancer. There are several potential clinical implications for healthy women having a high proneurotensin level. One would be to be considered for more frequent screening by mammography and possibly by regular magnetic resonance examination of the breasts. Another one, given the widespread and sometimes well-indicated use of HRT among postmenopausal women, would be to not prescribe this treatment to women with a high proneurotensin level as their increased breast cancer risk may override the beneficial effects of HRT.

We conclude that, as initially found in the MDCS study, fasting plasma proneurotensin was significantly and independently associated with the development of breast cancer in the MPP study as well. The replication suggests that proneurotensin may be of value for the identification of women at high risk of breast cancer development.

Disclosure of Potential Conflicts of Interest

G. Engström was formerly employed as an epidemiologist at AstraZeneca R&D. A. Bergmann is CEO of Sphingotec and has ownership interest (including patents) in the same. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: O. Melander, G. Engström, J. Struck, A. Bergmann, M. Orho-Melander
Development of methodology: O. Melander, A. Bergmann
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O. Melander, J. Manjer, B. Hedblad, P. Nilsson, J. Struck, A. Bergmann, M. Orho-Melander
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O. Melander, M. Belting, J. Manjer, A.S. Maisel, B. Hedblad, G. Engström, P. Nilsson, J. Struck, O. Hartmann, A. Bergmann, M. Orho-Melander
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): O. Melander, J. Manjer, B. Hedblad, A. Bergmann, M. Orho-Melander
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Acknowledgments

The authors would like to dedicate this article to their excellent and unforgettable former clinical researcher colleague, Dr. Jana Papassotiriou, who passed away much too early.

Grant Support

Funding was obtained from the European Research Council (StG-282255), the Swedish Heart and Lung Foundation, Swedish Research Council, Swedish Cancer Fund; the Novo Nordisk Foundation, the Skåne University Hospital donation funds; the Medical Faculty, Lund University; the governmental funding of clinical research within the national health services, the Albert Påhlsson Research Foundation, Region Skane, the King Gustaf V and Queen Victoria Foundation, and the Marianne and Marcus Wallenberg Foundation.

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Received November 18, 2013; revised May 20, 2014; accepted May 28, 2014; published OnlineFirst June 12, 2014.

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Published OnlineFirst June 12, 2014; DOI: 10.1158/1055-9965.EPI-13-1200

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Published OnlineFirst June 12, 2014; DOI: 10.1158/1055-9965.EPI-13-1200

Cancer Epidemiology, Biomarkers & Prevention

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Cancer Epidemiol Biomarkers Prev 2014;23:1672-1676. Published OnlineFirst June 12, 2014.

VOLUME 33 · NUMBER 24 · AUGUST 20 2015

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Stable Peptide of the Endogenous Opioid Enkephalin Precursor and Breast Cancer Risk

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Published online ahead of print at www.jco.org on July 13, 2015.

Supported by the European Research Council StG-282255, the Swedish Heart and Lung Foundation, the Swedish Research Council, the Skåne University Hospital donation funds, the Medical Faculty of Lund University, the Governmental funding of clinical research within the national health services, the Albert Påhlsson Research Foundation, Region Skane, the King Gustaf V and Queen Victoria Foundation, the Novo Nordisk Foundation, and the Marianne and Marcus Wallenberg Foundation.

Authors' disclosures of potential conflicts of interest are found in the article online at www.jco.org. Author contributions are found at the end of this article.

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0732-183X/15/3324w-2632w/$20.00

DOI: 10.1200/JCO.2014.59.7682

ABSTRACT

Purpose
In experimental studies, enkephalins (ENKs) and related opioids have been implicated as negative regulators of breast cancer development by enhancing immune-mediated tumoral defense as well as directly inhibiting cancer cells. We hypothesized that plasma levels of ENKs are predictive of the long-term breast cancer risk. Therefore, our objective was to measure pro-ENK A, a surrogate for mature ENK, and evaluate its predictive value for the development of breast cancer in a large population of middle-aged women and an independent study population.

Patients and Methods
We related pro-ENK in fasting plasma samples from 1,929 healthy women (mean age, 57.6 ± 5.9 years) of the Malmö Diet and Cancer study to breast cancer incidence (n = 123) during a median follow-up of 14.7 years. For replication, pro-ENK was related to risk of breast cancer (n = 130) in an older independent sample from the Malmö Preventive Project consisting of 1,569 women (mean age, 70.0 ± 4.4 years).

Results
In the Malmö Diet and Cancer study, pro-ENK was inversely related to the risk of incident breast cancer, with a hazard ratio per each standard deviation increment of logarithm-transformed pro-ENK of 0.72 (95% CI, 0.62 to 0.85; $P < .001$). The linear elevation of risk over pro-ENK quartiles 3, 2, and 1, with the fourth quartile as a reference, was 1.38 (95% CI, 0.73 to 2.64), 2.29 (95% CI, 1.26 to 4.15), and 3.16 (95% CI, 1.78 to 5.60; for the trend, $P < .001$), respectively. These results were replicated in the Malmö Preventive Project, where the continuous odds ratio for incident breast cancer was 0.63 (95% CI, 0.52 to 0.76; $P < .001$) and the risk over pro-ENK quartiles 3, 2, and 1, where the fourth quartile was the reference, was 2.48 (95% CI, 1.25 to 4.94), 2.94 (95% CI, 1.50 to 5.77), and 4.81 (95% CI, 2.52 to 9.18; for the trend, $P < .001$), respecitvely.

Conclusion
Low fasting plasma concentration of the opioid precursor peptide pro-ENK is associated with an increased risk of future breast cancer in middle-aged and postmenopausal women.

J Clin Oncol 33:2632-2638. © 2015 by American Society of Clinical Oncology

INTRODUCTION

Breast cancer is the most frequent type of tumor, accounting for approximately 25% of new cases, as well as the most common cause of cancer-related death among women worldwide.[1] Mammographic screening is broadly used to detect existing breast cancer at an early stage; however, lacking are biomarkers that can aid in predicting future development of breast cancer in healthy women to allow for pharmacologic primary prevention of breast cancer specifically directed at women with high risk.

Recently, the concept of the tumoral macroenvironment, that is, processes that operate at the systemic level, has gained increasing attention in the field of breast cancer.[2,3] What characterizes a protumorigenic or antitumorigenic macroenvironment remains an important question with relevance for primary prevention, early diagnosis, and treatment of breast cancer.

The level of neurobehavioral stress regulates the activity of key components of immune surveillance against cancer, including natural killer cells, cytotoxic T cells, and macrophages. Although our understanding of the mechanism of this phenomenon is far from complete, evidence for an important role of the hypothalamic-pituitary-adrenal signaling axis is convincing.[4-6] Results of independent studies

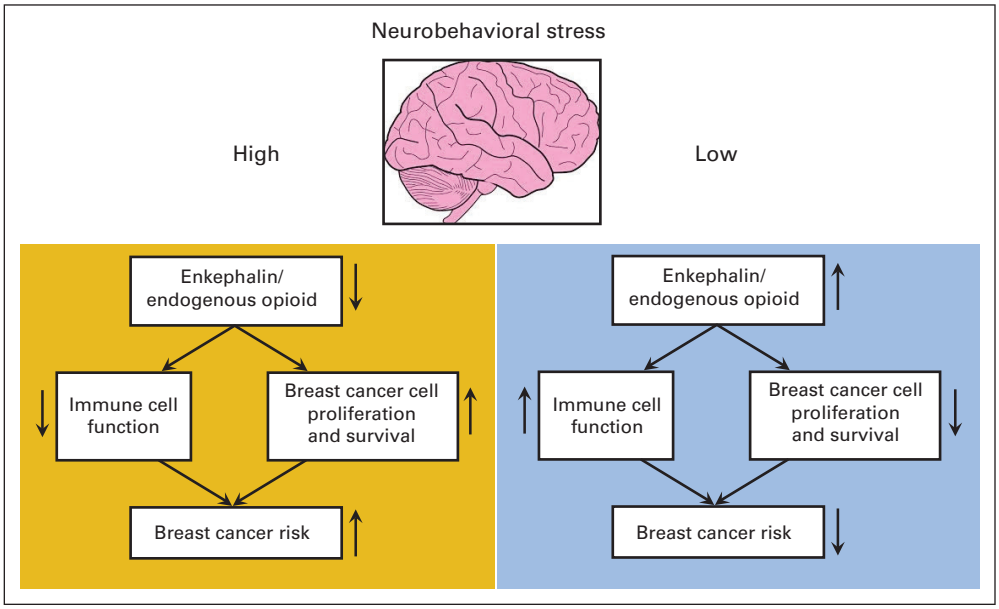

Fig 1. Schematic representation of the potential biologic relationships among neurobehavioral stress, levels of enkephalin endogenous opioids, tumor immune surveillance, and breast cancer cell proliferation and survival.

have linked breast-tumor induction and growth to negative stress exposure and to the immunologic phenotype of the host.[7,8] Enkephalins (ENKs), which, with endorphins and dynorphins, constitute the major classes of opioid neurohormones, provided an early molecular link between the neuroendocrine and immune systems.[9,10] ENK peptides, originally described as endogenous analgesics that bind to morphine receptors of the CNS,[11,12] have antianxiety and antidepressant effects and, therefore, are often referred to as feel-good hormones. These effects are mediated by modulation of, for example, the hypothalamic-pituitary-adrenal axis, and can enhance the immune response and tumor defense in animals as well as in humans.[13-17] In an experimental model of breast cancer, enhanced endogenous levels of closely related β-endorphin by neuronal transplantation suppressed stress levels, promoted immune function, and reduced tumor appearance.[18] Indeed, various immune-competent cells express opioids as well as their receptors.[19,20] Therefore, ENK has been considered a cytokine that integrates neurobehavioral endocrine signaling with immune defense activity at the systemic level.[21,22]

In addition to their immunoregulatory functions, ENKs and related opioids are known to negatively regulate the proliferation and survival of a wide variety of cancer cells in vitro and in vivo, including those derived from human breast tumors.[23-28] These effects may be mediated by both opiate and nonopiate receptors, for example, somatostatin receptors.[29,30] Further, an interaction between opioid and steroid receptors has been suggested because only estrogen-dependent breast cancer cells appear to be susceptible to the growth-inhibitory effect of opioids.[31,32] Therefore, enkephalins and related opioid peptides may negatively regulate carcinogenesis and the progression of breast tumors by means of a dual mechanism involving stimulation of the immune tumor defense system and direct inhibitory actions on transformed breast epithelial cells.

Given these potential links between ENKs and breast cancer (Fig 1), we hypothesized that plasma levels of ENKs are predictive of the long-term breast cancer risk because they regulate the onset and further development of breast tumors. Because mature ENKs are unstable both in vitro and in vivo, we instead measured a stable 41-amino-acid fragment of their precursor, pro-ENK A, a surrogate molecule for the release of mature ENKs.[33] We tested whether fasting plasma concentration of pro-ENK is predictive of the development of breast cancer during long-term follow-up in a large population of middle-aged women. This was followed by replication in an independent study population.

PATIENTS AND METHODS

Study Populations

The Malmö Diet and Cancer (MDC) study is a population-based, prospective cohort of 28,449 men (born from 1923 to 1945) and women (born from 1923 to 1950) from the city of Malmö in southern Sweden who underwent baseline examinations between 1991 and 1996. For methodologic details, see the Appendix (online only). From this cohort, 6,103 men and women were randomly selected to participate in the MDC Cardiovascular Cohort (MDC-CC), which was designed to investigate the epidemiology of carotid artery disease between 1991 and 1994.[34] Of women free from breast cancer at the time of baseline examination, 1,929 had fasting plasma samples available for analysis of pro-ENK and complete data for baseline covariates used in the fully adjusted model for incidence of breast cancer, as described in a recent previous study (Fig 2).[35] During a median follow-up of 14.7 years (interquartile range, 14.1 to 15.2 years), 123 incident cases of breast cancer occurred among these 1,929 women.[35]

Malmö Preventive Project

The replication cohort from the Malmö Preventive Project (MPP) was described recently.[36] The MPP is a Swedish single-center, prospective, population-based study. Between 1974 and 1992, a total of 33,346 men and women, who had a homogeneous ethnic background from the Malmö area, were recruited and screened for traditional risk factors of all-cause mortality and cardiovascular disease (see the Appendix). From 2002 to 2006, all survivors from the original

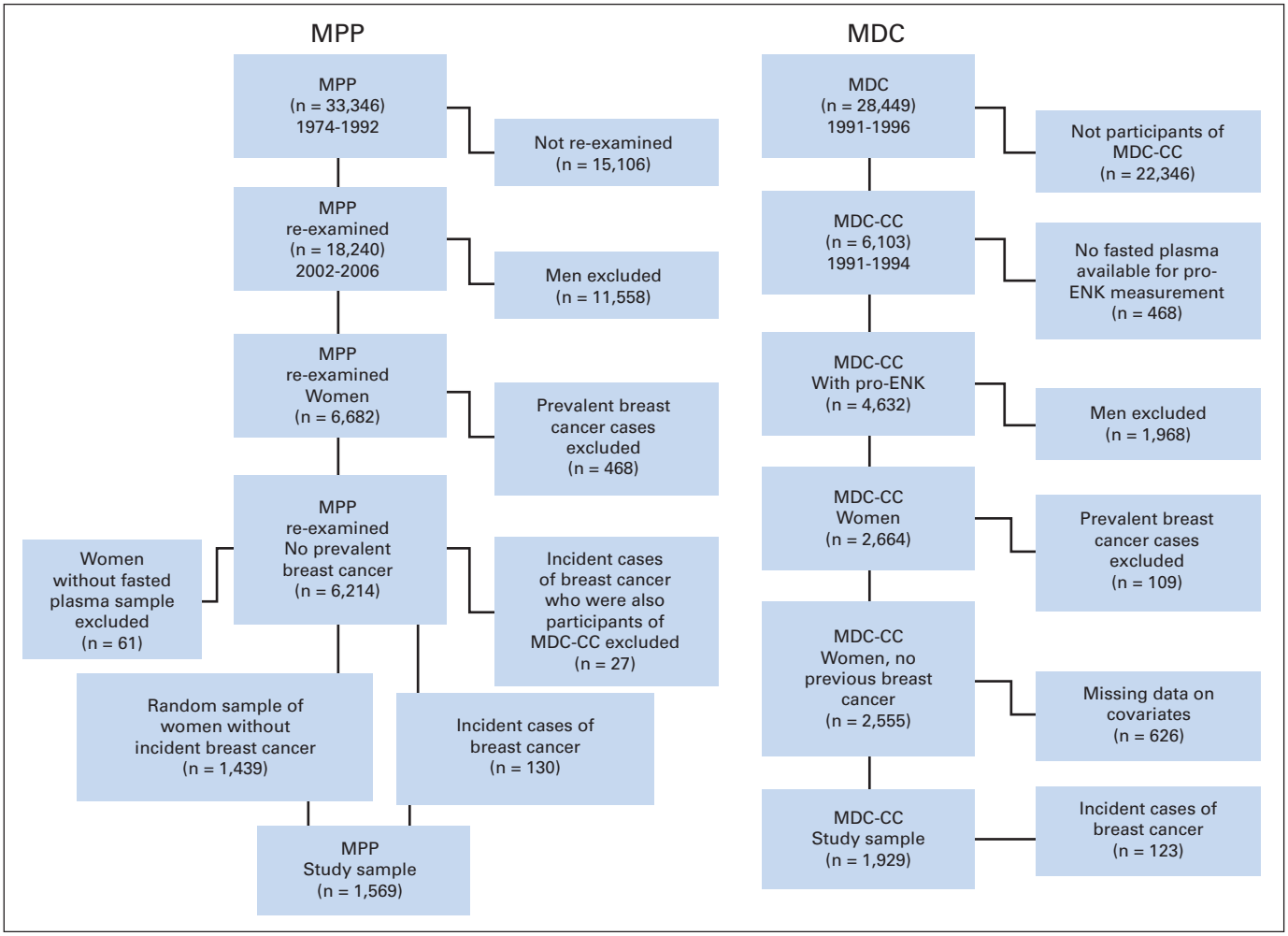

Fig 2. Diagram of the Malmö Diet and Cancer study (MDC) and the Malmö Preventive project (MPP). ENK, enkephalin; MDC-CC, MDC Cardiovascular Cohort.

MPP cohort were invited for repeat examination; 18,240 participants (6,682 women) responded. They were re-examined, and blood samples were obtained and immediately stored at −80°C in plasma aliquots containing 1-EDTA. This re-examination from 2002 to 2006 represents the baseline time point in the current study. Selecting from the women who participated in this examination who had not participated in the MDC-CC discovery study[35] and who had no history of breast cancer at this examination, we included all 130 who developed breast cancer during follow-up as of December 31, 2010. For a control group, we randomly selected 1,500 women among those participating in the MPP examination from 2002 to 2006 who had no history of breast cancer at that examination; who remained free from breast cancer during follow-up until December 31, 2010; and who did not participate in the MDC-CC discovery study.[35] Fasting plasma samples were available for 1,439 of these women for the measurement of pro-ENK. Therefore, 1,569 women were included by using a case-control design (Fig 1). Baseline characteristics of the study population are shown in Table 1.

Assays

Pro-ENK levels were measured in stored fasting plasma specimens that were immediately frozen to −80°C during the MDC-CC and MPP examinations. For this, we used a chemiluminometric sandwich immunoassay. The procedure is described in detail in the Data Supplement.

End-Point Retrieval

Breast cancer events were retrieved by means of record linkage with the Swedish Cancer Registry by using the unique 10-digit personal identification number. We included cancer in situ of the breast in our definition. Approximately 99% of all tumors diagnosed at Swedish hospitals are included in the registry, and 98% are morphologically verified. Tumor site was registered according to the International Classification of Diseases, 7th Revision, and the version of the International Classification of Diseases used at diagnosis.

Statistics

We calculated hazard ratios for the MDC-CC and odds ratios the MPP with 95% CIs relating pro-ENK levels at the baseline examinations to the risk of breast cancer using a Cox proportional hazards model for the MDC-CC and logistic regression for the MPP. Because the distribution was skewed, pro-ENK data were transformed with the natural logarithm (ln) throughout (see the Appendix for details).

Table 1. Clinical Characteristics of the Study Populations in the Analyses of Incident Breast Cancer

Characteristic	Plasma Proenkephalin Level: Below Median (n = 963) Mean ± SD	Plasma Proenkephalin Level: Above Median (n = 966) Mean ± SD
MDC-CC		
Age, years	57.2 ± 5.9	58.0 ± 5.8
Body mass index, kg/m^2	26.2 ± 4.4	24.8 ± 3.8
Age at menarche, years	13.5 ± 1.5	13.7 ± 1.5
No. of children	1.8 ± 1.1	1.8 ± 1.2
Current smokers, No. (%)	233 (24.2)	252 (26.1)
Menopausal hormone therapy, No. (%)	219 (22.7)	172 (17.8)
Use of oral contraceptives ever, No. (%)	435 (45.2)	415 (43.0)
Educational level, No. (%)		
Maximum of 9 years	712 (73.9)	727 (75.3)
9 to 12 years	66 (6.9)	75 (7.8)
Completed university degree	185 (19.2)	164 (17.0)
Menopausal status, No. (%)*		
Premenopausal	204 (21.2)	142 (14.7)
Perimenopausal	80 (8.3)	65 (6.7)
Postmenopausal	679 (70.5)	759 (78.6)
	Below Median (n = 785) Mean ± SD	Above Median (n = 784) Mean ± SD
Malmö Preventive Project		
Age, years	69.8 ± 4.5	70.1 ± 4.2
Body mass index, kg/m^2	27.2 ± 4.7	27.1 ± 4.8
Current smokers, No. (%)	154 (19.6)	153 (19.5)
Menopausal hormone therapy, No. (%)	99 (12.6)	87 (11.1)

Abbreviations: MDC-CC, Malmö Diet and Cancer study Cardiovascular Cohort; SD, standard deviation.
*At the time of baseline examination.

All participants gave written informed consent. The study was approved by the ethics committee at Lund University, Lund, Sweden, and followed the precepts established by the Helsinki Declaration.

RESULTS

Pro-ENK and Breast Cancer in the MDC-CC

Table 1 shows the baseline characteristics of the MDC-CC and MPP study participants. In MDC-CC, 123 incident breast cancer events occurred during a median follow-up of 14.7 years. No deviation was observed from the proportionality of the hazards assumption in the Cox proportional hazards models. After adjustment for age, body mass index (BMI), and smoking, a graded inverse association was observed between fasting plasma pro-ENK concentration and the risk of breast cancer development in the MDC-CC (Table 2). The Kaplan-Meier plot is shown in Figure 3. For women in the lowest quartile of pro-ENK values, the risk of developing cancer was more than three-fold higher than that for women in highest quartile (Table 2).

Pro-ENK and Breast Cancer in the MPP

The MPP sample was approximately 10 years older than the MDC-CC. We observed a highly significant age-, BMI-, and smoking-adjusted inverse association between fasting plasma pro-ENK concentration and risk of breast cancer. The lowest quartile of pro-ENK values was associated with an almost five-fold increase in the odds of breast cancer compared with the highest quartile (Table 2).

Pro-ENK, Reproductive Variables, Educational Level, and Further Adjustments

In a multivariable linear regression model in the MDC-CC with the *z* score of ln-transformed pro-ENK as the dependent variable, independent determinants of pro-ENK retained in the model at a *P* value of $< .10$ were BMI, current use of menopausal hormone therapy, age at menarche, menopausal status, and educational level (Table 3). Therefore, we entered current use of menopausal hormone therapy, age at menarche, menopausal status, and educational level as additional covariates in addition to age, BMI, and smoking in the multivariable Cox regression model for breast cancer incidence in the MDC-CC. However, this further adjustment only marginally changed the association between pro-ENK value and incident breast cancer. The hazard ratio per each standard deviation (SD) increment of ln-transformed pro-ENK was 0.75 (95% CI, 0.63 to 0.88; $P = .001$). Of note, of the added covariates, only current use of menopausal hormone therapy was significantly related to the risk of breast cancer (1.90; 95% CI, 1.30 to 2.78; $P = .001$).

We made an additional adjustment in the MPP for current use of menopausal hormone therapy in addition to age, BMI, and current smoking in the logistic regression model. The resultant odds ratio for incident breast cancer per 1 SD increment of ln-transformed pro-ENK remained unchanged (0.63; 95% CI. 0.52 to 0.76; $P < .001$; Table 2).

Finally, we assessed the MDC to ascertain whether self-reported high, intermediate, or low stress was related to pro-ENK values. The odds ratio of having a low level of pro-ENK in quartile 1 was 1.40 (95% CI, 1.04 to1.88; $P = .026$) for subjects reporting high versus low stress. Subjects with intermediate stress versus those with low stress had an odds ratio of 1.25 (95% CI, 0.98 to 1.60; $P = .073$) of having a low pro-ENK level (*P* value for the linear trend over strata of stress exposure = .02).

DISCUSSION

To our knowledge, this is the first study of the association between plasma concentration of pro-ENK and risk of future breast cancer in healthy women. We found an inverse and graded association between pro-ENK value and risk of breast cancer in a large, population-based female cohort, and also replicated the results in an independent population. The observed effect size was substantial, with an approximately three- to four-fold higher risk in the lowest versus highest quartile. This was independent of age, BMI, menopausal hormone therapy, and socioeconomic and reproductive factors.

Breast cancer is the most common cancer and cause of cancer-related death in women,[1] although huge advancements have been made in its screening, early detection, and treatment. In contrast, primary prevention of the disease is still hampered by insufficient risk-stratification tools for women without a history of breast cancer and without mammographic signs of existing breast cancer. Although clinically important for the prognosis of the individual patient, previous breast cancer or evidence of existing breast cancer, by definition, are not valid for prediction of a first breast cancer event and, therefore, not useful in the primary preventive setting. Lessons from prediction and primary prevention of cardiovascular disease suggest that identification of healthy individuals at high risk must be done many years,

Table 2. Fasting Plasma Concentration of Pro-ENK in Relation to Future Risk of Breast Cancer in the MDC Study and the MPP

	All Women	*P*	Quartile 1	2	3	4	*P* for Trend
MDC							
No.							
Participants	1,929		482	481	485	481	
First events	123		49	36	22	16	
Pro-ENK, pmol/L							
Median	47.3		36.2	44.0	50.1	60.9	
Range	9.00-215		9.00-40.3	40.4–47.2	47.3-54.1	54.2-215	
HR*		< .001	1.0†				< .001
Value	0.72			0.73	0.44	0.32	
95% CI	0.62 to 0.85			0.47 to 1.12	0.26 to 0.73	0.18 to 0.56	
HR‡		.001	1.0†				< .001
Value	0.75			0.73	0.45	0.34	
95% CI	0.63 to 0.88			0.47 to 1.13	0.27 to 0.75	0.19 to 0.60	
MPP							
No.							
Participants	1,569		392	393	392	392	
First events	130		53	35	30	12	
Pro-ENK, pmol/L							
Median	52.2		33.9	46.4	58.3	78.9	
Range	13.5-382		13.5-40.9	41.0-52.2	52.3-65.8	65.9-382	
OR*		< .001	1.0†				< .001
Value	0.63			0.61	0.52	0.21	
95% CI	0.52 to 0.76			0.39 to 0.96	0.32 to 0.83	0.11 to 0.40	
OR‡		< .001	1.0†				< .001
Value	0.63			0.62	0.52	0.21	
95% CI	0.52 to 0.76			0.39 to 0.97	0.33 to 0.84	0.11 to 0.40	

Abbreviations: HR, hazard ratio; MDC, Malmö Diet and Cancer study; MPP, Malmö Preventive Project; OR, odds ratio; pro-ENK, proenkephalin fasting plasma concentration.

*HR in the MDC and OR in the MPP are expressed per 1 standard deviation increment of natural logarithm–transformed pro-ENK. In analyses of quartiles, the lowest quartile, quartile 1, was defined as the reference category, and the HR in the MDC and OR in the MPP for each of quartiles 2, 3 and 4 were compared with the reference quartile. Analyses were adjusted for age, body mass index, and current smoking.

†Reference.

‡Analyses were additionally adjusted for menopausal hormone therapy, age at menarche, menopausal status, and educational level in the MDC and for menopausal hormone therapy in the MPP.

for example, 5 to 10 years, before a first event occurs in order for any pharmacologic primary preventive therapy to be effective. In this setting, pro-ENK appears to be a promising circulating biomarker, one which may aid in risk prediction during long-term follow-up. However, more studies are needed before its use is implemented in routine clinical practice because the homogeneous study populations in Sweden may limit generalizability of the results to other racial or ethnic groups in other geographic areas. Of interest, we recently observed strong prediction of breast cancer by using fasting plasma concentration of proneurotensin, which represents a different pathway related to metabolism. This finding suggests that an approach involving multimarkers may ultimately be the best tool for predicting breast cancer in the primary preventive setting.[35,36]

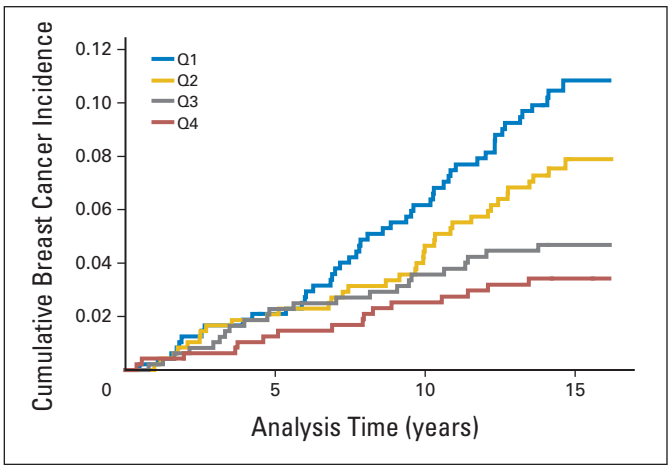

Fig 3. Kaplan-Meier plot shows 1 − cumulative breast cancer event-free survival during follow-up in quartiles 1 to 4, with quartile 1 representing lowest baseline fasting plasma concentrations of proenkephalin in the Malmö Diet and Cancer study.

Options for the prevention of breast cancer include pharmacologic prevention and interventions that have significant adverse effects, such as mastectomy. Identification of risk factors is fundamental to risk-benefit calculations in the selection of women for whom such treatments are appropriate and for their inclusion in upcoming trials of pharmacologic treatments, such as estrogen blocking therapy, aimed at the primary prevention of breast cancer.

Mammographic screening is performed every second year in Swedish women age 40 to 74 years to identify individuals with subclinical but existing breast cancer, whereas pro-ENK testing may identify higher-risk individuals several years before they develop detectable breast cancer. Unfortunately, we do not have data regarding the family history of breast cancer and the presence of benign breast disease. Therefore, we cannot determine whether the association between low levels of pro-ENK and breast cancer risk may be mediated through, or confounded by, these factors. Another limitation of our study is that

Table 3. Determinants of Fasting Plasma Concentration of Pro-ENK Retained in a Linear Regression Model With Backward Elimination in the Malmö Diet and Cancer Study

Independent Pro-ENK Determinants	β Coefficient*	95% CI	P
Body mass index, per kg/m^2	−0.04	−0.06 to −0.03	< .001
Educational level, per category†	−0.05	−0.11 to −0.01	.084
Age at menarche, per year	0.03	0.00 to 0.06	.038
Menopausal status, per category‡	0.16	0.10 to 0.22	< .001
Use of menopausal hormone therapy, yes or no	−0.22	−0.33 to −0.11	< .001

NOTE. The following variables were entered in the linear regression model with backward elimination at $P < .10$: age, body mass index, smoking, educational level, menopausal hormone therapy, use of oral contraceptives never or ever, age at menarche, number of children, and menopausal status.
Abbreviation: pro-ENK, proenkephalin.
*β is expressed as the per-unit, specified per variable, increase of the respective dependent variable in relation to per increment of the natural logartihm–transformed standardized values of pro-ENK.
†Expressed per category increment, where the first category is maximum of 9 years of education, the second category is 9 to 12 years of education, and the third category is completion of a university degree.
‡Expressed per category increment, where the first category is premenopausal, the second category is perimenopausal, and the third category is postmenopausal.

we do not have access to individual data from mammographic examinations actually performed, and we cannot exclude a confounding relationship between pro-ENK and mammographic screening density. However, the Kaplan-Meier curves of first breast cancer events versus pro-ENK data continued to separate throughout follow-up, and most evidently from 10 to 15 years after pro-ENK measurement (Fig 3). This observation is of interest when the potential biologic explanations of our findings are considered.

Epidemiologic findings regarding the relationship between exposure to stress and the risk of breast cancer are mixed, and, to date, no evidence of a causal link between stress and breast cancer has been reported. Still, the role of neurobehavioral stress as a negative regulator of immune surveillance against breast cancer by its effects on cytotoxic T cells, natural killer cells, and macrophages, for example, is gaining interest.[4-10] In a large cohort study of more than 10,000 women, breast cancer risk was mainly associated with life events that occurred more than 10 years before diagnosis.[37] The well-established immune-stimulatory function of ENK-derived peptides, perhaps most importantly methionine-ENK (MENK), makes it tempting to speculate that our findings reflect a role of low pro-ENK levels linked to increased neurobehavioral stress and decreased immune defense against the initial events of breast tumor development.[13-17] Our cross-sectional finding that self-reported stress was associated with a low level of pro-ENK may stimulate future intervention studies to determine if pro-ENK secretion can be increased by stress-modulating therapies, for example, provided that a causal link between pro-ENK and breast cancer actually exists. A recent study suggested that hypothalamic transplantation with neurons expressing the opioid peptide β-endorphin reduced tumoral incidence, growth, and metastasis in a rat model of chemically induced breast cancer.[18] These effects were correlated with increased activity of natural killer cells and macrophages, suggesting a protective role for β-endorphin by means of enhanced immune function. Moreover, a reduced risk of breast cancer was observed in individuals receiving maintenance treatment with the synthetic opioid methadone; this finding supports the notion of a protective effect of opioids against breast cancer in humans.[38] Our findings should motivate studies to determine the preventive effect of MENK therapy in experimental models of spontaneous breast tumor development. These results may provide a rationale for clinical interventional studies in which MENK is given to women with low pro-ENK levels and possibly other risk factors of breast cancer.

In conclusion, we show for the first time that a low fasting plasma concentration of the opioid precursor peptide pro-ENK is strongly associated with, and may therefore contribute to, the prediction of increased risk of future breast cancer in healthy middle-aged and postmenopausal women. Furthermore, our findings in large human populations support the results of several previous experimental studies and should encourage further research regarding the ENK system as a potential therapeutic target in the prevention and treatment of breast cancer.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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Data analysis and interpretation: All authors
Manuscript writing: All authors
Final approval of manuscript: All authors

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Stable Peptide of the Endogenous Opioid Enkephalin Precursor and Breast Cancer Risk

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Appendix

Study Populations

Malmö Diet and Cancer study. The Malmö Diet and Cancer (MDC) study is a population-based, prospective cohort of 28,449 men (born from 1923 to 1945) and women (born from 1923 to 1950) from the city of Malmö in southern Sweden who underwent baseline examinations between 1991 and 1996 (Berglund G: J Intern Med 233:39-79, 1993). From this cohort, 6,103 men and women were randomly selected to participate in the MDC Cardiovascular Cohort (MDC-CC) study, which was designed to investigate the epidemiology of carotid artery disease between 1991 and 1994.[34] Of the women who had no breast cancer at baseline examination, 1,929 had fasting plasma samples available for analysis of proenkephalin (pro-ENK) and complete data for the baseline covariates used in the fully adjusted model for the incidence of breast cancer, as described in a recent previous study.[35]

The baseline examination procedure has been described before (Belting M, et al: Cancer Epidemiol Biomarkers Prev 21:513-522, 2012; Enhorning S, et al: Circulation 121:2102-2108, 2010; Melander O, et al: JAMA 302:49-57, 2009). Blood sampling was performed in the fasted state, and plasma aliquots were stored at −80°C with EDTA. Body mass index (BMI) was defined as weight in kilograms divided by the square of height in meters. Data for smoking, educational level, current use of menopausal hormone therapy, use of oral contraceptives never or ever, age at menarche, and number of children were ascertained from a questionnaire (Manjer J, et al: Scand J Public Health 30:103-112, 2002). Educational level was divided into three categories: maximum of 9 years of education, 9 to 12 years of education, and university degree completed. Menopausal status was assessed with the help of a questionnaire and medical records, as described previously (Butt S, et al: Int J Cancer 125:1926-1934, 2009). Current smoking was defined as any cigarette smoking within the past year. During a median follow-up of 14.7 years (interquartile range, 14.1 to 15.2 years), 123 incident cases of breast cancer occurred among these 1,929 women.[35]

Malmö Preventive Project

The replication cohort from the Malmö Preventive Project (MPP) has been described recently.[36] MPP is a Swedish single-center, prospective, population-based study. Between 1974 and 1992, a total of 33,346 men and women with a homogeneous ethnic background from the Malmö area were recruited and screened for traditional risk factors of all-cause mortality and cardiovascular disease. Detailed description of baseline procedures may be found elsewhere (Fedorowski A, et al: Eur Heart J 31:85-91, 2010; Berglund G, et al: J Intern Med 239:489-497, 1996). From 2002 to 2006, all survivors from the original MPP cohort were invited for repeat examination. Of these, 18,240 participants (n = 6,682 women) responded to the invitation and were re-examined. Blood samples were obtained and immediately stored at −80°C in plasma aliquots containing EDTA. This re-examination from 2002 to 2006 represents the baseline time point in the current study. From the women participating in this examination who had not participated in the MDC-CC discovery study[35] and who had no history of breast cancer at this baseline examination, we included all 130 who developed breast cancer during follow-up as of December 31, 2010. For a control group, we randomly selected 1,500 women from among those participating in the MPP examination from 2002 to 2006 who had no history of breast cancer at this examination; who remained free from breast cancer during follow-up as of December 31, 2010; and who did not participate in the MDC-CC discovery study.[35] Of these 1,500 women, 1,439 had fasting plasma samples available for the measurement of pro-ENK. Therefore, 1,569 women were included by using a case-control design. Baseline characteristics of the study population are shown in Table 1.

Assays

Pro-ENK was measured by using a chemiluminometric sandwich immunoassay to test stored fasting plasma specimens that were frozen to −80°C immediately during the MDC-CC and MPP examinations. The assay for stable pro-ENK consisting of amino acids 119-159 of pro-ENK A has been previously reported[33] and was modified, as recently described (Ng LL, et al: J Am Coll Cardiol 63:280-289, 2014). In brief, two mouse monoclonal anti–pro-ENK antibodies were developed by immunizing them with pro-ENK peptide consisting of amino acids 119-159 of pro-ENK A. We used 2 μg of one antibody to coat polystyrene tubes. The other antibody labeled with methyl-acridinium ester served as the detector antibody. Standards, ie, pro-ENK peptide, amino acids 119-159 of pro-ENK A, and 50-μL samples were incubated in tubes with 150 μL of the detector antibody. After equilibration, tubes were washed, and bound chemiluminescence was detected with a luminometer LB952T/16 (Berthold, Bad Wildbad, Germany). The lower detection limit of the assay was 5.5 pmol/L. Intra-assay and interassay coefficients of variation were 6.4% and 9.5% at 50 pmol/L and 4.0% and 6.5% at 150 pmol/L.

Psychological Stress

A categorical variable representing psychological stress was constructed from questions measuring job strain by using items in the validated Swedish version (Sanne B, et al: Scand J Public Health 33:166-174, 2005) of Karasek's and Theorell's demand-control model and one question assessing non–work-related stress (Karasek RA: Adm Sci Q 24:285-308, 1979; Theorell T, et al: Scand J Work Environ Health 14:189-196, 1988). The psychological demands subscale consisting of five items and the decision latitude subscale consisting of six items from the job strain questionnaire were assessed using a four-point Likert scale. Scores from each subscale were summarized without weighting, and job strain was calculated by dividing psychological demands with decision latitude. The results dichotomized at the median to allow for a low-strain and a high-strain group.

Non–work-related stress was assessed by using one question: Have you suffered from stress or mental pressure lately because of problems or demands not related to your work? Answers of yes and no represented high stress and low stress levels, respectively. The dichotomized variables job strain and non–work-related stress were subsequently combined to create a categorical variable representing low, intermediate, or high psychological stress. Low psychological stress was assigned to individuals who belonged to the low-strain and low-stress group; intermediate stress, to those with low strain and high stress or high strain and low stress; and high stress, to those with high strain and high stress.

End-Point Retrieval

Breast cancer events were retrieved by means of record linkage with the Swedish Cancer Registry by using the unique 10-digit personal identification number. We included cancer in situ of the breast in our definition. Approximately 99% of all tumors diagnosed at Swedish hospitals are included in the registry, and 98% are morphologically verified (Garne JP: Doctoral thesis, Lund University, 1996; Swedish Cancer Registry: Stockholm, Sweden, The National Board of Health and Welfare, publication 2001-42-4, 2001). Tumor site was registered according to the International Classification of Diseases, 7th Revision, and the version of the International Classification of Diseases used at diagnosis.

Statistics

We calculated hazard ratios for the MDC-CC and odds ratios for the MPP with 95% CIs relating pro-ENK at the baseline examinations to risk of breast cancer in a model adjusted for baseline age, BMI, and smoking using Cox proportional hazards model for the MDC-CC and logistic regression for the MPP. We tested the proportional hazards assumption using weighted residuals. In the MDC-CC, for whom additional measures of estrogen exposure and socioeconomic factors at baseline were available, we subsequently used multiple linear regression with backward elimination (retention $P < .10$) to determine independent determinants of fasting plasma pro-ENK concentration, entering age, BMI, smoking, educational level, menopausal hormone therapy, use of oral contraceptives never or ever, age at menarche, parity, and menopausal status as independent variables and pro-ENK as the dependent variable. Variables retained in the model were subsequently included as covariates in the Cox proportional hazard model for breast cancer risk in addition to age, BMI, and smoking. Because of a skewed distribution, pro-ENK was transformed with the natural logarithm (ln) throughout, and its hazard ratio and odds ratio in relation to breast cancer risk are expressed as per 1 standard deviation (SD) increment of the ln-transformed value in continuous analyses. We also analyzed quartiles of pro-ENK in relation to breast cancer risk.

Results

Cox proportional hazards model instead of logistic regression in the MPP. As an alternative to logistic regression relating pro-ENK to breast cancer risk in the MPP, we applied a Cox proportional hazards model. Analysis on the basis of a Cox proportional hazards model, in which we accounted for the time to a breast cancer event with censoring at the time of death or emigration, was an alternative to logistic regression. This gave results similar to those of logistic regression. The hazard ratio per 1 SD increment of pro-ENK was 0.65 (95% CI, 0.55 to 0.78; $P < .001$).

Further adjustments, interactions, and stratifications. Analyses in the MDC-CC separating combined estrogen plus progestin and estrogen only revealed that the combination therapy of estrogen plus progestin, present in 207 women, was highly significantly associated with risk of breast cancer (2.02; 95% CI, 1.29 to 3.16; $P = .002$), whereas estrogen only, present in 156 women, had no significant association with breast cancer (1.01; 95% CI, 0.53 to 1.94; $P = .98$). However, pro-ENK per SD increment was significantly related to risk of breast cancer in both of these models (0.75; 95% CI, 0.63 to 0.88; $P = .001$) in the model with the combination and (0.73; 95% CI, 0.62 to 0.86; $P < .001$) in the model with estrogen only.

Adjustment in the MDC in addition to age, BMI, smoking, menopausal hormone therapy, age at menarche, menopausal status, and educational level for age at first childbirth and parity did not alter our results (0.75; 95% CI, 0.63 to 0.89; $P = .001$). None of these two additional reproductive variables (parity and age at first child birth) had a significant relationship with incident breast cancer in our study.

No interaction was observed between pro-ENK and menopausal hormone therapy with breast cancer risk ($P = .36$ in the MDC; $P = .67$ in the MPP). The association between pro-ENK and breast cancer was significant both in women with (hazard ratio, 0.65; 95% CI, 0.48 to 0.89; $P = .006$) and women without (hazard ratio, 0.78; 95% CI, 0.63 to 0.96; $P = .019$) menopausal hormone therapy in the MDC and in in women with (odds ratio, 0.57; 95% CI, 0.35 to 0.93; $P = .025$) and without (odds ratio, 0.64; 95% CI, 0.52 to 0.79; $P < .001$) menopausal hormone therapy in the MPP. Likewise, no significant interactions were found between pro-ENK and any other available covariates for breast cancer risk, in either the MDC or the MPP (data not shown).

Expression of Neurotensin and NT1 Receptor in Human Breast Cancer: A Potential Role in Tumor Progression

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Abstract

Emerging evidence supports neurotensin as a trophic and antiapoptotic factor, mediating its control via the high-affinity neurotensin receptor (NT1 receptor) in several human solid tumors. In a series of 51 patients with invasive ductal breast cancers, 34% of all tumors were positive for neurotensin and 91% positive for NT1 receptor. We found a coexpression of neurotensin and NT1 receptor in a large proportion (30%) of ductal breast tumors, suggesting a contribution of the neurotensinergic signaling cascade within breast cancer progression. Functionally expressed NT1 receptor, in the highly malignant MDA-MB-231 human breast cancer cell line, coordinated a series of transforming functions, including cellular migration, invasion, induction of the matrix metalloproteinase (MMP)-9 transcripts, and MMP-9 gelatinase activity. Disruption of NT1 receptor signaling by silencing RNA or use of a specific NT1 receptor antagonist, SR48692, caused the reversion of these transforming functions and tumor growth of MDA-MB-231 cells xenografted in nude mice. Our findings support the contribution of neurotensin in human breast cancer progression and point out the utility to develop therapeutic molecules targeting neurotensin or NT1 receptor signaling cascade. These strategies would increase the range of therapeutic approaches and be beneficial for specific patients. (Cancer Res 2006; 66(12): 6243-9)

Introduction

Breast cancer is the most frequent cause of cancer-related deaths among women in the western world (1). Today, early and systematic screening generates detection of early stages of breast cancer. In the last decade, improvement of adjuvant hormonal therapy and polychemotherapy has reduced the risk of recurrence and death from breast cancer (2, 3). Nonetheless, more accurate prognostic and predictive factors are needed to adjust the treatment and its aggressiveness.

For example, the overexpression of the HER-2/*neu* proto-oncogene was found to correlate with increased tumor aggressiveness, recurrence, and mortality in node-positive patients (4). The combination of chemotherapy and Herceptin, a blocker of the HER-2/*neu* growth factor receptor, provides benefits for metastatic breast cancer patients whose tumors overexpressed HER-2, suggesting that HER-2 signaling is critically involved in the carcinogenesis of the mammary gland (5). Several additional data and converging evidence support the concept that the HER family [epidermal growth factor (EGF) receptor (EGFR), HER-2, HER-3, and HER-4] and their ligands can contribute to breast cancer development and clinical course of the disease (6). Abnormal expression of HER members results in receptor hyperactivation due to abnormal transcriptional regulation ensuing in protein overexpression and stimulation enhancement by growth factors (7). Although the functionality of HER members, especially HER-2, has been extensively studied in breast cancer (6), the pathologic conditions resulting in HER member family activation is not fully understood. Emerging paradigms suggest that several growth-promoting peptides, such as angiotensin, endothelin, vasoactive intestinal peptide, bombesin, gastrin, and neurotensin, interact in a cross-talk with the EGFR signaling pathway (8, 9). Many of these peptide receptors, belonging to the G-protein-coupled receptor (GPCR) family, are often overexpressed in human solid tumors and activate several transforming functions, including cell proliferation, survival, invasion, tumor angiogenesis, and metastasis (10).

Several reports implicate neurotensin and its high-affinity neurotensin receptor (NT1 receptor) in several detrimental functions linked to the neoplastic progression, including proliferation of the pancreas, prostate, colon, and lung cancer cells (11), protection of breast cancer cells against apoptosis (12), and induction of the proinvasive potential of colon cancer cells (13). In colon and breast cancers, the NT1 receptor is a Wnt/β-catenin target gene (13).

The central and peripheral functions of neurotensin are mediated through its interaction with NT1, NT2, and NT3 receptors (14). NT1 and NT2 are GPCRs with high (subnanomolar) and low (nanomolar) affinity for neurotensin, respectively. *In vivo* and *in vitro* neurotensin effects are abolished by the specific antagonist of NT1 receptor SR48692, showing that NT1 receptor is the major mediator of these transforming actions (15).

Neurotensin is largely distributed along the gastrointestinal tract (16). Neurotensin is released into the blood circulation soon after a meal and persists for at least 10 hours, increasing its basal blood concentration from 10 to 20 up to 230 pmol/L (17). The physiologic functions of neurotensin include stimulation of pancreatic and biliary secretions, inhibition of small bowel and gastric motility, and facilitation of fatty acid translocation (18–20). In addition, neurotensin is a trophic factor on gastric antrum, small bowel, and colon tissue regeneration (11).

The potential malignant effect of neurotensin is further sustained by data showing increased NT1 receptor expression in human cancers versus the corresponding normal epithelium (21, 22). When NT1 receptor is challenged with neurotensin, phosphatidylinositols are hydrolyzed leading to Ca^{2+} mobilization, and extracellular signal-regulated kinase 1/2 (ERK1/2), Rho GTPases (RhoA,

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doi:10.1158/0008-5472.CAN-06-0450

Rac1, and Cdc42), and focal adhesion kinase (FAK) are activated, leading to immediate or delayed responses to neurotensin involving gene transcription activation, cell growth, death, or differentiation (23–25). More recently, it was shown that neurotensin via NT1 receptor transactivates EGFR by the shedding of transforming growth factor-α (TGF-α) in pancreas and heparin-binding EGF or amphiregulin in prostate cancer cells, both leading to ERK1/2 activation (26, 27).

In this article, we report on the coexpression of NT1 receptor and neurotensin in human breast ductal invasive adenocarcinomas. We showed the detrimental role of endogenous neurotensin and NT1 receptor activation on the tumor growth initiated by human breast cancer cells xenografted in nude mice. Neurotensin and NT1 receptor had a discernible malignant effect on human breast adenocarcinoma cell proliferation, migration, and invasion. This report is the first study to support the contribution of neurotensin and NT1 receptor in the progression of transformed human breast epithelial cells (HBEC) to malignancy.

Materials and Methods

Culture procedure. The human breast adenocarcinoma cell line, MDA-MB-231, was grown in DMEM (Invitrogen, Cergy Pontoise, France) supplemented with 10% FCS and 2 mmol/L glutamine. Normal HBECs were cultured as described previously in Gompel et al. (28). Briefly, breast tissue was obtained from 15 women undergoing reduction mammoplasty in ages between 15 and 25 years. Patients had no history of breast disease and pathologic studies revealed only normal breast tissue. Sampling of the tissues was done according to the French regulations on clinical experimentation. The tissue was digested with 0.15% collagenase and 0.05% hyaluronidase in Ham's F-10 (Invitrogen) and consecutively filtered through 300- and 150-μm sieves to retain undigested tissue and collected on 60-μm sieves. Cells were grown in Ham's F-10 with phenol red supplemented with 0.24% $NaHCO_3$, 1% penicillin-streptomycin, 5 ng/mL cortisol, 6.5 ng/mL T3, 10 ng/mL cholera toxin, 5 mg/mL transferrin, 5% compatible human serum, 0.12 units/mL insulin, and 10 ng/mL EGF in a humidified atmosphere of 5% CO_2, 95% air. Cells reached confluence after 15 to 20 days of primary culture.

Breast tumors. A total of 84 patients diagnosed for breast cancer and undergoing tumorectomy or mastectomy for complete resection of their primary tumors (Gynecology Department, Hôtel-Dieu Hospital, Paris, France) were studied. A series of 70 patients with invasive ductal breast cancers (IDC) with concomitant ductal carcinoma *in situ* (DCIS) were analyzed. The average age was 60.7 ± 12.1 years, with a mean tumor size of 2 ± 1.3 cm, and 28 patients exhibited invaded nodes (from a total of 67 cases studied). The malignancy of the infiltrating carcinomas was scored according to the Scarff-Bloom-Richardson (SBR) histoprognostic system (29); accordingly, 22, 26, and 22 patients were classified as grades 1, 2, and 3, respectively. Four DCIS and 10 invasive lobular breast carcinomas were also collected. Tumors were fixed in 10% formaldehyde and embedded in paraffin wax. Several sections (5 μm thick) were made for each case. The histologic diagnosis was routinely checked by microscopic examination of sections stained with H&E.

Immunohistochemistry. Immunostaining of NT1 receptor and neurotensin was carried out on deparaffinized sections using the avidin-biotin-peroxidase complex method. After inhibition of endogenous peroxidases with 3% hydrogen peroxide, slides were washed in TBS and incubated with 10% normal rabbit serum at room temperature for 30 minutes. NT1 receptor immunoreactivity was detected using a goat polyclonal antibody directed against the human COOH terminus of the receptor (1:100; C-20; Santa Cruz Biotechnology, Santa Cruz, CA). Neurotensin immunoreactivity was conducted using rabbit antibody directed against neurotensin (1:500; NA1230; Biomol International, Plymouth, PA) for 2 hours at room temperature in a humidified chamber. Neurotensin or NT1 receptor immunohistochemistry specificity was checked by omission of primary antibody and by displacement with neutralizing peptide (Santa Cruz Biotechnology) or neurotensin for 2 hours at room temperature. All slides were rinsed thrice with TBS; sections were incubated with biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. The antigen-antibody complex was revealed with avidin-biotin-peroxidase complex for 30 minutes according to the manufacturer's instructions for the Vectastain ABC kit (Vector Laboratories). Staining was done for 5 minutes with 3,3′-diaminobenzidine (Sigma-Aldrich, Lyon, France). All slides were counterstained with hematoxylin. A semiquantitative estimation of the number of positive cells was done by counting 1,000 reactive and nonreactive cells in 10 successive fields at the original ×250 magnification.

RNA extraction and reverse transcription-PCR. The protocols for total RNA extraction, reverse transcription, and PCR are documented by Souaze et al. (30). Reverse transcription was done on 2 μg total RNA using a specific NT1 receptor primer (5′-GCTGACGTAGAAGAG-3′) or 50 pmol oligo(dT) and oligo(dN). The PCR amplification was done on a 1:5 (v/v) of the reverse transcription reaction using 25 pmol of each primer 5′-CGTGGAGCTGTACAACTTCA-3′ and 5′-CAGCCAGCAGACCACAAAGG-3′ for NT1 receptor and 5′-TCCAATAGGTGATGTTGTCGT-3′ and 5′-TCCAATAGGTGATGTTGTCGT-3′ for matrix metalloproteinase (MMP)-9 and 1 unit Taq polymerase (Applied Biosystems, Courtaboeuf, France). The amplification profile consisted of denaturation at 94°C for 30 seconds, annealing at 57°C for 45 seconds, and extension at 72°C for 45 seconds. The PCR cycles were preceded by denaturation at 95°C for 5 minutes and were followed by a final extension at 72°C for 10 minutes. Amplification was done in a DNA thermal cycler 9700 (Perkin-Elmer Applied Biosystems, Courtaboeuf, France).

NT1 receptor small interfering RNA construction and transfection. Small interfering RNAs (siRNA) for human NT1 receptor (AAGAAGTTCATCAGCGCCATC) and scramble sequence (ATCGTCCGAACGTAAGTCAA) were prepared using pSilencer 3.1-H1 according the manufacturer's instruction (Ambion, Austin, TX). MDA-MB-231 cells were transfected using Lipofectamine reagent (Invitrogen). Stable transfectants were selected with hygromycin B (400 μg/mL) and colonies were screened using NT1 receptor reverse transcription-PCR (RT-PCR) and binding assay. Two MDA siNT1 receptor clones, MDA Si1 and Si2, and one pool of clones expressing the scramble sequence, MDA Scr, were chosen for further experiments.

Wound healing. For migration assays, MDA-MB-231 cells were plated in six-well dishes. Twenty-four hours after the cells reached confluence, scarification was done in a Moscona buffer with a pipette tip. Cells were maintained in culture in low-serum medium (0.1% FCS) and treated with 10^{-8} mol/L neurotensin agonist, JMV449, in the presence or absence of NT1 receptor antagonist, SR48692. Pictures were taken at the beginning of the treatment and 18 hours later.

Collagen invasion assays. The method was adapted from Bracke et al. (31). Petri dishes were filled with 2.5 mL neutralized type I collagen (0.18%; Upstate Biotechnology, Lake Placid, NY) and allowed to gel for 4 hours at 37°C and 5% CO_2. MDA-MB-231 cells were harvested and isolated using Moscona buffer and trypsin/EDTA, and 0.2×10^6 cells were seeded on top of the gel collagen. Dispersed cells were cultured for 24 hours at 37°C in the presence or absence of indicated effectors. Counting was done under code: invasive and superficial cells were counted in 12 fields of 0.157 mm^2. The invasion index was calculated as the percentage of cells invading the gel divided by the total number of cells.

Gelatin zymography. Metalloproteinase activity in supernatants of MDA-MB-231 cells cultured in serum-free medium and conditioned or not conditioned for 24 hours with neurotensin agonist, JMV449, and was tested by zymography. Supernatants were desalted on G25 Sephadex column and then concentrated by lyophilization. Total protein (50 μg) was analyzed under nonreducing conditions on 8% polyacrylamide gels containing 0.1% gelatin. For development, gels were washed twice in 2.5% Triton X-100, incubated overnight at 37°C in 50 mmol/L Tris-HCl (pH 7.6), 5 mmol/L $CaCl_2$, 0.02 mg/mL NaN_3, and stained with Coomassie blue.

Animals. Xenografts were initiated by s.c. injection of 3×10^6 MDA-MB-231 wild-type (MDA wt), their silenced NT1 receptor counterparts (MDA Si1 or Si2), or the control MDA scramble cells (MDA Scr). Cells were resuspended in 100 μL PBS and 100 μL Matrigel. Three days after injection, the mice were randomly divided in two groups of 10 animals each and

received the following treatments as i.p. injection: control, vehicle solution; NT1 receptor antagonist, SR48692 (1 mg/kg) resuspended daily in 0.5% Tween 20 in 9‰ NaCl. The treatment was continued for 27 days. The tumor volume was calculated using the formula: length $\times$ width^2 $\times$ 0.4. After the treatment period, mice were sacrificed and the tumors were dissected and weighed.

Statistical analysis. The biochemical data were compared by Student's *t* test or ANOVA followed by Neuman-Keuls' test.

Results

NT1 receptor is not expressed in normal human epithelial cells and breast mammary tissue. We first studied NT1 receptor mRNA expression in normal HBECs. As shown in Fig. 1*A*, the NT1 receptor is very poorly expressed or absent in normal cells (*lanes 2-6*) in comparison with MCF-7 cells (*lane 1*). NT1 receptor expression was also studied in 10 additional samples of HBEC (data not shown). In these patients, NT1 receptor expression was either feeble or absent. As confirmation of this result, immunohistochemistry in normal tissue exhibited no staining in the epithelial and in the myoepithelial cells of ducts or in the lobular cells (Fig. 1*B*).

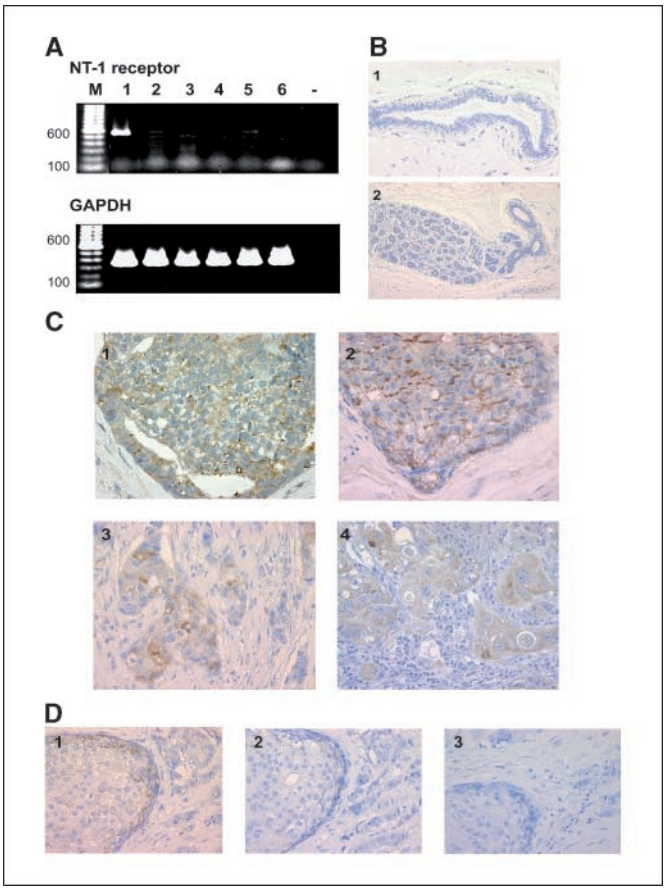

Figure 1. NT1 receptor expression in normal human breast cells, normal human breast tissues, and human breast carcinomas. *A,* 1 μg total RNA from HBEC and MCF-7 cells was reverse transcribed. A PCR experiment was done using specific primers for NT1 receptor and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *Top,* NT1 receptor amplicons from MCF-7 cells (*lane 1*) and HBEC (*lanes 2-6*); *bottom,* GAPDH amplicons from the same samples. For both gels, *lane* - corresponds to PCR from reverse transcription without mRNA. *B,* normal duct (*1*) and lobule (*2*) from reduction mammoplasty samples exposed to NT1 receptor antibody at 1:100 dilution. *C,* typical staining of DCIS or IDCs exposed to 1:100 of COOH-terminal NT1 receptor antibody: DCIS (*1*), ductal component of SBR histologic grade 2 (*2*), and invasive component of SBR histologic grade 2 (*3*) or grade 3 (*4*). *D,* IDCs exposed to NT1 receptor antibody at 1:200 dilution (*1*) after preincubation with the antigen peptide for 2 hours at 1:20 (*2*) or without primary antibody (*3*). Original magnification, ×200 (*B*) and ×400 (*C* and *D*).

NT1 receptor is expressed in human breast cancers. NT1 receptor expression was studied in patients diagnosed for lobular carcinoma, DCIS, or IDC. Globally, NT1 receptor immunoreactivity was observed in the ductal or invasive compartment or both and with variable intensity. In DCIS (Fig. 1*C, 1*) or in the ductal component of IDC with SBR histologic grade 2 (Fig. 1*C, 2*), the staining of the cancer cells was granular and located at the cell surface. Interestingly, a polarized cell staining was often observed as shown in Fig. 1*C, 2*. Concerning the invasive component, the staining was mainly restricted to the cytoplasm. As shown in Fig. 1*C, 3* and *4*, no major difference could be noticed within the SBR histologic grades. We confirmed the specificity of the COOH-terminal antibody directed against human NT1 receptor; the labeling observed when incubated with NT1 receptor was totally suppressed by a preincubation with the antigen peptide (Fig. 1*D, 2*) or by omission of the primary antibody (Fig. 1*D, 3*).

Among the breast carcinomas studied, the majority exhibited a high proportion of NT1 receptor–positive cells (50-100%). Nonetheless, patients were considered positive with $\geq$10% positive cells. According to this assessment, we observed that 9 of 10 invasive lobular breast cancers, 4 of 4 DCIS, and 64 of 70 (91%) of the patients with IDCs were positive. Interestingly, NT1 receptor expression was not homogeneous in the ductal or the invasive compartment and varied according to the SBR grade. We observed that in 59% of patients with histologic grade 1 NT1 receptor reactivity was restricted to the intraductal compartment compared with only 4% for grade 3. In sharp contrast, the NT1 receptor–positive patients in the invasive component were predominantly in the histologic grade 3 (90%), with only 36% in grade 1.

Neurotensin is expressed in human breast carcinomas. Neurotensin expression was studied by immunohistochemistry on 50 patients previously studied for NT1 receptor expression to establish the potential NT1 receptor activation via neurotensin. As shown in Fig. 2, neurotensin labeling was detected in the IDCs. The labeling was cytoplasmic in both ductal and invasive compartments. Neurotensin reactivity was either absent or intense in both compartments (Fig. 2, *1* and *2*). Neurotensin antibody specificity was assessed by antibody preincubation with neurotensin as well as by the omission of primary antibody; in both cases, no labeling was observed (Fig. 2, *3* and *4*). Among the 50 patients, 44 (88%) were NT1 receptor positive, 17 (34%) neurotensin positive, and 15 (30%) positive for both NT1 receptor and neurotensin.

Tumor growth in the human breast cancer cell line MDA-MB-231 is affected by NT1 receptor expression. We next investigated the expression of NT1 receptor in the highly tumorigenic and metastatic breast cancer cell line MDA-MB-231. A specific PCR amplicon with a size of 590 bp, corresponding to the NT1 receptor, was detected (Fig. 3*A*). To establish the contribution of NT1 receptor on tumor breast progression, expression vectors encoding siRNA to silence NT1 receptor gene expression were stably introduced into parental MDA-MB-231 cells (MDA-wt). As expected, NT1 receptor transcripts were depleted in the two clones Si1 and Si2 (Fig. 3*A*). As a control, we verified that the NT1 receptor amplicon was unchanged in MDA-MB-231 cells stably transfected by the expression vector encoding the scrambled NT1 receptor silencing RNA sequence (Scr). MDA wt and Scr cells bound 2.89 $\pm$ 0.4 and 2.98 $\pm$ 0.3 fmol [^{125}I]neurotensin/mg crude

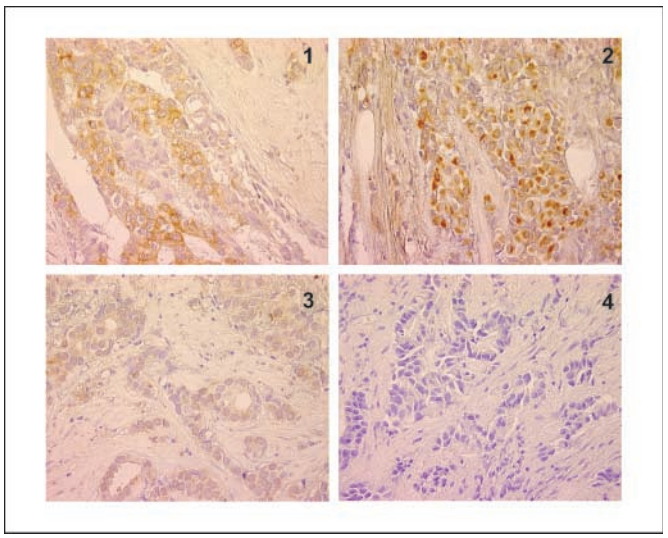

Figure 2. Neurotensin is expressed in human breast carcinomas. Typical staining of IDCs exposed to 1:500 of neurotensin antibody: ductal (*1*) and invasive (*2*) component of SBR histologic grade 2. IDCs exposed to neurotensin antibody at 1:500 dilution after preincubation with 10^{-8} mol/L neurotensin (*3*) or without primary antibody (*4*). Original magnification, ×400.

membrane protein, respectively, whereas MDA Si1 and Si2 cells did not bind [^{125}I]neurotensin.

Using these cellular models, we examined the effect of NT1 receptor depletion on the tumorigenic potential of MDA-MB-231 cells xenografted in nude mice. Silenced, scrambled, and parental cells were injected s.c. in the right flank of nude mice, and the tumor size was measured 5 weeks after inoculation. As shown in Fig. 3*B*, depletion of NT1 receptor in MDA Si1 and Si2 cells is accompanied by a significant 40% and 70% fold decrease in tumor volume, 25% and 70% fold decrease in the final tumor weight, and 35% and 80% lengthening of tumor doubling time compared with MDA wt cells. The strength of the two siRNAs (Fig. 3*A*) correlated with their ability to inhibit tumor growth in MDA cells, with Si2 being the more affected clone.

This result substantiates NT1 receptor involvement in promoting breast tumor growth. To validate the *in vivo* effect, xenografted nude mice with MDA wt cells were administrated with daily doses of the specific NT1 receptor antagonist, SR48692 (15), for 27 days. Five days after inoculation, the initial breast tumor volume was the same in control and treated groups (191 ± 10 and 192 ± 3.4 mm^3, respectively). As shown in Fig. 3*C*, SR48692 reduced by 25% and 30% the volume and weight of the MDA-MB-231 breast tumor xenografts and, accordingly, lengthened the tumor doubling time by 35%.

Neurotensin-induced motility and cellular invasion of breast cancer cell lines through NT1 receptor activation. Because active migration and invasive growth of transformed cells are characteristics of cancer progression, we examined the effect of neurotensin and NT1 receptor in the wound closure and collagen type I invasion assays using MDA wt and Si1 and Si2 cells. As shown in Fig. 4, the weakly degradable neurotensin agonist, JMV449, remarkably increased the migration and wound healing of parental MDA-MB-231 cell layers. The residual gap size after 18 hours was smaller in cells treated with JMV449 compared with control cells (35% of the original open gap in the treated cells versus 50% in control cells). The specific NT1 receptor antagonist, SR48692, completely inhibited the agonist-induced wound healing effect in MDA wt (Fig. 4). The Scr cells exhibited the same response as the parental cells (data not shown). In contrast, wound repair was not accelerated in the presence of JMV449 for the silenced cell lines. Compared with the original gap, after 18 hours, the MDA Si1 residual gap was 64% for treated cells and 67% for nontreated control cells, respectively (Fig. 4). The residual gap sizes after 18 hours were similar for control and treated MDA Si2 cells (42% and 38%; data not shown).

As shown in Fig. 5*A* (and *inset*), neurotensin and JMV449 were both found to promote collagen I invasiveness in MDA wt cells. The induced proinvasive effects were dose dependent and abolished by cotreatment with the specific antagonist, SR48692. Neurotensin proinvasive effects were abolished in the MDA Si1 and MDA Si2 cell lines (Fig. 5*B*), whereas silenced cells remained responsive to TGF-α. As control, MDA Scr cells were shown to be responsive to neurotensin, JMV449, and TGF-α (Fig. 5*B*).

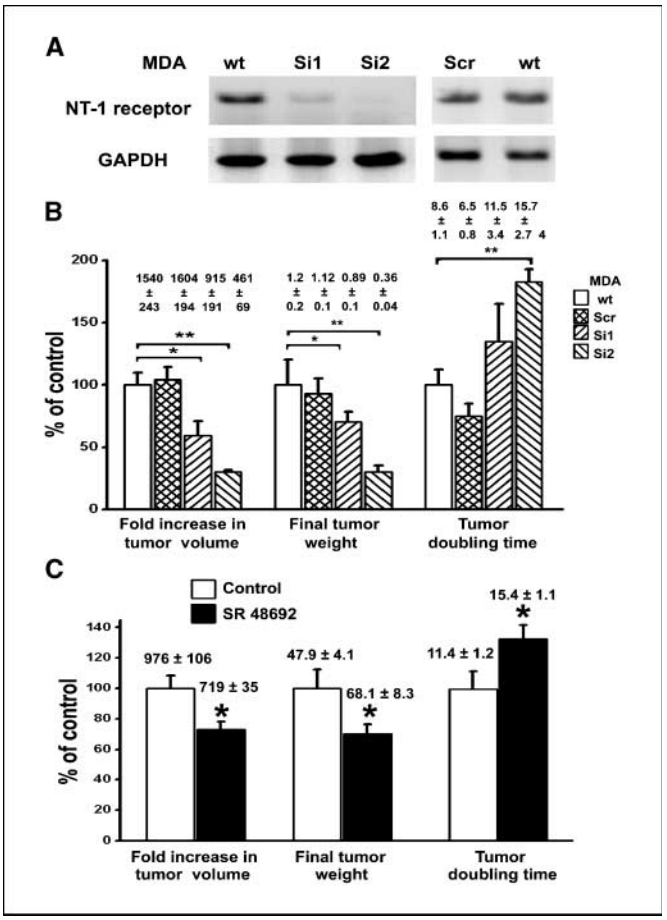

Figure 3. NT1 receptor depletion and pharmacologic blockade by SR48692 reduced the tumorigenic potential of MDA-MB-231 cells xenografted in nude mice. *A,* 1 μg total RNA from MDA wt, MDA Si1 or Si2 for NT1 receptor, or scramble (Scr) was reverse transcribed. A PCR experiment was done using specific primers for NT1 receptor and GAPDH. Amplicons were electrophoresed on agarose gel and stained with ethidium bromide. *B,* nude mice were xenografted with 3 × 10^6 MDA wt, Scr, Si1, or Si2 cells. Five weeks after inoculation, the mice were killed and tumor size and weight were measured. Representative of three independent experiments. *Columns,* mean percentage of values (g, mm^3, or days) from MDA wt cells; *bars,* SE. *, $P < 0.05$; **, $P < 0.01$, ANOVA and Student's-Neuman-Keuls' test. *C,* MDA wt cells were xenografted in nude nice and treated or not with 1 mg/kg SR48692 for 27 days. Representative of two experiments. *Columns,* mean (in g, mm^3, or days); *bars,* SE. *, $P < 0.05$, Students' *t* test.

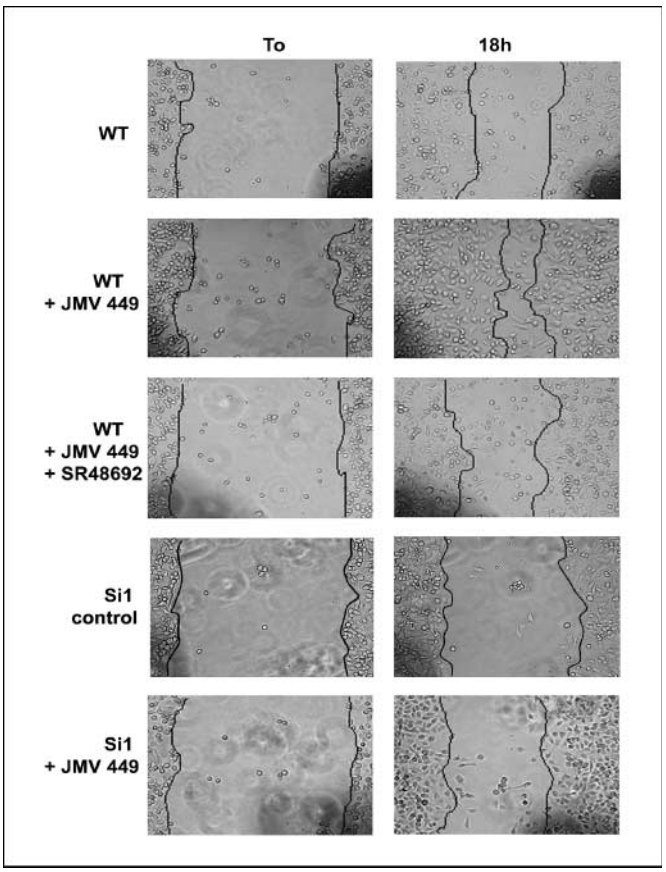

Figure 4. Neurotensin agonist promotes cellular migration of MDA-MB-231 cells. The wound repair process was studied in MDA wt and Si1 cells. Cell monolayers were incubated in the presence of 0.5% serum. Initial gap (*To*) and remaining gap after 18-hour incubation (*18h*) at 37°C. *Top,* control MDA wt cells treated with JMV449 (10^{-8} mol/L) alone or combined with SR48692 (10^{-6} mol/L); *bottom,* MDA Si1 control and treated with JMV449. Representative of two to three independent experiments.

Activation of cellular invasion in MDA-MB-231 cells treated by JMV449 was blocked by selective pharmacologic inhibitors, including the mitogen-activated protein kinase (MAPK) activation blockers, PD098059 and SB203580, and by drugs targeting phospholipase C (U73122) and protein kinase C (PKC; Gö6976 and GF109). The two latter indicated the participation of the Gαq/Gβγ and PKC signaling pathways in this cellular response. Equally interesting, the proinvasive activity of the NT1 receptor remained in the presence of pharmacologic inhibitors for phosphatidylinositol 3-kinase (PI3K; wortmannin), Rho GTPases and Rho kinase (C3T and Y27632), and Gαo/i subunits (pertussis toxin) and protein kinase A (KT5720), two signaling elements involved in the cyclic AMP (cAMP) pathways.

Neurotensin-induced cell invasion requires MMP-9 activation. Tumor cell invasion through matrix and tissue barriers requires the combined effects of increased cell motility and regulated proteolytic degradation of the matrix. In breast tumors, the MMP-9 has been detected in vascular pericytes, cancer cells, and tumor stromal cells (32, 33). Elevated levels of the MMP-9 in tumor tissue have been generally correlated with cancer cell invasion and metastasis (34). To show the requirement for MMP-9 activation in neurotensin induced invasion, we preincubated MDA-MB-231 cells with a specific MMP-9 inhibitor for 30 minutes (35). The invasive activity induced by the NT1 receptor agonist JMV449 was selectively abolished because these breast cancer cells remained sensitive to the EGFR agonist TGF-α (Fig. 6*A*). We further confirmed that neurotensin induced MMP-9 expression by raising the levels of MMP-9 mRNA (Fig. 6*B*). As a consequence, the MMP-9 gelatinase activity was elevated as visualized by zymography in MDA-MB-231 cells (Fig. 6*C*).

Discussion

Because GPCRs can be exposed to an excess of locally produced or circulating agonist, increasing evidence argues for a direct correlation between aberrant GPCR signaling and cancer progression (9). The current study documents the overexpression of NT1 receptor in most human ductal breast cancers and the frequent concomitant expression of its specific ligand. We also confirm the participation of neurotensin in several deleterious characteristics associated with breast cancer progression, including cellular migration, invasion, and tumor growth. The tumorigenic potential observed here on human breast cancer tissue and cells was shown to be reversible by silencing the NT1 receptor by RNA interference or by NT1 receptor pharmacologic blockade. Building on previous reports showing the neurotensin trophic and survival function of neurotensin in breast cancer cells, the present results strengthen the argument for the involvement of neurotensin in breast cancer growth and progression (12).

Cancer progression involves a sequential series of critical genetic and molecular alterations inducing the deregulation of cell proliferation, adhesion, migration, and invasion and leading to the lethality associated with metastatic spread of malignant tumors. It has been shown previously that NT1 receptor gene is a target of the Wnt/APC oncogenic pathways connected with the β-catenin/Tcf transcriptional complex, known to activate genes involved in cell proliferation and transformation (13). Because an exact correlation was found between NT1 receptor expression and β-catenin cytoplasmic or nuclear localization in colonic adenomas, we concluded that NT1 receptor overexpression in cancer is an early event in colonic cell transformation (13).

Wnt genes are frequently up-regulated in breast cancer and are associated with mammary tumorigenesis (36). We observed previously that Wnt factors activate NT1 receptor gene expression in normal human epithelial breast cells (13), indicating a possible up-regulation of NT1 receptor in breast cancer. In the present study, we observed NT1 receptor expression mainly located in the ductal compartment within histologic grade 1, reinforcing the concept that NT1 receptor expression is an early event of breast epithelial cell transformation. In addition, we also detected intracellular NT1 receptor localization as well as neurotensin labeling predominantly in the invasive compartment in histologic grades 2 and 3, suggesting that intensive internalization follows receptor activation by intense and sustained ligand exposure. This event is associated with a chronic self-activation loop between neurotensin and the NT1 receptor and is a mechanism driving constitutive activation of the MAPK signaling pathways coupled with cell division (37). These critical agonist exposure conditions would seem to occur in breast cancer patients expressing both ligand and NT1 receptor and consequently enable the potential action of neurotensin/NT1 receptor in human breast cancer.

The molecular mechanisms involved in neurotensin gene up-regulation observed in human colon tumors (38), carcinoid tumors

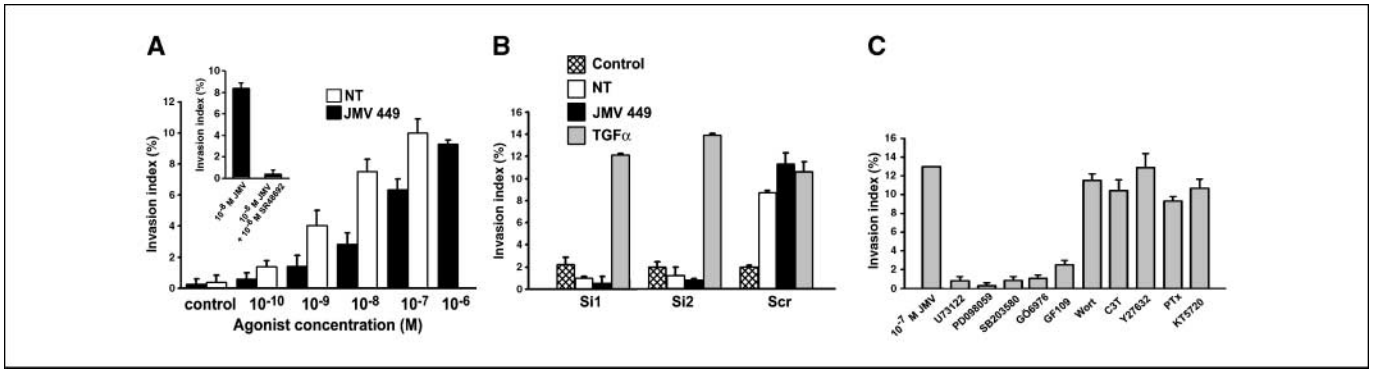

Figure 5. Neurotensin agonist promotes cellular invasion of MDA-MB-231 cells. *A,* neurotensin or JMV449 dose-dependent induction of cellular invasion in type I collagen by MDA wt cells. *Inset,* neurotensin (10^{-8} mol/L) induced cellular invasion blockade by SR48692 (10^{-6} mol/L). *B,* neurotensin or JMV449 (10^{-7} mol/L) induced invasion in type I collagen was compared in MDA wt, S1, S2, or Scr cells. TGF-α (10 ng/mL) was used as positive control. *C,* JMV449 (10^{-7} mol/L) induced cellular invasion blockade by phospholipase C inhibitor, U73122 (10^{-5} mol/L); MAPK inhibitors, PD098059 (5×10^{-5} mol/L) and SB203580 (10^{-5} mol/L); PKC inhibitors, Gö6976 (10^{-5} mol/L) and GF109 (10^{-6} mol/L); PI3K inhibitor, wortmannin (*Wort*; 10^{-5} mol/L); Rho GTPase inhibitor, C3T (5 μg/mL); ROCK inhibitor, Y27632 (10^{-5} mol/L); Gi component of adenylate cyclase inhibitor, pertussis toxin (*PTx*; 200 ng/mL); and cAMP-dependent protein kinase (PKA) inhibitor, KT5720 (10^{-5} mol/L).

(39), and pancreatic tumors (40) are not fully clarified. Evidence shows that neurotensin gene hypomethylation (41), along with the activation of transcription factors activator protein-1 (AP-1) and cAMP-responsive element-binding protein and the oncogenic signaling factors RAS and Src, are potential mechanisms leading to neurotensin overexpression and secretion during cancer progression (42). It is noteworthy that estrogens can also activate neurotensin expression in the brain through the cAMP cascade and through an additional, nonconventional, steroid receptor pathway (43). A future challenge will be to evaluate the possible participation of estrogens in neurotensin expression in breast cancer.

No clear correlation has been described today between circulating neurotensin and the stages of pancreas, prostate, or medullar thyroid tumors (44) probably due to its high degradability and rapid clearance by the liver. Nevertheless, neurotensin was found to be released from gut and pancreas cancers and has been identified as a gene associated with enhanced metastasis in a lung carcinoma cell line (40, 45). Additionally, it has been shown that the NT1 receptor antagonist SR48692 inhibited the growth of human colon and lung cancer cells xenografted in mice (46). In this report, we further establish the contribution of endogenous or tumoral neurotensin within the breast cancer progression by showing the reversibility of its tumorigenic effects through silencing RNA and direct pharmacologic blockades. Nevertheless, direct determination of the local neurotensin concentration, at the tumor vicinity, would provide the clearest evaluation of neurotensin secretions by the tumor and its sequential placement within cancer progression.

Neurotensin signaling-associated effectors, such as nuclear factor-κB, ERK1/2, AP-1, Ras, Src, Rho family protein, and FAK, substantiate the potential neurotensin/NT1 receptor oncogenic role (23–25, 47, 48). These signaling factors are correlated with cell and tissue growth, cell death, and differentiation. Here, we illustrated the proinvasive activity of neurotensin and the NT1 receptor in MDA-MB-231 cells mediated by MMP-9. Interestingly, expression of MMP-9 has been also associated with high potential of metastasis in several human carcinomas, including breast carcinomas. MMP-9 immunohistochemistry revealed MMP-9 in malignant and stromal cells, predicting a poor survival index when expressed in stromal cells within a hormone-responsive tumor, whereas it has a favorable survival index when expressed in carcinoma cells (49).

This article is the first report on neurotensin/NT1 receptor in human breast cancer and brings new data on neurotensin malignant effects in breast cancer cells, suggesting that NT1 receptor may not only be a marker but also participates as part of the process of breast cancer progression. Further analysis will

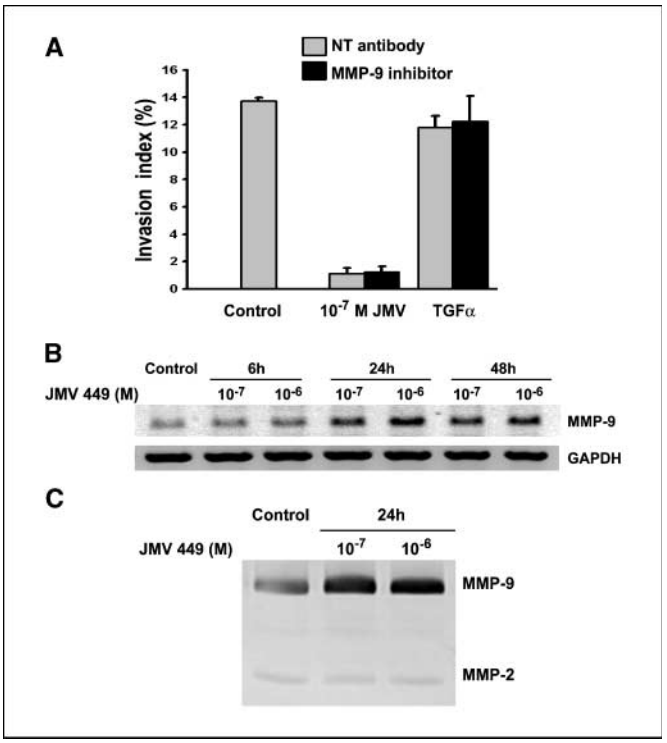

Figure 6. Neurotensin agonist activates MMP-9 expression and activity in MDA-MB-231 cells. *A,* neurotensin agonist (10^{-8} mol/L) induced cellular invasion blockade induced by MMP-9-specific inhibitor I (25×10^{-9} mol/L). *B,* accumulation of endogenous MMP-9 transcripts detected by RT-PCR following treatment of MDA wt cells with JMV449 (10^{-7} or 10^{-6} mol/L) for 6, 24, and 48 hours. Representative of one of three independent experiments. *C,* gelatinolytic activities assessed by zymography on lyophilized medium of MDA wt cells treated or not with JMV449 (10^{-7} or 10^{-6} mol/L) for 24 hours. Representative of one of three independent experiments.

detail the position and the participation of neurotensin in breast tumorigenesis, particularly the signaling, its partner (HER, EGF-like ligands, interleukin, etc.), and its place in the clinical course. The data described here should aid in the search for neurotensinergic-blocking agents to be joined to the adjuvant antihormonal or the chemotherapy treatments for breast cancer.

Acknowledgments

Received 2/7/2006; revised 3/31/2006; accepted 4/18/2006.

Grant support: Institut National de la Sante et de la Recherche Medicale, Association pour la Recherche sur le Cancer grant 3543, Ligue national contre le cancer grant 75/00-RS/40 France, and Belgian Federation against Cancer Research Belgium.

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We thank Dr. D. Gully (Sanofi-Synthelabo) for providing the NT1 receptor antagonist (SR48692), Dr. D. Pélaprat (U 773 EP 11 Hôpital Bichat) and Dr. N. Insdorf for very useful discussions, Isabelle Renault (Hôpital Saint-Antoine) for assistance with the animals, and Dr. D. Hugol (Laboratoire d'Anatomie Pathologique, Hôtel-Dieu de Paris) for reviewing the pathology of breast cancer biopsies.

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Expression of Neurotensin and NT1 Receptor in Human Breast Cancer: A Potential Role in Tumor Progression

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Cancer Res 2006;66:6243-6249.

available at www.sciencedirect.com

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Proneurotensin 1–117, a stable neurotensin precursor fragment identified in human circulation

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ARTICLE INFO

Article history:
Received 20 December 2005
Received in revised form
25 January 2006
Accepted 26 January 2006
Published on line 7 March 2006

Keywords:
Prohormone
Neurotensin
Neuromedin N
Pro NT/NMN 1–117
Mass spectrometry
Sandwich immunoassay
Half-life

ABSTRACT

Proneurotensin/neuromedin N (pro NT/NMN) is the common precursor of two biologically active peptides, neurotensin (NT) and neuromedin N (NMN). We have established antibodies against peptide sequences of the NT/NMN precursor and developed a sandwich immunoassay for the detection of pro NT/NMN immunoreactivity in human circulation. Endogenous pro NT/NMN immunoreactivity was enriched by affinity chromatography using antibodies against two different pro NT/NMN epitopes, and further purified by reversed phase HPLC. Mass spectrometry analysis revealed pro NT/NMN 1–117 as major pro NT/NMN immunoreactivity in human circulation. Pro NT/NMN 1–117 is detectable in serum from healthy individuals (n = 124; median 338.9 pmol/L). As known for NT, the release of pro NT/NMN 1–117 from the intestine into the circulation is stimulated by ingestion of an ordinary meal. Investigation of the pro NT/NMN 1–117 in vitro stability in human serum and plasma revealed that this molecule is stable for at least 48 h at room temperature. Since pro NT/NMN 1–117 is theoretically produced during precursor processing in stoichiometric amounts relative to NT and NMN, it could be a surrogate marker for the release of these bioactive peptides.

1. Introduction

Neurotensin (NT) and neuromedin N (NMN) are two related peptides, primarily localized in the central nervous system [7,27] and the gastrointestinal tract [23], where they excert neurotransmitter/neuromodulator [24,34] and endocrine/paracrine functions, respectively. Moreover, expression of NT was demonstrated in the heart [32] and the adrenals [19].

Intestinal NT is produced and stored by specific enteroendocrine mucosal cells (N-cells) in the jejunum and ileum and to a lesser extent in the colon and duodenum [21,31,39]. The release of NT into the circulation is triggered after ingestion of food [25], and fat has been shown to be the strongest stimulus [33,16,40]. NT exhibits various digestive functions: stimulation of pancreatic and biliary secretions, inhibition of gastric acid secretion and motility, stimulation of colon motility, and inhibition of jejuno-ileum motility (for review see [17]). In the central nervous system NT excerts diverse effects including hypothermia [5], antinociception [12], stimulation of anterior pituitary hormone secretion [26,36], and modulation of the dopaminergic transmission [30,34].

The human prepro NT/NMN precursor molecule, containing the peptide sequences of NT and NMN, consists of 170 amino acids [2] and its structure is illustrated in Fig. 1. Cleavage of the N-terminal signal sequence by specific signal peptidases [3] results in the formation of pro NT/NMN (147 amino acids) [14]. The N- and C-termini of NT and NMN are flanked by Lys–Arg-sequences which represent potential

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0196-9781/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2006.01.021

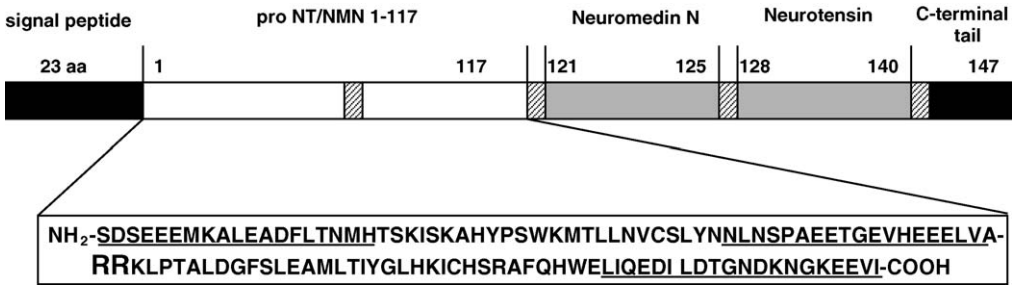

Fig. 1 – Symbolic sequence structure of the human prepro-NT/NMN precursor. The signal sequence consists of 23 amino acids. Dibasic amino acids representing targets for proteolytic processing are shown as dashed boxes. The amino acid sequence of pro NT/NMN 1–117 is indicated. Peptide sequences pro NT/NMN 1–19, 44–62 and 98–117, used for antibody production, are underlined. The potential prohormone convertase cleavage site Arg^{63}–Arg^{64} is highlighted.

cleavage sites for prohormone convertases [15,35]. A fourth dibasic site consisting of an Arg-doublette is present within the N-terminal region of the precursor. Processing of the NT/NMN precursor is tissue-specific. In brain processing results in the formation of NT and NMN [10,13], whereas in the gut and in the adrenals processing leads to the production of NT and two other potentially bioactive peptides, large NT and large NMN, which are N-terminal extended peptides of NT and NMN, respectively [8–10].

However, the reliable measurement of both, mature NT and NMN as well as large NT and large NMN in biological fluids, is limited due to the instability of these molecules in vivo [1,11] as well as in vitro [18].

In this report, we describe the identification of a new neurotensin precursor fragment in human blood, characterized as pro NT/NMN 1–117. We developed an immunoassay for the detection of pro NT/NMN 1–117 and were able to show, that this molecule is completely stable in human serum and plasma for at least 48 h when stored at room temperature. In addtion, we demonstrate, that the secretion of pro NT/NMN 1–117 into the blood stream is stimulated by ingestion of an ordinary meal, as it was reported for neurotensin. Since pro NT/NMN 1–117 is produced in equimolar amounts to neurotensin and neuromedin N, released amounts of pro NT/NMN 1–117 should represent those of NT and NMN, respectively. The high stability of pro NT/NMN 1–117 is a significant advantage and a main prerequisite for its use in laboratory and clinical routine.

2. Materials and methods

2.1. Chemicals

If not stated otherwise, chemicals were obtained at p.a. grade from Merck (Darmstadt, Germany). Non-specific rabbit immune globulin, bovine serum albumin and horse serum H1270 were purchased from SIGMA (Deisenhofen, Germany).

2.2. Peptides

Three peptides chemically related to the NT/NMN precursor (1–147), pro NT/NMN 1–19 (SDSEEEMKALEADFLTNMH), pro NT/NMN 44–62 (NLNSPAEETGEVHEEELVA) and pro NT/NMN 98–117 (LIQEDILDTGNDKNGKEEVI), were supplied by JPT Peptide Technologies GmbH (Berlin, Germany). These peptides were synthesized with an additional N-terminal cystein-residue for conjugation of the peptides to Keyhole limpet hemocyanin (KLH) and covalent immobilization on Sulfolink coupling gel (Perbio Science, Bonn, Germany). The peptide pro NT/NMN 1–62 (SDSEEEM KALEADFLTNMHTSKI-SKAHVPSWKMTLLNVCSLVNNLNSPAEETGEVHEEELVA) was synthesized as standard material for the sandwich immunoassay using antibodies against the peptides pro NT/NMN 1–19 and 44–62.

2.3. Antibodies

Antibodies against the pro NT/NMN-peptides 1–19, 44–62 and 98–117 were raised in rabbits by InVivo BioTech Services GmbH (Hennigsdorf, Germany). Peptide-specific antibodies were purified from rabbit antisera by affinity chromatography using Sulfolink gel according to the suppliers instructions.

2.4. Serum and plasma samples

Serum and plasma samples (EDTA and heparin) from healthy blood donors used for determination of stability, frequency distribution and purification of pro NT/NMN, were obtained from InVent Diagnostica GmbH (Hennigsdorf, Germany). These samples were taken randomly without any information about eating behaviour or daytime. For the purification of pro NT/NMN immunoreactivity serum samples from healthy blood donors were pooled (final volume: 1000 mL), filtrated through a 0.22 μm Millipore filter. Subsequently, the serum pool was diluted 1:2 in PBS, pH 7.2 containing 10 mmol/L Na-EDTA and pro NT/NMN immunoreactivity was isolated as described later.

To determine the level of pro NT/NMN 1–117 in humans before and after an ordinary meal (about 1200 kcal), three men and three women, volunteer employees of a local biotechnology center, fasted overnight (14 h), consumed a 1-L water load the next morning and a standardized meal for lunch. Fifteen serum samples of each study participant obtained with peripheral venipuncture at several time points were subsequently centrifuged and frozen at −20 °C until measurement.

2.5. Immunoassay

2.5.1. Coating and labelling of antibodies

Initially, two immunassays were designed, to evaluate potential further fragmentation of pro NT/NMN. Immunoassay 1 employed the affinity-purified antibodies to amino acids 44–62 as solid phase and antibodies to amino acids 1–19 as tracer. Immunoassay 2 employed antibodies to amino acids 44–62 as solid phase and antibodies to amino acids 98–117 as tracer. Coating of the solid-phase antibody was done for 20 h on polystyrene tubes (Greiner, Frickenhausen, Germany) (2 μg/tube) in 0.3 mL of coating-buffer (20 mmol/L sodium phosphate buffer, pH 7.8, 50 mmol/L NaCl). The tubes were blocked with 5 g/L bovine serum albumin and vacuum dried. The polyclonal rabbit-antibodies specific for the peptides pro NT/NMN 1–19 and 98–117 were labelled with acridiniumester-*N*-hydroxy-succinimid obtained from InVent Diangostica GmbH. Briefly, 200 μg antibody in 200 mmol/L sodium phosphate buffer, pH 8.0, was incubated for 20 min at room temperature with 5 μL of acridiniumester (1 g/L in acetonitrile). Labelled antibodies were purified by HPLC using a BioSil SEC 400-5 column from BioRad (Munich, Germany). Each HPLC run was carried out with a buffer containing 50 mmol/L Tris–HCl and 250 mmol/L NaCl (pH 7.2) and a flow rate of 0.8 mL/min.

2.5.2. Immunoassay performance

Immunoassays 1 and 2 were performed by incubating 50 μL of samples/standards and 150 μL assay buffer (PBS containing 10 mmol/L Na-EDTA, pH 7.2) 16 h at room temperature. After washing the tubes four times with 1 mL of PBS (pH 7.2), 200 μL tracer was added for 2 h followed by four washing steps and measurement of bound chemiluminescence with a LB952T luminometer (Berthold, Wildbad, Germany).

2.5.3. Calibration

Immunoassay 1 was calibrated using the synthetic standard peptide pro NT/NMN 1–62 diluted in horse serum (20–7250 pmol/L). With this calibration curve, a large pool of human serum was calibrated as reference standard for subsequent measurements, resulting in a concentration of 995.1 pmol/L. Immunoassay 2 was calibrated using the human reference serum pool and serial dilutions in horse serum, resulting in calibrators between 6.0 and 995.1 pmol/L (Fig. 2).

Subsequently, 85 human serum samples were measured in both immunoassays. As shown in Fig. 3, linear regression revealed that both assays had practically identical results. Serum samples containing pro NT/NMN 1–117 concentrations above the highest standard solution were pre-diluted in horse serum, measured again and concentration was subsequently extrapolated. The analytical assay sensitivity, as determined with horse serum (mean relative light units of 10 determinations plus 2 S.D.), was 45.3 pmol/L for immunoassay 1 and 9.95 pmol/L for immunoassay 2. Therefore, we decided to use immunoassay 2 for further measurements.

2.6. Purification, mass spectrometric analysis and N-terminal sequencing of serum pro NT/NMN immunoreactivity

Pro NT/NMN immunoreactivity was enriched from the serum pool solution by affinity chromatography using a mixture of anti-pro NT/NMN 44–62 and anti-pro NT/NMN 98–117 antibodies. One millilitre of each antibody in 100 mmol/L sodium phosphate, pH 7.0 (1 mg/mL) were pooled and oxidised by addition of 10 mg sodium periodate, incubated for 30 min at room temperature and desalted using a NAP25 column (Amersham Pharmacia Biotech, Freiburg, Germany). The desalted antibody solution (3.5 mL) was mixed with 2.0 mL Carbolink gel material (Perbio Science, Bonn, Germany). The mixture was slightly shaken for 6 h at room temperature and filled into a polycarbonate column (diameter 2.8 cm, BioRad,

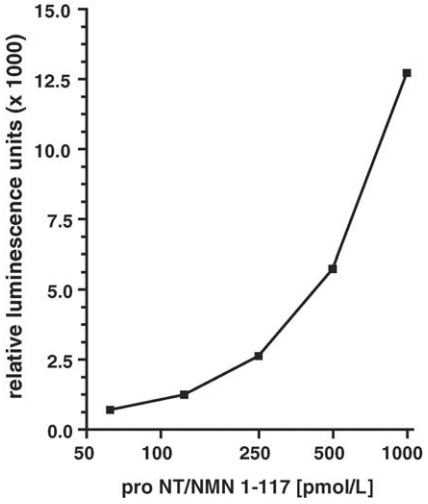

Fig. 2 – Representative dose–response curve of the pro NT/NMN 1–117 assay using antibodies against pro NT/NMN 44–62 and 98–117 (immunoassay 2).

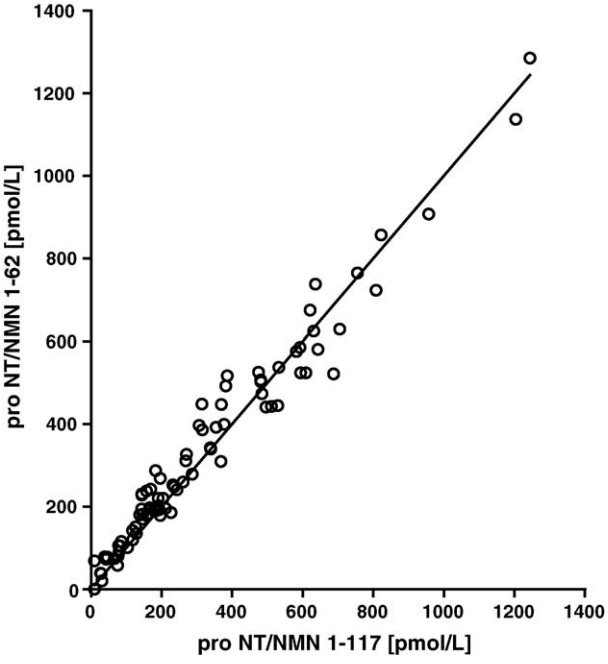

Fig. 3 – Linear regression of pro NT/NMN 1–62 and 1–117 concentrations of serum samples from healthy blood donors ($n = 85$) measured with immunoassays 1 and 2 (slope of linear regression line 1.0 ± 0.014; $P < 0.001$).

Munich, Germany). Unbound antibodies were removed by washing with 40 mL of 1 mol/L NaCl and subsequently with 50 mL of 100 mmol/L sodium phosphate, pH 7.0. The pro NT/NMN-containing serum solution (2000 mL, containing 795 pmol in total) was passed two times through the column at a flow rate of 1 mL/min at 4 °C. After washing the column with 20 mL PBS, pH 7.2, bound peptide was desorbed by elution with 50 mmol/L glycine/HCl containing 5% methanol, pH 2.0 at 1 mL/min. Column efflux was continuously detected for UV absorption at 280 nm. Fractions of 0.5 mL were collected, protein containing fractions were pooled (final volume 3.0 mL), and further purified by reversed phase HPLC.

The material eluted from the affinity column was diluted 1:2 in eluent A (10% acetonitrile, 0.1% trifluoroacetic acid [TFA]). A μ-bondapak RP-C18 column (Waters, Eschborn, Germany) was equilibrated with eluent A at a flow rate of 1 mL/min. Eluent B was 90% acetonitrile with 0.1% TFA. After injection of sample (6 mL) the column was eluted at 1 mL/min using a linear gradient (5 min) from 0% B to 33% B, followed by four successive step gradients (3 min each) to 36%, 39%, 42% and 45%, and finally a linear gradient (5 min) to 100% B. Column efflux was continuously detected for UV absorption at 214 nm. Fractions of 1 mL were collected, a 1:50 dilution in horse serum of each fraction (10 μL) was prepared and immediately measured for pro NT/NMN immunoreactivity using assay 2. RP-HPLC purified serum pro NT/NMN immunoreactivity (fraction 20) was dried in a SpeedVac concentrator (ThermoSavant, Egelsbach, Germany). Peptides were analyzed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and N-terminal Edman sequencing employing standard procedures (performed by Proteome Factory AG, Berlin, Germany).

2.7. *Stability of pro NT/NMN 1–117 in human serum and plasma*

To determine the stability of endogenous pro NT/NMN 1–117 in human serum, EDTA- and heparin-plasma, five freshly prepared samples of each matrix from healthy blood donors were stored at room temperature for up to 48 h. Aliquots were taken immediately after preparation of sample (for reference) and after 2, 4, 6, 24 and 48 h incubation at room temperature and stored frozen at −20 °C. For measurement of pro NT/NMN 1–117 all samples were thawed together and concentration was determined. The average concentration of the references for each matrix was defined to be 100% and all other average concentrations were referred to this value.

3. Results

To elucidate the identity of pro NT/NMN immunoreactivity in human circulation, we isolated the peptide molecule from a serum pool of healthy blood donors containing 795 pmol in total. After affinity chromatography and RP-HPLC 31.9% pro NT/NMN immunoreactivity was recovered. The RP-HPLC chromatogram is shown in Fig. 4, illustrating the elution of the main pro NT/NMN immunoreactivity in fraction 20. No other pro NT/NMN peaks were obtained. MALDI-TOF mass spectrometry, performed without previous trypsinization of the peptide material, was used to identify the nature of the pro NT/NMN immunoreactivity. The mass analysis revealed one mass peak of 13295.9 $(M + H)^+$ ±0.1% compatible with the theoretical mass of pro NT/NMN 1–117 of 13291 Da (Fig. 5). The mass peak at 6629.01 resemble the higher protonated form $(M + 2H)^+$ of the 13295.9 $(M + H)^+$ peptide. In addition, the peptide was analyzed by N-terminal Edman sequencing of the first five amino acid residues. The N-terminal amino acid sequence of the peptide was SDSEE, which is identical with the first five amino acid residues of the pro NT/NMN 1–117 sequence.

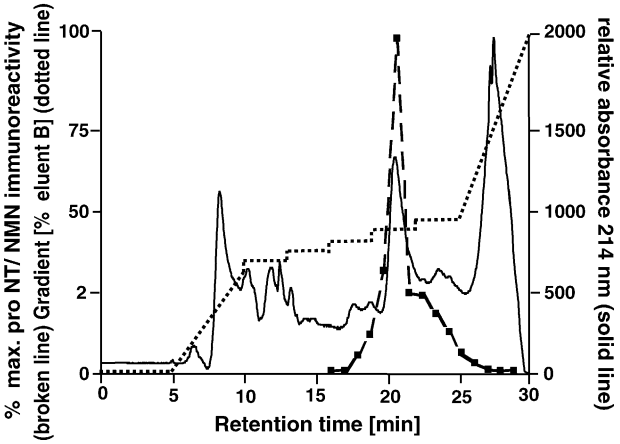

Fig. 4 – RP-HPLC elution profile of a serum pool fraction from healthy blood donors after enrichment of pro NT/NMN immunoreactivity by affinity chromatography. The running buffer gradient (dotted line), the recorded absorption profile at 214 nm (solid line) and the pro NT/NMN immunoreactivity measured in 15 fractions (broken line) are shown. Pro NT/NMN immunoreactivity was measured using immunoassay 2 (see Section 2). The illustration of the running buffer gradient was corrected by the dead volume of the HPLC-system. The fraction containing the pro NT/NMN-peak (fraction number 20) was subjected to mass spectrometric analysis.

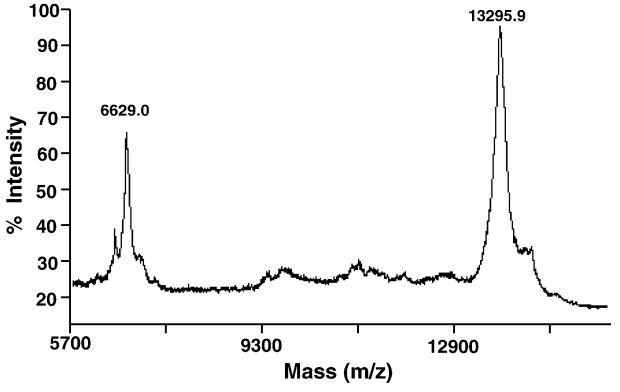

Fig. 5 – MALDI-TOF mass spectrum of fraction 20. After RP-HPLC separation of affinity purified serum pro NT/NMN immunoreactivity fraction 20 was investigated by MALDI-TOF mass spectroscopic analysis. The mass peak 6629.0 resemble the higher protonated form $(M + 2H)^+$ of the 13295.9 Da peptide $(M + H)^+$.

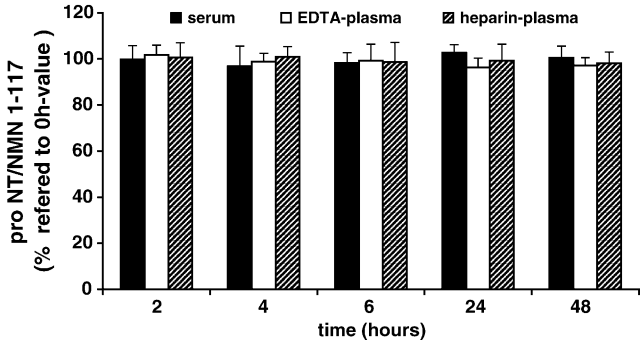

Fig. 6 – Stability of pro NT/NMN 1–117 in human serum and plasma. Serum, EDTA-plasma and heparin-plasma (n = 5, for each matrix), from healthy blood donors were tested for pro NT/NMN 1–117 stability at room temperature for up to 48 h. The concentration of the reference value (at 0 h) for each matrix was defined 100%. Average concentrations at the various time points were referred to this value (mean ± S.E.M.).

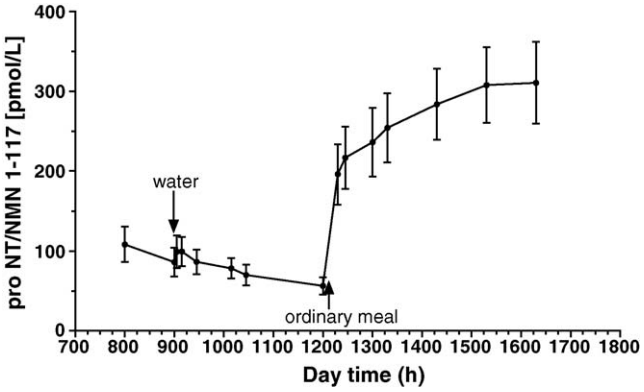

Fig. 8 – Pro NT/NMN 1–117 concentrations before and after water intake and an ordinary meal. Six volunteer blood donors were monitored and measured for pro NT/NMN concentrations from 08:00 to 16:30 h in 15 consecutive samples collected during this period with a 1-L water load and an ordinary meal (~1200 kcal) indicated by arrows. Mean ± S.E.M. is illustrated for each time point.

A sandwich immunoassay for the detection of pro NT/NMN 1–117 was used employing antibodies against the peptides pro NT/NMN 44–62 and 98–117 (referred to as immunoassay 2). Stability of pro NT/NMN 1–117 was assessed in serum and plasma samples from healthy blood donors (n = 5 for each matrix). Pro NT/NMN 1–117 appeared to be stable with a mean recovery of the initial pro NT/NMN 1–117 immune reactivity after storage for 48 h at 22 °C of 100.4% for serum, 97.2% for EDTA-plasma, and 98.1% for heparin-plasma, respectively (Fig. 6).

One hundred and twenty-four healthy blood donors were included to determine the frequency distribution of serum pro NT/NMN 1–117 independent of the participant's nutritional status and daytime of blood sampling. A median concentration of 338.9 pmol/L pro NT/NMN 1–117 (range 53.6–1473 pmol/L; Fig. 7) was found.

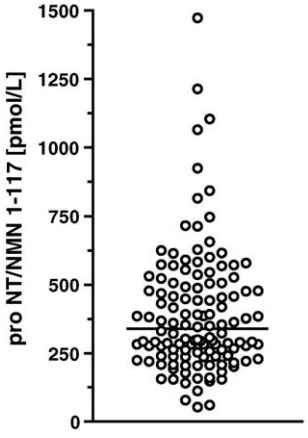

Fig. 7 – Frequency distribution of pro NT/NMN 1–117 of healthy subjects. Absolute concentrations of pro NT/NMN 1–117 were measured in serum samples of healthy blood donors with no regard to nutritional status or daytime of sample taking (n = 124).

To test whether the uptake of water or of an ordinary meal (~1200 kcal) has an effect on serum pro NT/NMN 1–117 concentrations, six healthy volunteers were monitored from 08:00 to 16:30 h for their serum pro NT/NMN 1–117 (Fig. 8). The first serum sample was drawn from the participants being without water and food for 14 h. Subsequent water intake did not change the serum pro NT/NMN 1–117 concentration. In addition, serum pro NT/NMN 1–117 of all six participants was significantly decreased after 18 h fasting as compared to 14 h being without food ($P < 0.022$). Compared to 18 h fasting, postprandial serum pro NT/NMN 1–117 concentrations were increased up to three-fold ($P < 0.006$) with a consistent increase within the next 4 h after food intake.

4. Discussion

Endoproteolytic cleavage of the NT/NMN precursor generates the tridecapeptide neurotensin and the hexapeptide neuromedin N as well as two other potentially bioactive peptides, large NT and large NMN [8–10]. As it has been shown for a number of other peptide hormones, NT and NMN are rapidly cleared from the circulation with an in vivo half-life time of 2–6 min [1,11]. Moreover, NT and NMN are degraded in vitro in cell culture medium containing 8% fetal calf serum with a half-life of less than 30 min [18]. In contrast, large NT and large NMN are more stable in vitro than NT and NMN, but showed a loss of immune reactivity of more than 50% after 5 h incubation in cell culture medium [18]. Hence, the measurement of these peptides in biological fluids is difficult and often underestimated due to its high instability.

It has been successfully shown for other peptide hormones that the measurement of presumably non-functional precursor sequences of these peptides is much more reliable than the measurement of the mature peptides, due to their stability and their equimolar generation. This has been demonstrated so far for the natriuretic peptides of the

A- and B-type [6,22] as well as for adrenomedullin [37,28] and vasopressin [38,29]. For this reason, we developed an immunoassay for the detection of N-terminal proneurotensin precursor fragments and were able to measure N-terminal pro NT/NMN immunoreactivity in healthy individuals. We hypothesized that, in contrast to the mature peptides NT and NMN as well as large NT and large NMN, N-terminal pro NT/NMN could be more stable in human serum or plasma. Actually, there was no decline in N-terminal pro NT/NMN immunoreactivity after storage of serum and plasma samples for up to 48 h at room temperature, indicating that the measurement of pro NT/NMN immunoreactivity could be a suitable alternative method for the quantification of mature neurotensin and neuromedin N, respectively.

In this study, we present mass spectroscopy and N-terminal Edman sequencing evidence that the main circulating pro NT/NMN immunoreactivity is N-terminal pro NT/NMN 1–117. Moreover, the data obtained by MS analysis as well as the results of linear regression of immunoassays 1 and 2, suggest that the potential prohormone cleavage site between Arg^{63}–Arg^{64} is not processed in human blood. Bidard et al. have shown that the rat medullary thyroid carcinoma 6–23 cell line (rMTC 6–23) is producing pro NT/NMN 1–116, a molecule which is analogue to the human pro NT/NMN 1–117 fragment [4]. In addition, they demonstrated that the Lys^{63}–Arg^{64} in the rat pro NT/NMN, which corresponds to the Arg^{63}–Arg^{64} moiety in the human NT/NMN precursor, is not processed. In contrast, this dibasic residue has been shown to be partially processed in selective brain regions in rats [13,42].

It is well known that ingestion of food is a strong stimulus for the release of neurotensin from enteroendocrine N-cells into circulation [33,16,40]. Basal circulating NT-like immunoreactivity levels between 30 and 50 pmol/L have been determined in a number of studies [20,33,41]. In contrast, we detected two- to five-fold higher basal levels of pro NT/NMN 1–117, suggesting that the in vivo half-life time of the prohormone fragment is elevated compared to mature NT/NMN. The intestinal release of NT is known to be stimulated by food ingestion and up to two-fold elevated postprandial NT concentrations were reported [20,33,41]. Here we demonstrate a rapid up to three-fold postprandial pro NT/NMN 1–117 increase in all study participants, without a decline 4 h after food ingestion. These results suggest an extended in vivo half-life of the prohormone fragment compared to NT.

In conclusion, we demonstrate that the NT/NMN precursor fragment representing the amino acids 1 to 117 is circulating in detectable amounts in human blood. Moreover, postprandial serum concentrations of pro NT/NMN 1–117 increased significantly, similar to neurotensin. Due to its in vivo stability of at least 48 h, pro NT/NMN 1–117 measurement could be used as an alternative for the quantification of mature NT/NMN and large NT/large NMN, respectively.

Acknowledgements

The underlying project of this report was aided by grants of the ''Ministerium für Wirtschaft des Landes Brandenburg''. The authors are responsible for the content of this publication. Furthermore, we wish to thank Sandra Mueller for excellent technical assistance and Dr. Nils G. Morgenthaler for consultancy.

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Proenkephalin A 119–159, a stable proenkephalin A precursor fragment identified in human circulation

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ARTICLE INFO

Article history:
Received 17 January 2006
Received in revised form
27 February 2006
Accepted 6 March 2006
Published on line 18 April 2006

Keywords:
Prohormone
PENK A 119–159
Enkephalin
Enkelytin
Sandwich-immunoassay
Half-life
CSF
Concentration gradient

ABSTRACT

In this report, we describe a newly developed sandwich immunoassay using antibodies against the proenkephalin A 119–159 peptide (PENK A 119–159). PENK A 119–159 immunoreactivity was detectable in the circulation of human blood donors and in cerebrospinal fluid (CSF) of patients without a neurologic disorder. The concentration was about 100 times higher in CSF than in serum. Analytical reversed phase HPLC revealed that PENK A 119–159 is the main immunoreactivity in human circulation and CSF. Moreover, PENK A 119–159 is stable in vitro for at least 48 h at room temperature as compared to the low stability of the peptides methionine- and leucine-enkephalin. This suggests the use of PENK A 119–159 measurement as surrogate molecule for the release of the mature peptides derived from proenkephalin A.

1. Introduction

Human preproenkephalin A consisting of 267 amino acids (aa) [7,28], is distributed throughout the nervous system [14,31,39] as well as in the adrenal medulla [42], bone derived cells [36,37] and different cells of the immune system [16,32]. Removal of the N-terminal signal-sequence by a specific signal peptidase [4] gives rise to the proenkephalin A (PENK A) precursor protein (243 aa) (Fig. 1), which is further cleaved at paired basic amino acid residues [8] leading to active enkephalin peptides, namely four copies of methionine-enkephalin (Met-Enk) and one copy each of leucine-enkephalin (Leu-Enk), methionine-enkephalin-Arg-Gly-Leu (Met-Enk-RGL), methionine-enkephalin-Arg-Phe (Met-Enk-RF) and enkelytin [12,18]. Processing of the PENK A precursor has been shown to be tissue-specific and more advanced in brain than in adrenal chromaffin cells, resulting in different processing intermediates [5,11,20,45].

Enkephalin peptides exhibit various functions, including the involvement in stress response mechanisms [9], antinociception [33,34], and immune stimulation [32,38]. They have been implicated in certain neuropathologies such as Alzheimer's [27], Parkinson's [3,6] and Huntington's disease [2,15], schizophrenia [44], severe head injury [40], cerebral ischemia [17] and migraine [10,19,24]. Since mature enkepha-

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0196-9781/$ – see front matter
doi:10.1016/j.peptides.2006.03.008

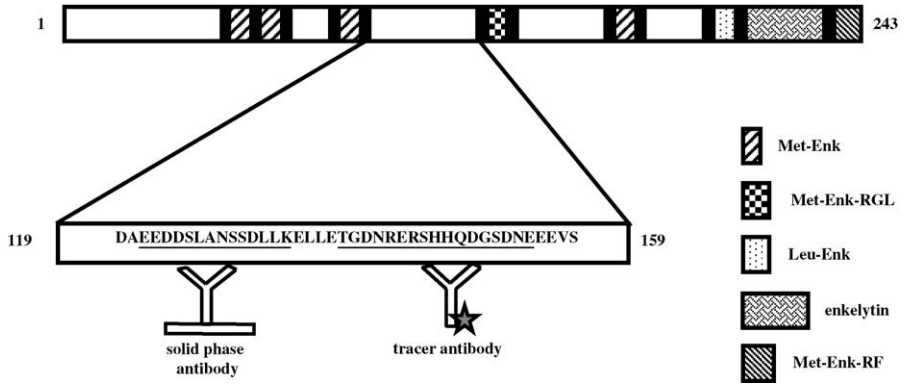

Fig. 1 – Schematic organization of sequence and structure of the human proenkephalin A precursor (amino acids 1–243). Paired basic amino acids representing targets for endoproteolytic processing are shown as black boxes. The amino acid sequence of PENK A 119–159 is shown. Underlined amino acids indicate the sequences of the synthetic peptides PENK A 121–134 and 139–155, used for the production of the solid-phase and tracer antibody, respectively.

lins, as Met- and Leu-Enk, are instable in vitro, possessing a half-life time in human plasma of less than 15 min [25,26], their reliable quantification for routine application is limited.

In this study we demonstrate, that a proenkephalin A precursor fragment, termed PENK A-peptide 119–159 (PENK A 119–159), which has been identified in human cerebrospinal fluid [41], is also present in human circulation. With a newly developed sandwich immunoassay we measured PENK A 119–159 concentrations in human serum and CSF samples, revealing a concentration gradient between CSF and serum of nearly 100. Moreover, we show that the peptide PENK A 119–159 is resistant against degradation in vitro, representing a stability of more than 48 h in human blood and lumbar CSF preparations. The measurement of PENK A 119–159 rather than that of the enkephalins should be more eligible for laboratory and clinical investigations, due to its high stability and stoichiometric generation with mature PENK A-derived peptides.

2. Materials and methods

2.1. Chemicals

If not denoted otherwise, chemicals were obtained at p.a. grade from Merck (Darmstadt, Germany). Bovine serum albumin, goat-anti-mouse immune globulin and horse serum H1270 were purchased from SIGMA (Deisenhofen, Germany).

2.2. Peptides and antibodies

Three peptides chemically related to the PENK A-precursor (aa 1–243) were supplied by JPT Peptide Technologies GmbH (Berlin, Germany). The peptides PENK A 121–134 (EEDDSLANSSDLLK) and PENK A 139–155 (TGDNRERSHHQDGSDNE) were synthesized with an additional N-terminal cysteine-residue. A peptide comprising the amino acids 119–159 of the PENK A precursor was produced as standard material.

A monoclonal antibody against the peptide PENK A 121–134 was produced by InVivo BioTech Services GmbH (Hennigsdorf, Germany) and supplied purified in PBS, pH 7.2. Polyclonal antibodies against the peptide PENK A 139–155 were raised in sheep by Micropharm (Carmarthenshire, Great Britain). For affinity purification of anti-PENK A 139–155 antibodies, the peptide PENK A 139–155 was covalently linked to Sulfolink coupling gel from Pierce (Boston, USA) according to the suppliers instructions. The gel material was mixed with the sheep antiserum, incubated over night at room temperature and loaded on a polycarbonate-column. Excessive serum was discarded. The gel material was washed with 100 mmol/L potassium phosphate buffer, pH 6.8, containing 0.1% Tween 20 to eliminate serum proteins. Bound antibodies were eluted in fractions of 1 mL with 50 mmol/L citric acid (pH 2.2). The protein content in each fraction was determined with the BCA protein assay kit from Perbio (Bonn, Germany) and fractions with a protein concentration of more than 1 mg/mL were pooled.

2.3. Serum, plasma and lumbar cerebrospinal fluid samples

Serum, EDTA-plasma or heparin-plasma samples from healthy blood donors were collected following ethical guidelines and stored at −20 °C until further use. Human lumbar cerebrospinal fluid samples from patients without a known neurological disorder were obtained from InVent Diagnostica GmbH (Hennigsdorf, Germany).

2.4. Immunoassay

A chemiluminescence-label coated-tube immunoassay was set up using the monoclonal anti-PENK A 121–134 antibody as coated tube and the polyclonal anti-PENK A 139–155 antibody as tracer antibody, respectively. Assay components were prepared as described by Morgenthaler et al. [23]. The coating of the antibodies was slightly modified by incubating the anti-PENK A 121–134 antibody (0.5 μg per tube) on polystyrene tubes, which were pre-coated with goat-anti-mouse antibodies (2 μg per tube). Dilutions of the synthetic peptide PENK A 119–159 were used as calibrators. The peptide was diluted in horse serum for the calculation of PENK A 119–159 immuno-

reactivity in human serum and plasma samples, and in assay buffer (PBS containing 10 mmol/L Na-EDTA, pH 7.2) for the calculation of PENK A 119–159 immunoreactivity in human lumbar CSF. Serum samples from control subjects were used undiluted whereas CSF-samples were diluted 1:20 in assay buffer. The immunoassay was performed by incubating 100 μL of sample/sample dilution or standard peptide solution and 100 μL tracer for 20 h at room temperature. The tubes were washed five times with 1 mL of PBS (pH 7.2) and bound chemiluminescence was detected with a luminometer LB 952T/16 (Berthold, Wildbad, Germany).

The lower detection limit of the immunoassay was determined by a 10-fold measurement of horse serum and assay buffer and calculation of the respective mean plus 2 standard deviations. The corresponding PENK A 119–159 concentration was extrapolated from the calibration curve.

2.5. RP-HPLC

Fractionation of a serum sample was performed by RP-HPLC. 600 μL sample (c = 314 pmol/L) was mixed with 2.4 mL 5% acetonitrile, 0.1% TFA, and cleared by filtration through a 0.45 μm filter (Millipore, Schwalbach, Germany). 2.8 mL of the filtrate was applied to a μBondapak RP-C18-column (Waters, Eschborn, Germany) and eluted with a linear acetonitrile gradient up to 95% acetonitrile within 35 min. The flow rate was 1.0 mL/min and 1.0 mL fractions were collected. The fractions were dried in a SpeedVac concentrator (Thermo-Savant, Egelsbach, Germany) and dissolved and neutralized in 220 μL assay buffer for measurement of PENK A 119–159 immunoreactivity.

In addition, 50 μL CSF sample (c = 6600 pmol/L) diluted in 450 μL horse serum and 200 μL of a PENK A 119–159 standard dilution in horse serum (c = 2500 pmol/L) were mixed with 5% acetonitrile, 0.1% TFA, in a 1:5 ratio and filtrated. 2.3 mL of the CSF solution and 0.8 mL of the standard peptide solution were analyzed by RP-HPLC as described above.

2.6. Stability of PENK 119–159 in serum, plasma and CSF samples

To determine the stability of endogenous PENK A 119–159 in human serum, EDTA-plasma and heparin-plasma, 5 freshly prepared samples of each matrix from healthy blood donors were stored at room temperature for up to 48 h. Aliquots were taken immediately after preparation of sample (for reference) and after 2, 4, 6, 24 and 48 h incubation at room temperature and stored frozen at −20 °C. The same procedure was performed with lumbar CSF samples (n = 3) from patients with no known neuropathological disease. For the determination of stability, all samples were thawed together and PENK A 119–159 concentration was measured. The value of each reference was defined as 100%. Mean percentage values (±S.D.) for each time point and each matrix were calculated.

3. Results

An one-step sandwich immunoassay was developed for the detection of PENK A 119–159, employing the chemiluminescence-label and coated-tube technique. Dilutions of the synthetic peptide PENK A 119–159 in horse serum and assay buffer, respectively, served as calibrator for the measurement of PENK A 119–159 concentrations in serum and CSF. A typical calibration curve for the measurement of PENK A 119–159 is shown in Fig. 2. The lower detection limit of the assay was determined to be 20 pmol/L.

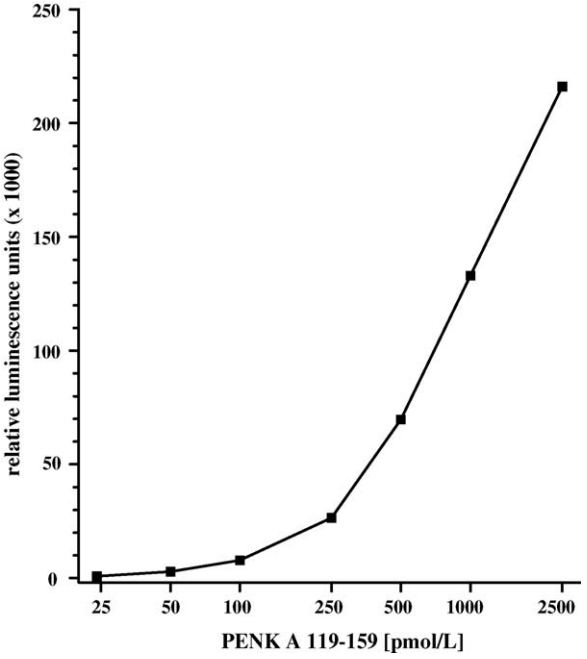

Fig. 2 – Representative dose–response curve for the detection of PENK A 119–159.

With this assay PENK A 119–159 concentrations between 41.8 and 131 pmol/L (n = 50, median 62.3 pmol/L) were detected in serum of healthy blood donors (Fig. 3). In contrast, in lumbar CSF samples from patients with no known neurological disorder (n = 9), we measured a median concentration of 6240 pmol/L (range between 5780 and 9480 pmol/L; Fig. 3).

The stability of PENK A 119–159 was assessed in serum and plasma samples from healthy blood donors. PENK A 119–159 emerged to be stable in all of these matrices with a mean recovery of initial PENK A 119–159 immunoreactivity (after storage for 48 h at room temperature) of 100.0% in serum, 101.2% in EDTA-plasma and 98.0% in heparin-plasma (Fig. 4). Moreover, PENK A 119–159 immunoreactivity was stable in lumbar CSF exhibiting a mean recovery of 102.7% after 2 days storage at room temperature (Fig. 4).

To characterize the structure of PENK A 119–159 immunoreactivity we used an analytical reversed phase chromatographic method. 61.0 and 70.3% of the PENK A 119–159 immunoreactivity from serum and CSF, respectively, that was applied to the μBondapak column, was recovered. The prominent PENK A 119–159 immunoreactivity in serum and in lumbar CSF co-eluted with the synthetic PENK A 119–159 peptide (Fig. 5). However, in addition PENK A 119–159-containing fragments, which exhibit increased or decreased hydrophobic features compared to PENK A 119–159, were detectable in healthy blood donor serum, but to a minor extent

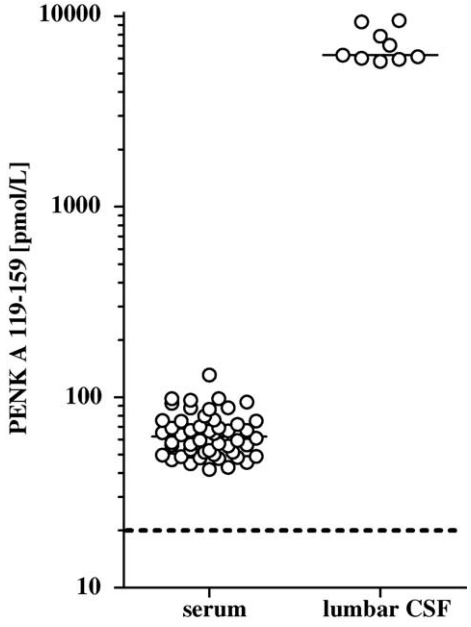

Fig. 3 – Concentration of PENK A 119–159 immunoreactivity in serum samples of healthy blood donors (n = 50) and lumbar CSF samples of patients without a known neurologic disorder (n = 9). The analytical assay sensitivity of 20 pmol/L (determined as described in Section 2) is indicated as a dotted line.

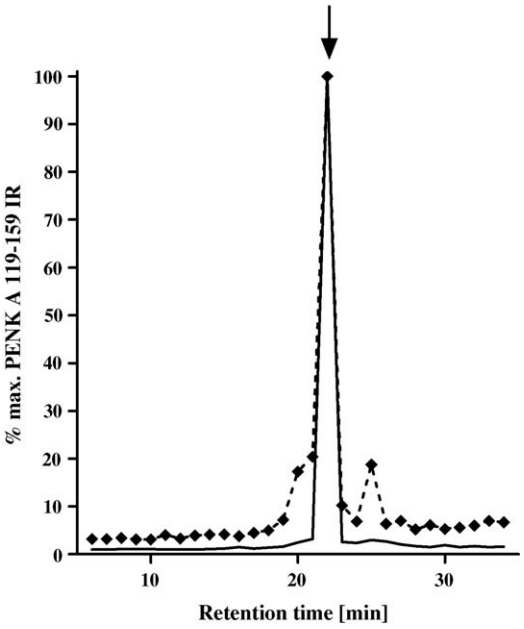

Fig. 5 – Analytical RP-HPLC of a serum (broken line) and CSF sample (solid line). The retention time of synthetic PENK A 119–159 is indicated by an arrow. PENK A 119–159 immunoreactivity was measured in fractions 5–35. The maximum measured immunoreacitivity for each sample was defined as 100%.

compared to PENK A 119–159. In contrast, only PENK A 119–159, but no other PENK A 119–159-containing fragments could be detected in human lumbar CSF.

4. Discussion

With a newly developed sandwich immunoassay, proenkephalin A 119–159 immunoreactivity was detectable in every serum, plasma and CSF sample. As mature enkephalins are encoded by the PENK A-precursor, the measurement of PENK A 119–159 is supposed to reflect the Leu-Enk-, enkelytin-, Met-Enk-RGL and Met-Enk-RF-release in a one-to-one and the Met-Enk-release in a one-to-four ratio. However, Leu- and Met-Enk are extremely unstable in plasma, showing a half-life time of 11.8 [26] and 12.8 min [25], respectively. In this study, we demonstrate that PENK A 119–159 immunoreactivity is stable in human blood preparations as well as lumbar CSF for at least 48 h at room temperature, suggesting the measurement of this robust molecule in laboratory and clinical application instead of the unstable bioactive PENK A-derived peptides. Enkelytin and the C-terminal extended Met-Enk-peptides, Met-Enk-RGL and Met-Enk-RF, are exclusively derived from the proenkephalin A precursor molecule. However, the pentapeptide Leu-Enk is encoded and released not solely by PENK A but also by the prodynorphin (PDYN)-precursor [13]. Hence, measurement of PENK A 119–159 can merely serve as surrogate molecule for PENK-A-derived Leu-Enk.

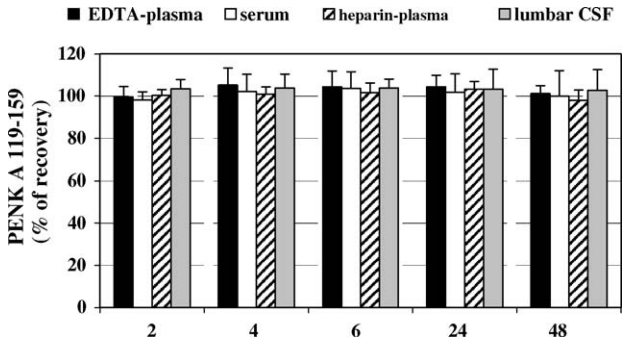

Fig. 4 – Stability of PENK A 119–159 immunoreactivity in human serum, plasma and CSF samples. Serum, EDTA- and heparin-plasma (n = 5, for each matrix), from healthy blood donors as well as lumbar CSF samples (n = 3) were tested for PENK A 119–159 stability at room temperature for up to 48 h. The concentration of the reference value (at 0 h) for each matrix was defined as 100%. Mean (+S.D.) recovery values for each time point were calculated.

The major immunoreactivity in human circulation and lumbar cerebrospinal fluid, detectable with the immunoassay developed against the PENK A peptide sequence 119–159, is PENK A 119–159. This peptide has been previously identified in CSF [41] but not in blood. Synthetic PENK A 119–159 peptide was shown to co-elute with the peptide measured in serum and CSF by means of analytical reversed phase HPLC. However, the elution pattern of PENK A 119–159 immunoreactivity in serum revealed the presence of PENK A 119–159-containing peptides in blood (Fig. 5). Recombinant

PENK A is detectable with our immunoassay developed against amino acids 119–159 (data not shown), indicating that the antibodies can cross-react with PENK A 119–159-containing peptides. In contrast to serum, we could not detect PENK A 119–159-containing peptides in cerebrospinal fluid. It has been reported that PENK A is only partially processed in CSF and larger PENK A processing intermediates are present in concentrations exceeding those of the active enkephalin peptides [29,30]. However, our results demonstrate that the potential prohormone cleavage sites Lys^{117}-Lys^{118} and Lys^{160}-Arg^{161} of the PENK A precursor are completely processed in human CSF.

We detected an approximately 100 times higher PENK A 119–159 concentration in lumbar CSF than in the circulation, supporting the finding that proenkephalin A-derived peptides are mainly produced in the central nervous system. Levels of the CSF-specific protein beta-trace protein (prostaglandin D synthase) were reported to be 32–76-fold higher in CSF than those in serum [1,21,22,35,43], representing the highest known concentration gradient of a CSF protein so far. However, the results of the present study show a more elevated concentration gradient of PENK A 119–159 immunoreactivity between CSF and serum as compared to that of beta-trace protein.

Changes of enkephalin levels in the brain of patients with Alzheimer's [27], Parkinson's [3,6] and Huntington's [2,15] disease as well as in schizophrenia [44], severe head injury [40], cerebral ischemia [17] and migraine [10,19] were reported. As we suggest the measurement of PENK A 119–159 as surrogate molecule for mature PENK A-derived peptides, this could provide a tool to investigate possible changes of the enkephalin release rate into CSF in these neuropathological conditions.

Acknowledgements

The underlying project of this report was aided by grants of the "Ministerium für Wirtschaft des Landes Brandenburg". Furthermore this report will be part of a dissertation work (AE) realized at the Charité Universitätsmedizin Berlin. The authors are responsible for the content of this publication. We thank Sandra Mueller (SphingoTec GmbH) for excellent technical assistance and Christine Alonso and Joachim Struck (BRAHMS AG) for consultancy.

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PLoS one

The Neurotensin Receptor-1 Pathway Contributes to Human Ductal Breast Cancer Progression

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Abstract

Background: The neurotensin (NTS) and its specific high affinity G protein coupled receptor, the NT1 receptor (NTSR1), are considered to be a good candidate for one of the factors implicated in neoplastic progression. In breast cancer cells, functionally expressed NT1 receptor coordinates a series of transforming functions including cellular migration and invasion.

Methods and Results: we investigated the expression of NTS and NTSR1 in normal human breast tissue and in invasive ductal breast carcinomas (IDCs) by immunohistochemistry and RT-PCR. NTS is expressed and up-regulated by estrogen in normal epithelial breast cells. NTS is also found expressed in the ductal and invasive components of IDCs. The high expression of NTSR1 is associated with the SBR grade, the size of the tumor, and the number of metastatic lymph nodes. Furthermore, the NTSR1 high expression is an independent factor of prognosis associated with the death of patients.

Conclusion: these data support the activation of neurotensinergic deleterious pathways in breast cancer progression.

Citation: Dupouy S, Viardot-Foucault V, Alifano M, Souazé F, Plu-Bureau G, et al. (2009) The Neurotensin Receptor-1 Pathway Contributes to Human Ductal Breast Cancer Progression. PLoS ONE 4(1): e4223. doi:10.1371/journal.pone.0004223

Editor: Syed A. Aziz, Health Canada, Canada

Received October 1, 2008; **Accepted** November 6, 2008; **Published** January 19, 2009

Funding: This work has been supported by INSERM, UPMC and a Grant from ARC (Association pour la Recherche sur le Cancer # 3543) and LNC (Ligue Nationale Contre le cancer) # 75/00-RS/40 and 07/75-85 France. Sandra Dupouy was supported by the 'Ligue nationale contre le cancer'.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Breast cancer is the most frequent cause of cancer-related deaths among women in the western world [1]. Among these patients, one of four women dies from breast cancer, despite improvements in diagnosis, surgery, chemotherapy and the new targeted therapies. Death is associated with the metastatic development of the disease. The discovery and characterization of new contributors remain necessary in order to develop appropriate and highly specific treatments targeted to metastasis initiation and progression processes.

Neurotensin (NTS) is a 13 amino acids peptide formed from a large precursor, cleaved by convertases. NTS is commonly known for its distribution along the gastrointestinal tract [2]. Typical physiological functions for NTS include stimulation of pancreatic and biliary secretions, inhibition of small bowel and gastric motility, and facilitation of fatty acids translocation [3–5]. NTS was equally reported in functions linked specifically to neoplastic progression, including proliferation of the pancreas, prostate, colon, and lung cancer cells [6]. We have previously described detrimental effects, caused by NTS, on xenografted breast tumor growth as well as migration, invasion, and survival of breast cancer cells [7,8].

NTS expression is also found in endocrine tumors and is linked to tissue differentiation [9]. NTS is expressed in fetal colon, repressed in newborn and adult colon, and re-expressed in approximately 25% of human colon cancers due to epigenetic mechanisms linked to NTS gene hypomethylation [10].

NTS peripheral functions are mediated through its interaction with the NTSR1 [11]. When NTSR1 is challenged with NTS, phosphatidyl inositols are hydrolyzed leading to Ca^{2+} mobilization and PKC, ERK1/2, RhoGTPases, NFkappa-B, and focal adhesion kinase (FAK) activation [12–15]. The NTSR1 gene is a target of the Wnt/APC oncogenic pathways connected with the β-catenin/Tcf transcriptional complex, known to activate genes involved in cancer cell proliferation and transformation [7].

In this report, we investigate the expression of NTS and NTSR1 in a cohort of 106 women diagnosed for invasive ductal breast cancer (IDCs). We conclude that NTSR1 regulation may occur in breast cancer and participates in the neoplastic progression in up to 35% of all patients.

Results

NTS expression in normal epithelial breast cells is regulated by estradiol

The NTS gene was previously described as an estradiol target gene [16] with estradiol increasing NTS transcription in the preoptic area and neurosecretory cells of the hypothalamic arcuate

nucleus [17,18]. We hypothesized that NTS is also expressed in normal human breast tissue, and studied NTS transcript on normal mammary glands, and on eight different human breast epithelial cells (HBEC) cultures. We consistently detected NTS amplicon with low to medium intensity. Typical examples are shown in figure 1A *left*. In order to evaluate if NTS gene is also regulated by estradiol in human breast, HBEC were exposed to estradiol. As shown in figure 1A *right*, an enhancement of NTS transcripts was observed. This effect was abolished when ICI 182780, a pure anti-estrogen, was added concomitantly to estradiol (Figure 1A *right*) suggesting that estrogen receptors participate in the NTS gene regulation in human breast tissue. Corroborating these results, NTS expression was positively detected by immunohistochemistry in 19 (76%) biopsies of normal breast tissues from 25 premenopausal women. We observed NTS labeling within sparse epithelial cells of ducts and lobules (Figure 1B, 1 and 4). On the same slide we noticed that the lobular structures were labeled with a more intense staining than the duct structures. We also noticed that the normal adjacent tissue of invasive ductal breast carcinomas (IDCs) was very often labeled by NTS antibody, with similar intensity and cellular distribution as in the tissue from healthy women (Figure 1B, 5 and 6).

NTS expression in invasive ductal breast carcinomas

We previously demonstrated the presence of NTSR1 and NTS expression in breast ductal carcinomas, along with NTS induced effects on tumor growth, cellular mobility and collagen invasion of

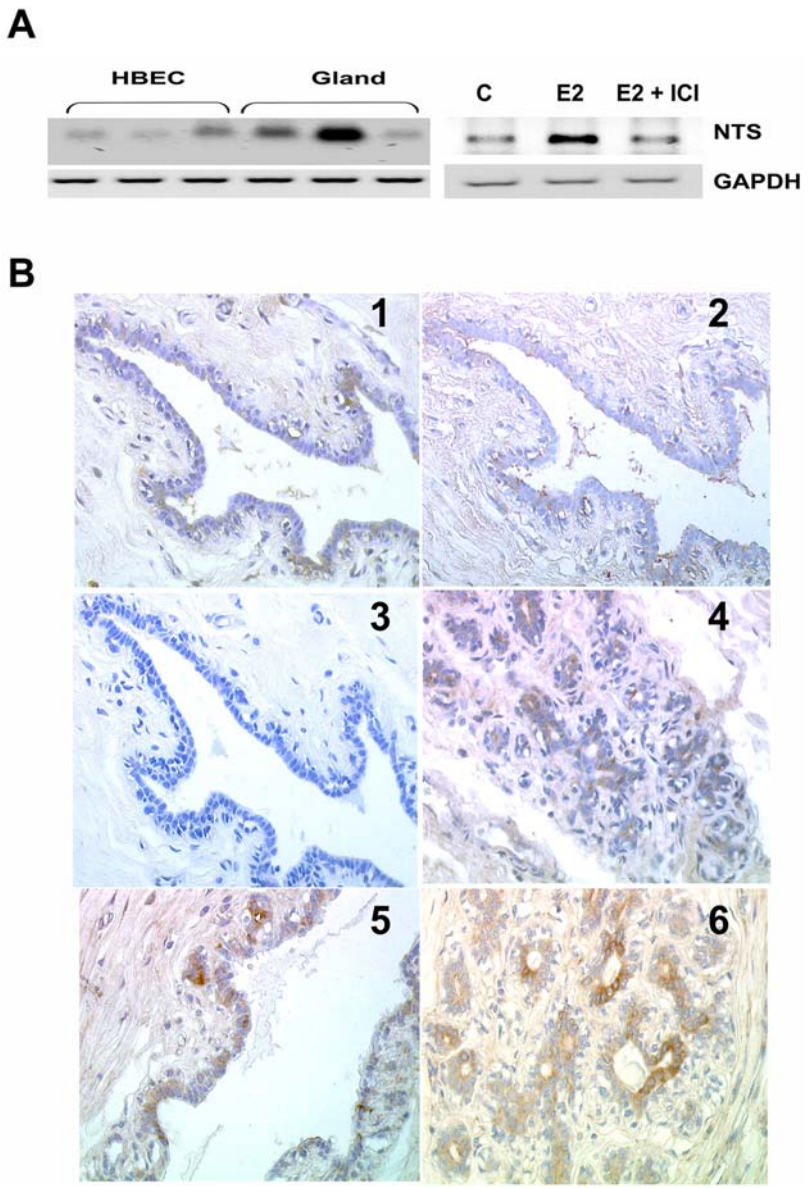

Figure 1. Neurotensin expression in normal breast tissue. a) *Left*, one μg of total RNA from HBEC or whole gland were reverse-transcribed and a PCR experiment specific for NTS was performed. *Right*, one μg of total RNA from HBEC cells (control, treated with 10 nM estradiol (E2) with or without 1 μM ICI 182780) was reverse-transcribed. A PCR experiment was performed using specific primers for NTS and GAPDH. **b**) Normal duct exposed to NTS antibody at 1/500 dilution (1), after pre-incubation with the antigen peptide for 2 h at 10 nM (2), or without primary antibody (3), and lobule exposed to NTS antibody (4). Normal tissue exposed to NTS antibody at 1/500 dilution adjacent to tumor duct (5), lobule (6). The original magnification was 200×.
doi:10.1371/journal.pone.0004223.g001

Table 1. Patients clinical characteristics.

	IDCs
	n = 106
Age in years [mean±SD]	57.96±14.04
Menopausal status	
Post menopausal patients [n (% of patients)]	69 (65%)
Age at menopause [mean±SD]	50.96±3.05
Family history of breast cancer [n/number of cases studied (% of patients)]	18/79 (23%)
HRT use [n/number of cases studied (% of patients)]	17/92 (18%)
Follow-up in months [mean±SD]	67.9±41.93
Positive estrogen receptor [n/number of cases studied (% of patients)]	69/100 (69%)
Positive progesterone receptor [n/number of cases studied (% of patients)]	71/99 (71.7%)
Tumor size (cm) [mean±SD]	2.3±1.43
SBR grade [n]	
1	33
2	49
3	24
Number of patients N+ [n/number of cases studied (% of patients)]	42/104 (40.4%)
Number of invaded nodes [mean±SD]	1.32±2.55
Recurrence during follow-up [n (% of patients)]	26 (25%)
Deaths during follow-up [n (% of patients)]	11 (10.4%)
Adjuvant therapy (number of cases studied)	102
Radiotherapy [n (% of patients)]	96 (94%)
Chemotherapy [n (% of patients)]	36 (35.5%)
Tamoxifen use [n (% of patients)]	78 (76.4%)

SBR; Scarff Bloom and Richardson, n = number of patients, SD = standard deviation.

doi:10.1371/journal.pone.0004223.t001

a cancer mammary cell line [7]. In order to further evaluate the status of NTS and NTSR1 in breast cancer we studied their respective expression in 106 IDCs. Details of clinical data, pathological characteristics, and treatment modulations are shown in table 1. NTS was graded in the invasive and ductal components in the patients' IDCs. In most cases a large amount of cells were positively labeled with NTS antibody (Figure 2A). NTS positive labeling in invasive component is significantly correlated with the positive labeling in the ductal component ($P = 0.004$). In both cases, NTS labeling was cytosolic (Figure 2A). Using RT-PCR, we confirmed the high expression of NTS transcript in 9 of 11 breast cancer tissues (Figure 2B). Five patients exhibited a very strong expression of NTS transcript (Figure 2B, lane 2–6) and four others displayed a weaker expression (Figure 2B, lane 7–9 and 11). No correlation was observed with prognosis factors and disease progression (tumor size, grade, number of invaded nodes, recurrence, and death) with NTS expression, neither in the ductal nor in the invasive components (Table 2). The only correlation found, was between PR and NTS expression in the invasive component. NTS is neither a marker nor associated with tumor progression in breast cancer.

NTSR1 expression in IDCs

NTSR1 staining in IDCs showed that NTSR1 expression was spread throughout many tumor cells in the invasive and ductal components. The labeling was granular and mostly cytosolic. In the invasive component of studied IDCs, the majority exhibited a high proportion of NTSR1 positive cells (from 50 to 100%). We hypothesized that the deleterious effects of NTS previously described should occur in tumors containing a very high proportion of NTSR1 expressing cells [7,19]. We focused on the 35% of patients in which 80% or more of the tumor cells expressed the NT1 receptor. Expression of NTSR1 was verified by RT-PCR on frozen tissues from 11 patients. As shown in Figure 2B, three patients expressed NTSR1 (lane 3, 6, 9) with two showing a very high amplicon amount (Figure 2B lane 6, 9).

The characteristics of the women exhibiting high NTSR1 expression (≥80% of tumor cells) are shown in table 3. High NTSR1 expression was associated with a larger tumor size ($p<0.01$), SBR grade 3 ($p<0.05$), the number of positive lymph nodes ($p<0.05$), and as a consequence it was also associated with chemotherapy ($p<0.01$). Using univariate analysis we found that patients with high expression of NTSR1 had a significantly worse prognosis than those with low NTSR1 expression (ten years survival rate of 66.2% versus 96.5%; $p = 0.01$). Kaplan-Meier survival graph up-to 10 years, and number of patients at risk during this period of time are shown in figure 3. Multivariate analysis with a Cox model adjusted for major prognosis risk factors, age, tumor size, SBR grade, positive ER status and lymph nodes, showed that high NTSR1 expression remained an independent prognosis marker. The relative risk of dying in women with expression of NTSR1≥80% compared to women with expression of NTSR1<80% was significantly increased (RR = 5.29, 95% confidence interval [1.04–26.88], $p = 0.044$).

NTSR1 paracrine regulation

Within the 48 patients expressing NTS in the invasive component, 20 (42%) exhibited high expression of NTSR1 (≥80%), corresponding to 20% of the whole population. Examining adjacent tissue sections of these patients, a clear regional co-localization of the ligand and its receptor was detected in all IDCs (Figure 2C). Within the population co-expressing NTS and NTSR1 the distribution among the SBR grades was altered as compared to the total population, with few patients in the grade 1 and most patients in the grade 3 ($p<0.05$). The size of the tumor, the recurrence and the number of death were higher in this subpopulation than in the total population. In addition, the ER alpha receptor positivity, characterized as a differentiated and good prognosis marker, was correlated with the NTS and low NTSR1 expression ($p<0.05$) (Table 4).

NTSR1 and NTS gene expression in breast cancer microarray studies

We sought to compare our findings with those from publically available breast gene array analysis. A correlation between up regulated expression of NTSR1 with the higher grades was found studying 55 and 125 breast carcinomas, from the Ginestier and Sotiriou databases ($p = 0.028$ and 0.04), respectively [20,21]. In the Chin gene profile, containing 118 frozen primary breast carcinomas, NTSR1 was found over expressed in stage IV carcinomas as compared to stage I with $p = 0.003$. In the same study, a correlation between NTSR1 over expression and the positive lymph node status was found ($p = 0.04$) [22]. No correlation was detected in the available databases between the over expression of NTSR1 and 5-year survival, noting that data were not available for longer time periods. The latter result is unsurprising, because of the very low number of deaths registered in these small populations during a survival time frame which is shorter than that observed in breast cancer patients managed with

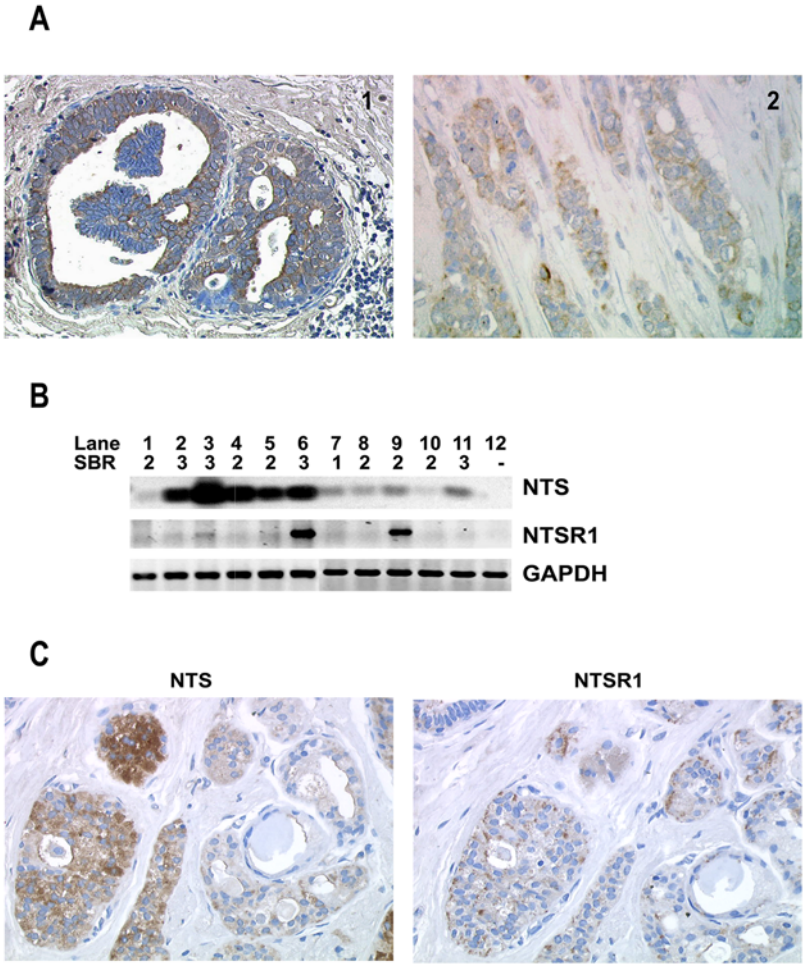

Figure 2. Neurotensin expression in IDCs. a) NTS immunohistochemistry was performed on IDCs, ductal (1) and invasive (2) components, magnification 200× for (1) and 400× for (2). **b**) NTS and NTSR1 transcripts in IDCs. One µg of total RNA from 11 patients with IDCs were reverse-transcribed, and specific PCR was performed for NTS, NT-1 receptor, or GAPDH (control). The SBR grade is indicated below each line. **c**) Example of NTS and NTSR1 regional co-localization by immunohistochemistry for NTS (right) and NTSR1 (left) at the original magnification 400×.
doi:10.1371/journal.pone.0004223.g002

the currently available multimodality treatments. In our study the correlation between survival and NTSR1 expression was strongest at the 10-year follow-up ($p = 0.01$) and decreased with shortening of it, arriving at a value of 0.052 (NS) at the 5-year follow-up. Of note, studies using other parameters confirm that the NTSR1 expression is a poor prognosis marker. in the "Ma" database studying 54 patients with breast carcinomas, NTSR1 is correlated with the recurrence at 5 years ($p = 0.04$) [23]. In Chang's study, dealing with 24 breast ductal carcinomas classified according to the docetaxel response, NTSR1 was more intensively expressed in the group resistant to this chemotherapy agent [24].

A high correlation was found between NTS and estrogen receptor expression in the Sotiriou and Chin gene arrays ($p = 7.9$ E-5 and 0.002, respectively [21,22]). In the Chin gene array, NTS expression was also correlated with progesterone receptor expression ($p = 0.003$). As in our study, no other correlation was detected. The results reported here and those from the gene array profiles lead, therefore, to similar conclusions.

Discussion

This paper evaluates the status of NTSR1 as a contributor in human breast cancer progression. One approach to address this question is to determine if paracrine NTS regulation is associated with the patients' poor outcome. We suspected that the contribution of NTSR1 in tumorigenesis occurred from local and sustained activation of the receptor rather than from circulating NTS, because NTS is a highly degradable peptide and its blood concentration rapidly drops after its release. It has been demonstrated that sustained activation of NTSR1 results in persistent NTSR1 recycling as well as signalization activation, including ERK1/2 [19], and causes sustained gene activation of MMP9 and Bcl-2 [7,8]. In human tumor these conditions would be satisfied if NTS is synthesized and released within the vicinity of NTSR1 expressing cells. NTSR1 expression is an early event of cell transformation, because of the resulting NTSR1 promoter activation by the Wnt/β-catenin pathway [25]. Here we showed that NTSR1 is highly expressed (≥80%) in 35% of the patients with a granular labeling mostly cytosolic suggesting an intense receptor endocytosis.

The data concerning NTS expression in human cancer are sparse. Hypomethylation, or NTS regulation by Ras or Src oncogenes were described as possible mechanisms leading to expression of NTS gene in cancer [10]. Hormonal regulation was also described more specially in specific areas of the hypothalamus, and in the preoptic area, where NTS mRNA is stimulated by

Table 2. Prognosis factors and deaths stratified by NT expression in the ductal and invasive components of IDCs.

	NT			NT		
	Ductal component			Invasive component		
	n = 87			n = 103		
	NO	YES	P	NO	YES	P
	n = 31	n = 56		n = 55	n = 48	
Positive estrogen receptor [n/number of cases studied (SD)]				30/49	36/47	NS
Positive progesterone receptor [n/number of cases studied (SD)]				32/51	37/45	0.034
Tumor size (cm) [number of cases studied]	30	52		54	44	
[mean±SD]	2.36±1.7	2.22±1.25	NS	2.43±1.7	2.03±0.9	NS
SBR grade [number of cases studied]	31	56		52	47	
1	7	21		16	17	
2	13	26		25	22	
3	11	9	NS	14	9	NS
Number of Invaded nodes [number of cases studied]	30	54		52	48	
[mean±SD]	1.17±2.3	1.66±3	NS	1.23±2	1.49±3	NS
Recurrence during follow-up [n/n of patient studied]	6/31	14/56	NS	15/55	11/47	NS
Deaths during follow-up [n/n of patients studied]	2/31	7/56	NS	5/55	6/48	NS
Adjuvant therapy	30	53		51	47	
Radiotherapy [n]	29	50	NS	48	46	NS
Chemotherapy [n]	12	19	NS	19	16	NS
Tamoxifen use [n]	25	38	NS	41	34	NS

doi:10.1371/journal.pone.0004223.t002

estrogen [26]. This effect is transcriptional and involves cAMP/protein kinase A-dependent signaling mechanisms [16]. In this paper we observed the expression of NTS and NTS mRNA in HBEC and demonstrated that NTS is an estrogen target gene in those cells. We also observed expression of NTS in tumor cells of the ductal and invasive components in breast IDCs, with a strong statistical correlation of NTS expression in both components. This latter finding, with a similar NTS repartition within the low or high NTSR1 expressing patients, suggests that NTS gene remains constitutively expressed during the neoplastic process, rather than being deregulated. In parallel, we observed a frequent regional co-localization of both markers in adjacent tissue sections from the same tumor, suggesting NTSR1 activation. Together with the expression of NTSR1, these data validate our hypothesis of the NTS paracrine regulation of transformed epithelial cells during the neoplastic process, with NTS released from the surrounding normal breast tissue or from the breast tumor.

High NTSR1 expression was significantly associated with the SBR grade, the size of the tumor, and the number of metastatic lymph nodes, and ultimately with death of the patients. These findings support the deleterious effects of NTS found in breast cancer cells [7]. NTS and NTSR1 are implicated in several detrimental functions linked to the neoplastic progression, including proliferation of the pancreas, prostate, colon and lung cancer cells [6], protection of breast cancer cells against apoptosis [8], and induction of the proinvasive potential of colon cancer cells [25]. More recently, it was shown that NTSR1 activation results in EGFR transactivation by the shedding of transforming growth factor-α (TGF-α) in pancreas, and HB-EGF or amphiregulin in prostate cancer cells, both leading to ERK1/2 activation [27], but also EGFR expression [28]. These findings point out that the poor prognosis attributed to patients with highly NTSR1 expressing IDCs may be directly related to the expression of its natural ligand NTS, with the continuous activation of the NTSR1, leading to enhanced cancer cell survival, invasiveness potential, and metastasis [7,8].

In conclusion, based on a series of 106 patients with invasive ductal breast cancer, we provide evidence for NTS/NTSR1 as a contributor to breast cancer progression. Identification of breast cancer patients characterized by paracrine NTS/NTSR1 signaling pathway, as evidenced in the present study, will provide alternative strategies to improve the treatment of IDCs. These findings support the therapeutic potential of NTS/NTSR1 inhibition or drug cellular targeting through NTSR1 in advanced stages of human breast cancers.

Materials and Methods

Breast Biopsies

Clinical file of 106 patients completely resected for invasive ductal breast cancers (IDCs) by tumorectomy or mastectomy, at the Gynecology Department, Hôtel-Dieu Hospital, Paris, were studied. Patients were diagnosed by the same oncologist (Dr Y. Decroix) for a period from June 1984 through May 1998. Clinicopathological information was derived retrospectively from patient records. Survival and follow-up durations were measured as the time between the first histological confirmation of breast cancer and the last consultation in the department, or death. Patient records were reviewed retrospectively for demographical characteristics, clinical data, outcome, and survival. The histological diagnosis was routinely checked by microscopic examination of sections stained with hematoxylin-eosin.

Ethics

The following studies were conducted on tissues obtained from patients between 1984 and 1998. The experiments reported here were carried out under the current French ethical regulations as

Table 3. Prognosis factors and deaths stratified by NT-1 receptor expression in the invasive component of IDCs.

	NT1 receptor		
	n = 106		
	<80%	**>80%**	**P**
	n = 68	**n = 38**	
Positive estrogen receptor [n/number of cases studied]	46/64	23/36	NS
Positive progesterone receptor [n/number of cases studied]	46/63	25/36	NS
Tumor size (cm) [number of cases studied]	68	38	
[mean (SD)	2.08±1.35	2.71±1.4	0.007
SBR grade [number of cases studied]	68	38	
1	26	7	
2	31	18	
3	11	13	0.036
Number of invaded nodes[number of cases studied]	66	38	
[mean±SD]	0.86±1.7	2.11±3.4	0.05
Recurrence during follow-up [n/number of cases studied]	13/67	13/38	0.09
Deaths during follow-up [n/number of cases studied]	2/68	9/38	0.0025
Adjuvant therapy (102 cases studied)	65	37	
Radiotherapy [n]	61	35	NS
Chemotherapy [n]	17	19	0.01
Tamoxifen use [n]	49	29	NS
NT [n/number of cases studied]	28/65	20/37	NS

doi:10.1371/journal.pone.0004223.t003

Table 4. Correlation of the subpopulation co-expressing NT and NT1 receptor with the major prognosis factors.

	NT invasive component		
	n = 48		
	NT-1 receptor	**NT-1 receptor**	**P**
	<80%	**>80%**	
	n = 28	**n = 20**	
Positive estrogen receptor [n/number of cases studied]	24/27	12/20	0.049
Tumor size (cm) [mean±SD]	28	20	
[number of cases studied]	2.08±1.15	2.37±1.16	
SBR grade [number of cases studied]	28	20	
1	14	3	
2	12	10	
3	2	7	0.011

doi:10.1371/journal.pone.0004223.t004

defined by the Huriet-Sérusclat act of 12/20/1988. Under this act, institutional review board approval was not required. Accordingly, patients were specifically asked for a verbal informed consent only, and consequently no IRB number approval was requested. The study was carried out according to the Declaration of Helsinki principles and in agreement with the French laws on biomedical research.

Immunohistochemistry

Breast tumor sections of 5 μm thickness were analyzed by immunohistochemistry for NTSR1, NTS, ER, and PR staining, using the following antibodies: a NTSR1 goat polyclonal antibody (C-20 Santa Cruz USA), a NTS rabbit antibody (NA1230 Biomol, USA), a ER-α monoclonal antibody (Santa Cruz), a PR monoclonal antibody (Santa Cruz). Immunostaining was carried out on deparaffinized sections using the streptavidin biotin peroxidase complex method as described previously by Souazé et al [7]. All slides were counterstained with hematoxylin. A semi-quantitative estimation of the number of positive cells was performed by counting 1 000 reactive and non-reactive cells in ten successive fields at the original 200× magnification.

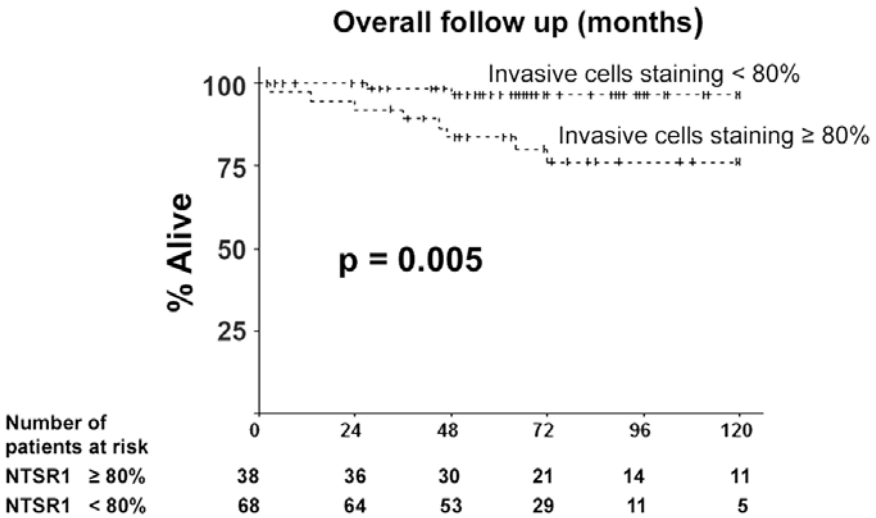

Figure 3. NTSR1 expression in IDCs and global survival duration. Kaplan-Maier analysis for global survival duration in both groups with low (<80%) and high (≥80%) NTSR1 expression. Probability of overall death for patients with high NTSR1 expression (n = 38) versus patients with low NTSR1 expression (n = 68).
doi:10.1371/journal.pone.0004223.g003

Normal breast tissues

Normal human breast epithelial cells (HBEC) were cultured as previously described in Gompel et al. [29]. Additional biopsies of normal breast tissues from 25 premenopausal women of various ages (18–50 years) undergoing plastic surgery were obtained according to the French regulations on clinical experimentation.

RNA extraction and RT-PCR

The protocols for total RNA extraction, reverse-transcription reaction (RT), and PCR are documented in Souazé et al [30]. RT was performed on 1 µg of total RNA using a specific NTSR1 primer (5′-GCTGACGTAGAAGAG-3′) or 50 pmol of oligo dT and oligo dN. The PCR amplification was performed on a 1:5 v/v of the RT reaction using 25 pmol of each primer 5′-CGTG-GAGCTGTACAACTTCA-3′ and 5′-CAGCCAGCAGACCA-CAAAGG-3′ for NT1 receptor, and 5′-AAGCACATGTTCC-CTCTT-3′ and 5′-CATACAGCTGCCGTTTCAGA-3′ for NTS, and 1 unit of Taq polymerase (Applied Biosystems, Courtaboeuf, France). The amplification profile consisted of denaturation at 94°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 45 s. The PCR cycle were preceded by denaturation at 95°C for 15 min and were followed by a final extension at 72°C for 7 min.

Hormonal treatments

HBEC were synchronized for 40 h in Ham F10 phenol red free medium containing 20 µM lovastatin. Synchronization was stopped by adding 2 mM mevalonate to the hormone-containing medium. Subsequently, cells were treated 48 h in a phenol red free medium containing 5% of compatible human serum with 10 nM estradiol (E2) with or without 1 µM ICI 182780.

Statistics

Analyses were processed with StatView Version 5 (Abacus Concepts, Berkeley, CA., USA). Descriptive statistics were performed for each variable; quantitative results are presented as mean±SD and qualitative results are presented as a distribution of a number of patients. To compare the two groups, χ^2 test was used for qualitative variables and t test for quantitative variables. A p value<0.05 was accepted as significant. Survival analysis was performed by Kaplan-Meier method and comparison with Log-Rank test. For multivariate analysis, Cox model was performed using R statistical package.

Acknowledgments

The authors wish to express many thanks to Dr Neil Insdorf for its help in the writing of the manuscript and for helpful discussions.

Author Contributions

Conceived and designed the experiments: SD FS AG PF. Performed the experiments: SD VVF FS. Analyzed the data: MA GPB DH. Contributed reagents/materials/analysis tools: MC Al DH. Wrote the paper: MA CG AG PF.

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The differential processing of proenkephalin A in mouse and human breast tumour cell lines

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Abstract

We have carried out an investigation into the processing of the enkephalin-like immunoreactivity reported in breast tissue using two human breast tumour cell lines and a mouse tumour cell line. A 46 kDa form of proenkephalin (PE) has been observed in the cell lysates of two human breast tumour cell lines (MCF-7, ZR-75–1) and the mouse androgen-responsive Shionogi breast carcinoma cell line (SC115). PE processing in the cell lysates of these cells was assessed by a specific met-enkephalin RIA. The basal levels of processed PE in the MCF-7, ZR-75–1 and SC115 cell lysates were 30, 30 and 76% respectively. The processing enzymes PC1 and PC2, which have been implicated in the differential processing of PE, were detected by immunoblot analysis in these cells. PC1 was found within the cell extracts of all three cell lines. PC2 was only observed in the SC115 cell line, which may account for the higher percentage of processed PE measured. The cDNA of PC2 has been transfected into ZR-75–1 cells and this was accompanied by an increase in the level of processed PE from 30 to 76%. These breast tumour cell lines may provide a useful insight into the function of enkephalin-containing peptides in breast cancer.

Journal of Endocrinology (1999) **161,** 475–484

Introduction

The induction and growth of human breast cancer appear to be directly related to the endocrine status of the host. The implication of ovarian hormones in breast cancer has been extensively reported (Sirbasku *et al.* 1980). In addition to these steroid hormones, various other factors, such as glucocorticoids, thyroid hormones, vitamin D, insulin, prolactin, growth hormone, prostaglandins, epidermal growth factors and endogenous neuroendocrine peptides have also been shown to affect breast tissue (Medina *et al.* 1987). One such family of peptides are the opioids, which include the enkephalins, endorphins and dynorphin. These peptides are derived by enzymic processing of three separate precursors; proenkephalin A (PE), proopiomelanocortin (POMC) and prodynorphin. Enzymic processing of the prohormone precursors usually occurs at selected dibasic or monobasic amino acid cleavage sites by specific prohormone-processing enzymes with which they are co-localised.

These opioid peptides and their precursors are widely distributed throughout the body but are generally localised within the tissues of the brain and the central and peripheral nervous system (Cooper *et al.* 1996). The co-expression of endogenous opioid peptides and the putative opiate receptors, through which their wide spectrum of effects are mediated, have been described in several benign and malignant tumours from humans and other animals by immunohistochemical techniques (Zagon *et al.* 1987). Immunoreactive opioid peptides were also identified in 56% of invasive ductal carcinomas of the breast from 61 premenopausal women (Scopsi *et al.* 1989). A recent immunocytochemical study of β-endorphin and enkephalin expression in primary breast cancers, adrenofibromas and peritumoral non-neoplastic tissue showed around 90% of all tumours to be positive (Chatikhine *et al.* 1994). Leu-enkephalin and met-enkephalin occurred predominantly in the epithelial cells of both benign and malignant tumours, whereas β-endorphin was found mainly in the stroma. In non-neoplastic tissues all three opioids were predominantly expressed in the stroma. Although the role of opioids in the neoplasms is unclear, they have been implicated in the controlling of analgesic, behavioural (Morley 1986) and endocrine responses (Olson *et al.* 1986) and are also thought to have effects on the humoral and cellular immune system (Chang 1984, Teschemacher & Schweigerer 1985). With regard to the action of opioids on the growth of tumour cells, reports in the literature are contradictory, since they

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exert both stimulatory and inhibitory effects on the growth of experimental tumours *in vivo* and *in vitro* depending on the type of opioid peptide, the dosage of the opioid and the type of cancer cell and tissue (Zagon & McLaughlin 1981, 1984, Lewis *et al.* 1983, Murgo 1985, Scholar *et al.* 1987, Maneckjee *et al.* 1990). Neuropeptides may therefore act as endocrine, paracrine or autocrine stimulatory growth factors on the cancer cell (neoplasia) or on the surrounding stroma (desmoplasia).

Although much work has been performed on the effects of opioid peptides on breast cancer cell growth, the mechanisms by which the bioactive peptides are enzymically released from their precursors in this tissue have not yet been investigated. The prohormone-processing enzymes responsible for processing opioid prohormone precursors were first identified in mammalian tissues at the end of 1989 (Fuller *et al.* 1989) and the first half of 1990 (Seidah *et al.* 1990, 1991*a*). In these early studies three enzymes were recognised as the mammalian Kex-2-like convertases, namely furin (Van den Ouweland *et al.* 1990), PC1 (Smeekens *et al.* 1991) and PC2 (Smeekens & Steiner 1990). This was followed by the complete elucidation of the cDNA sequence of mouse and human PC1 (Seidah *et al.* 1992). Furin cleaves proproteins that *in vivo* are normally expressed in cells devoid of secretory granules (constitutively secreting cells). In contrast, PC1 and PC2 demonstrate a selectivity of cleavage at paired basic residues that is best suited to the activation of precursors synthesised in cells containing secretory granules (Seidah *et al.* 1991*a*,*b*). This functional specialisation of these enzymes correlates with their observed cellular and tissue distribution. Thus furin is ubiquitously expressed in constitutive secreting cells whereas PC1 and PC2 are mostly found in neuroendocrine and endocrine cells and tissues that are endowed with a regulatory secretory pathway (Smeekens & Steiner 1990, Seidah *et al.* 1991*a*,*b*, 1992, Smeekens *et al.* 1991). In addition to the differential subcellular distribution of the subtilisin-like enzymes, the levels of PC1 and PC2 transcripts are often co-regulated with those of their substrates (Day *et al.* 1992), whereas furin does not exhibit such regulation.

PC1 and PC2 are implicated in the processing of PE in selected tissues of the central and peripheral nervous system (Seidah *et al.* 1990, 1991*b*, Schafer *et al.* 1993) (Fig. 1). In a preliminary study we had observed different processing patterns of PE and enkephalin-containing peptides (ECPs) in MCF-7 and ZR-75–1 human metastatic cells and the mouse androgen-responsive breast tumour cell line known as SC115. The present study was designed to investigate whether the distinct processing patterns of the ECPs were the result of co-localisation and activity of specific prohormone-processing enzymes such as PC1 and PC2 in these breast tumour cells, which could alter the spectrum of activity of the final products and thus tumour pathogenicity.

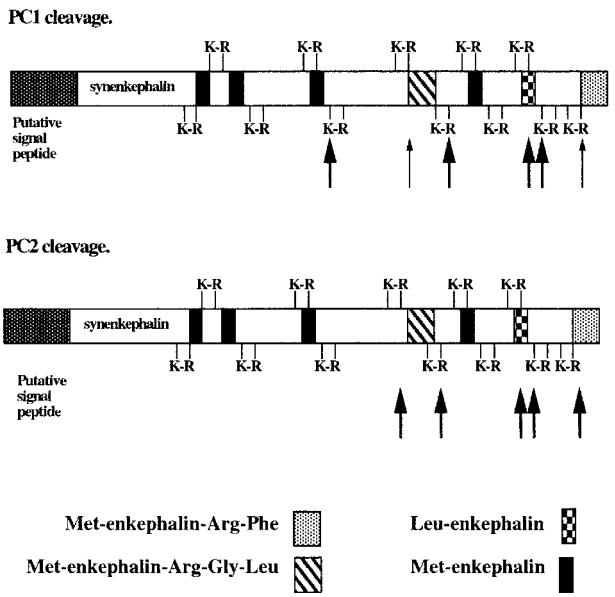

Figure 1 Representation of PE and some of the processing sites of PC1 and PC2. The main processing sites are indicated by thick arrows and the minor by thin. Adapted from Breslin *et al.* (1993).

Experimental procedures

Cell culture

Culture media, gentamicin, trypsin–EDTA solution, trypan blue, fetal calf serum (FCS) and horse serum were purchased from Gibco-BRL, Uxbridge, Middlesex, UK. Tissue culture flasks and apparatus were obtained from Falcon Marathon Lab. Supplies, London, UK.

The human MCF-7 breast carcinoma cell line was originally isolated from a pleural effusion of a primary breast cancer patient (Soule *et al.* 1973). MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with gentamicin (50 μg/ml), 1×10^{-8} M 17β-estradiol (Steraloids Ltd, London, UK) and 10% (v/v) heat-inactivated FCS.

The ZR-75–1 human breast cancer cell line was derived from a pleural effusion from a breast cancer patient (Engel *et al.* 1978) and was maintained in the supplemented DMEM medium (as the MCF-7 cells) with the addition of 0·1 unit/ml insulin (Sigma Chemical Co. Ltd, Poole, Dorset, UK).

The SC115 tumour originated spontaneously in a female mouse of the DD/S strain (Minesita & Yamaguchi 1965). Cells were maintained in DMEM supplemented with $3{\cdot}5 \times 10^{-8}$ M testosterone (Steraloids), gentamicin (50 μg/ml) and 10% heat-inactivated FCS.

A Chinese hamster ovary (CHO) cell line stably transfected with the cDNA of rat preproenkephalin (DL1/50) was kindly donated by Dr I Lindberg, Department of Biochemistry and Molecular Biology, Louisiana State

University Medical Center, New Orleans, LA, USA (Lindberg *et al.* 1991). The DL1/50 cells were grown in alpha modified Minimal Essential Medium, without nucleotides or ribonucleotides, with 10% (v/v) well-dialysed FCS, 50 μg/ml gentamicin and 50 μM methotrexate (Sigma). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air and were grown in 10 cm culture dishes at an initial density of $\sim 45 \times 10^3$ cells per dish. Growth medium was replaced every 2 days until the cells reached 80% confluence. At this point the cells were ready for transfection or experimentation.

Antibodies

The sheep polyclonal anti-PE antiserum (W346) was raised to recombinant full-length rat PE (purified from DL1/50 cell media by Q-Sepharose anion exchange chromatography (Hannah 1993)). Purified rat PE (3 mg) was conjugated to purified protein derivative (PPD) of avian tuberculin (MAFF, Central Veterinary Laboratory) at a ratio of 2:1 (w/w) of PPD to PE. Sheep were immunised with 400 μg rat PE conjugate added to 2 ml Freund's incomplete adjuvant (Sigma). Thereafter, 200 μg of peptide conjugate in 2 ml of incomplete adjuvant were given every 4 weeks and the sheep were bled at similar time intervals.

The anti-rat-PC2 antiserum was generously supplied by Dr J Hutton of the Department of Clinical Biochemistry, University of Cambridge, UK and was raised to a bacterial fusion protein containing rat PC2 amino acids 168–380 (Bennett *et al.* 1992). Anti-PC1 antiserum, developed against peptides representing the segment of amino acids 84–100 at the amino terminus of mouse PC1 was a generous gift from Dr N G Seidah of the Clinical Research Institute Montreal, Montreal, Quebec, Canada (Benjannet *et al.* 1993).

Secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-sheep and anti-rabbit IgG (DAKO A/S, Glostrup, Denmark). The SM2–2 antiserum, used for the met-enkephalin sulphoxide (MEO) RIA (a gift from Dr S Medbak of the Department of Chemical Endocrinology, St Bartholomew's Hospital, London, UK) was raised against MEO and has a similar specificity to that described previously (Clement-Jones *et al.* 1980).

A monoclonal antiserum that was raised previously in this laboratory to human PE (PE-1) was used at a dilution of 1:1000 for SDS-PAGE immunoblot detection of PE within breast tumour cells (Spruce *et al.* 1989). This antiserum was generated to a chimeric peptide of *Escherichia coli* β-galactosidase fused to the amino acid sequence 69–207 of human preproenkephalin. Sera from BALB/c mice immunised with the β-galactosidase–preproenkephalin A (69–207) hybrid polypeptide were tested for anti-PE activity and the binding domains of PE-1 were broadly located with respect to the primary translation product, within amino acid sequences 152–207. The anti-PE-1 antiserum was used to confirm the specificity of the W346 antiserum in detection of PE.

Transfection of human PC2 (hPC2) into the ZR-75–1 cells

All the buffers and most of the methods used during this work were taken from Maniatis (Sambrook *et al.* 1988). Genetic manipulation reagents and restriction endonucleases were obtained from Promega (Southampton, Hants, UK) and New England Biolabs (Bishop's Stortford, Herts, UK). Full-length cDNA for hPC2 was a generous gift from Drs Steiner and Smeekens at the Department of Biochemistry and Molecular Biology, Howard Hughes Medical Institute, University of Chicago, IL, USA. The multiple cloning site from Bluescript (Stratagene, Cambridge, Cambs, UK) (19 restriction sites) was ligated into the BamHI site (blunt ended ligation) of pSRα (obtained from the Department of Biochemistry, Imperial College, London, UK). pSRα possesses a promoter which is a fusion between the simian virus (SV40) early promoter and part of the long-term repeat of type 1 human T-cell leukaemia virus (Takebe *et al.* 1988) and additionally incorporates a pBr322 origin of replication and an ampicillin resistance gene. The full-length cDNA for hPC2 was subcloned from the cloning vector Bluescript II (Stratagene) into the multiple cloning site of the pSRα expression vector. The expression vector containing the cDNA of hPC2 was stably transfected by calcium phosphate co-precipitation into the ZR-75–1 cells and transfected cells were selected on the basis of neomycin resistance (G418 sulphate, geneticin, Schering, purchased from Gibco). Expressing transfectants of PC2 were assessed using SDS-PAGE immunoblot analysis.

Cell lysate preparation

The cells were lysed with 1 ml ice-cold lysis buffer (50 mM HCl, 0·1% (v/v) 2-mercaptoethanol, 0·001% (v/v) Triton X-100). The lysate was then sonicated (3×20 s bursts), heat treated (85 °C for 15 min) and subsequently centrifuged (12 000 ***g*** for 15 min). The supernatant was retained and the processed enkephalin peptides were analysed by the MEO RIA and by SDS-PAGE immunoblot analysis (see below). An aliquot (50 μl) of cell lysate was retained for cell protein content determination using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd, Hemel Hempstead, Herts, UK).

Trichloroacetic acid (TCA) extraction of proteins for SDS-PAGE immunoblot analysis

To extract cellular proteins, 50% (w/v) of ice-cold TCA was added to 1 ml cell lysate at a final dilution of 10% (w/v) TCA and incubated overnight at 4 °C. The protein precipitate was pelleted by centrifugation

(12 000 ***g*** for 10 min) and traces of TCA were removed by ether extraction (3 × 1 ml). Precipitated proteins were resuspended in 100 µl SDS-PAGE sample treatment buffer (8% (w/v)) with an equal volume of sterile water. The pH of the samples was then adjusted to pH 7·6 with 1 M Na_2HCO_3. Proteins were analysed with 16% polyacrylamide gel using the 0·75 mM mini gel Tris/Tricine Schagger and Von Jagow buffer systems (Schagger & Von Jagow 1987). The electrophoresed proteins were subsequently transferred onto polyvinyldifluorine Flourotrans transfer membrane (PALL Europe Ltd, Portsmouth, UK) using a semi-dry blotter (Biotech Instruments Ltd, Luton, UK). Non-specific protein binding sites were blocked using 0·05 M PBS (0·8% (w/v) NaCl, 0·02% (w/v) KCl, 0·181% (w/v) $Na_2HPO_4.2H_2O$, 0·024% (w/v) KH_2HPO_4, pH 7·4) containing 1% (w/v) BSA and 1% (w/v) non-fat dry milk and then probed with W346 sheep anti-PE antiserum and the mouse anti-human PE (PE-1) antiserum. The cell lysate proteins of the breast tumour cells were additionally probed with anti-PC1 and anti-PC2 antiserum. PE immunoreactive bands were visualised using the enhanced chemiluminescence (ECL) system (Amersham Corp., Arlington Heights, IL, USA). The immunoblot was exposed to pre-flashed Fuji RX film in a film cassette (Genetic Research Instrumentation, Gene House, Feltsted, Essex, UK) and the film was developed using Kodak D-19 and fixed with Kodak Unifix.

RIA for MEO

MEO, TPCK-treated trypsin, carboxypeptidase B (CPB) and other miscellaneous chemical reagents were purchased from Sigma. One millilitre samples of both cell lysates were digested for 2 h at 37 °C with 1 mg/ml N-tosylphenyl-alanine chloromethyl-ketone (TPCK)-treated trypsin that was dissolved in RIA buffer which contained 0·2% (w/v) human serum albumin (HSA), 0·05 M phosphate pH 7·4, 0·01% (w/v) sodium azide. Trypsin digestion was terminated by heat inactivation (85 °C for 15 min) and has been described previously (Hannah *et al.* 1993). A series of control digestions of 0 to 1 mg/ml of TPCK-treated trypsin and 0·2% (w/v) HSA (i.e digestion buffer alone) failed to detect measurable quantities of met-enkephalin immunoreactive peptides using the MEO RIA following a 2 h digestion at 37 °C. Samples were treated with CPB (40 µg/ml diluted in RIA buffer) for 30 min at 37 °C and CPB activity was then terminated by heat inactivation (85 °C for 15 min). Met-enkephalin released from PE and ECPs in samples was converted to the immunoreactive sulphoxide by oxidation with H_2O_2 (100 volumes, Fisons Scientific Co., Crawley, Sussex, UK) by the addition of a final concentration of 1% (v/v) H_2O_2 and incubation at room temperature for 2 h. MEO standards were serially diluted from 2000 to 1·9 pg/ml in RIA buffer. The samples were assayed in triplicate 200 µl aliquots and were incubated overnight at 4 °C with 50 µl rabbit polyclonal anti-MEO, SM2–2 antiserum at a final dilution of 1:64 000 in 300 µl with 50 µl radiolabelled MEO (^{125}I-MEO, 12 000 c.p.m.). MEO immunoreactivity was pelleted following a 1 h incubation at 4 °C with 200 µl pre-precipitated sheep anti-rabbit fragment crystalline (10% (v/v) in 1% (v/v) normal rabbit serum, 4% (w/v) polyethylene glycol in (0·05 M phosphate buffer (pH 7·4)). Ice-cold wash buffer (2 ml 9% (w/v) NaCl, 0·001% (v/v) Triton X-100) was added to each tube. Following centrifugation (4200 ***g***, 30 min at 4 °C), the supernatants were aspirated and the MEO radioactivity retained in the pellets was assessed using a 1261 Multigamma counter (LKB, Malmo, Sweden). The lower sensitivity limit of the assay was between 5 and 10 pg/ml MEO. To assess the intracellular proteolytic activity of prohormone-processing enzymes in the processing of PE, not all samples were treated with trypsin or CPB. Trypsin and CPB treatment (treatment I), measures total met-enkephalin and by inference total PE. CPB treatment only (treatment II) measures PE partially processed by trypsin-like endogenous enzymes to met-enkephalin containing carboxy-terminal basic amino acid residues and PE that has been completely processed to met-enkephalin by endogenous trypsin-like and CPB-like enzymes. 'No treatment' (treatment III) measures PE that has been processed to met-enkephalin by specific trypsin-like and CPB-like endogenous proteolytic enzymes. To calculate the percentage of processed PE by endogenous trypsin-like enzymes, the CPB treatment (treatment II) is calculated as a percentage of the cellular PE content (treatment I).

Statistical analysis

Values of MEO content were corrected to cell protein content and expressed as mean ± S.E.M. pg per µg cell protein (n=3). Student's t-test was used when applicable.

Results

SDS-PAGE immunoblot analysis detected PE within the cell lysates of the ZR-75–1, MCF-7 and SC115 breast tumour cell lines (Fig. 2a and b). Figure 2a and b show the immunoblot detection of PE using the sheep anti-rat (W346) and mouse anti-human PE (PE-1) antisera respectively. Both antisera detected a 46 kDa form of PE in the breast tumour cell lines, suggesting that this protein is PE. In contrast to the breast tumour cell lines, the recombinant rat PE expressed in the Chinese hamster ovary CHO/Dl150 cells migrated with molecular masses of 30 and 32 kDa. The 32 kDa form of PE has been previously characterised by Lindberg *et al.* (1991) as a glycosylated form of the 30 kDa PE.

To measure the intracellular processing of PE in each of the breast tumour cell lines, the cell lysates were subjected

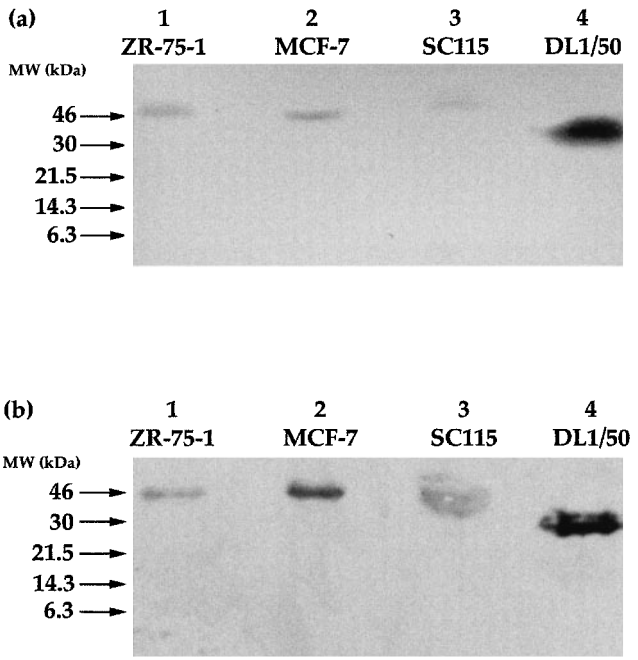

Figure 2 (a) Immunoblot of the TCA-precipitated protein extracts from the cell lysates of the human breast tumour cell lines ZR-75–1 (lane 1) and MCF-7 (lane 2), the mouse breast tumour cell line SC115 (lane 3) and CHO cells transfected with the cDNA encoding rat PE (CHO/DL150 cells (lane 4)), using a 1:1000 dilution of W346 sheep anti-PE antiserum. The methods and reagents for detection of PE were by ECL using an HRP-conjugated secondary antibody. PE that migrated with a molecular mass of 46 kDa was detected in all three breast tumour cell lines (lanes 1–3) in contrast to the rat PE expressed by the DL1/50 cell line that migrated with molecular masses of 30–32 kDa. (b) The same experiment as in (a) but using a 1:1000 dilution of mouse anti-human PE (PE-1) antiserum. The methods, reagents and results were the same as in (a).

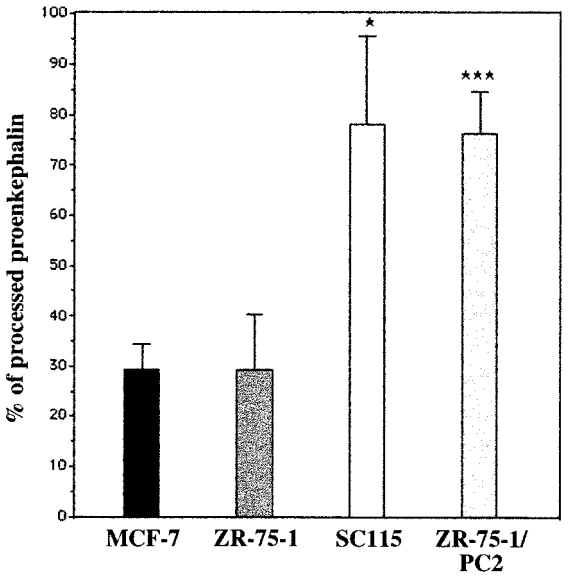

Figure 3 Histogram of the percentage of intracellular processed PE (calculated by MEO RIA analysis) contained within the cell lysate extracts of the MCF-7, ZR-75–1 and SC115 breast tumour cells. The percentage of PE processed by the SC115 cell line is significantly greater than that by both the MCF-7 and ZR-75–1 wild type cell lines (*P<0·05). The percentage of PE processed to met-enkephalin immunoreactive peptides is significantly greater within the cell lysates of the ZR-75–1/PC2 cells when compared with the ZR-75–1 wild type cells (***P<0·01).

to three different enzymic treatments (as described in the Experimental procedures), and the resultant peptides were analysed by the MEO RIA. Treatment I corresponds to the total amount of met-enkephalin and by inference is the total amount of PE. Treatment II (an addition of CPB only) corresponds to the amount of PE processed to met-enkephalin and carboxy-terminally elongated met-enkephalins which are formed by the action of cellular trypsin-like enzymes on the precursor. The total amount of PE processed to peptides containing the met-enkephalin sequence is calculated by expressing treatment II as a percentage of treatment I. As shown by Fig. 3 the MCF-7, ZR-75–1 and SC115 cells processed 28 ± 5·0, 29 ± 11 and 78 ± 17% of the total cellular PE respectively, indicating that the human and mouse breast tumour cell lines differentially process PE. As shown by Fig. 4, the amount of met-enkephalin (pg per μg cell protein) varies between each breast tumour cell line. The majority of all the enkephalin peptides present in the SC115 cell extracts were detected as processed met-enkephalin.

The differential PE processing patterns between the MCF-7, ZR-75–1 and the SC115 breast tumour cells suggested that these cell lines may contain different complements of the PE-processing enzymes. To determine whether the differential processing profiles of PE were due to the co-expression of prohormone-processing enzymes, such as the prohormone-converting enzymes PC1 and/or PC2, TCA-precipitated cell proteins were separated by SDS-PAGE and the immunoblots were probed with anti-PC1 and anti-PC2 antisera. PC1 was detected in all three breast tumour cell lines; however, variant molecular mass forms of PC1 were observed in each of the cell lines (see Fig. 5). The MCF-7 and the ZR-75–1 breast tumour cells both contained a 68 kDa form of PC1. The most predominant form of PC1 detected within the SC115 cells exhibited a molecular mass of 69 kDa. In contrast to the SC115 cells the MCF-7 cells contained multiple forms of the PC1 enzyme. The five forms of PC1 detected within the cell lysates of the MCF-7 cells exhibited molecular masses of 100, 96, 88, 68 and 52 kDa. It is possible that the 100 kDa form of PC1 can be identified as the unactivated mature proenzyme. The 52 kDa form of PC1 is not noticeable within the cell lysates of the ZR-75–1 and SC115 cells. Two 96 and 68 kDa forms of PC1 were found within the cell lysates of ZR-75–1 cells.

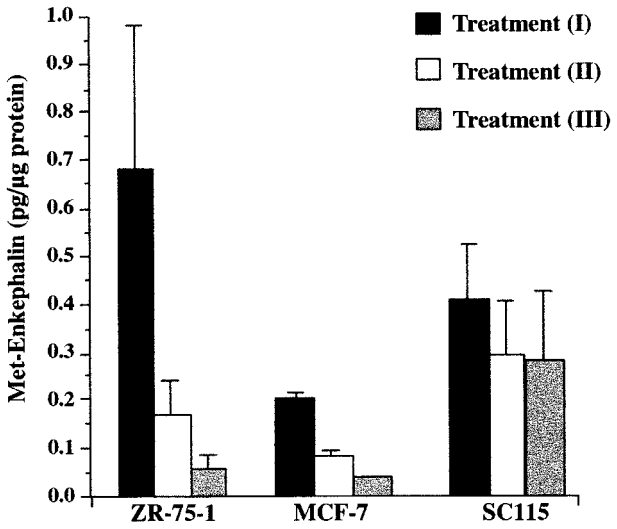

Figure 4 MEO RIA analysis of the relative amounts of PE and ECPs contained within the cell lysates of the MCF-7, ZR-75–1 and SC115 breast tumour cells. The cell lysates were subjected to three CPB or trypsin treatments. Trypsin and CPB treatment (treatment I), measures total met-enkephalin and by inference total PE. CPB treatment only (treatment II) measures PE partially processed by trypsin-like endogenous enzymes to met-enkephalin containing carboxy-terminal basic amino acid residues and PE that has been completely processed to met-enkephalin by endogenous trypsin-like and CPB-like enzymes. 'No treatment' (treatment III) measures PE that has been processed to met-enkephalin by specific trypsin-like and CPB-like endogenous proteolytic enzymes. The values of PE-derived peptides and PE (pg per µg cell protein) varies significantly between each of the breast tumour cell lines. Statistical significance ($P<0{\cdot}05$) between the three treatments was achieved with ZR-75–1 and MCF-7 cells. There was no difference between the three treatments in the case of the SCll5 cells.

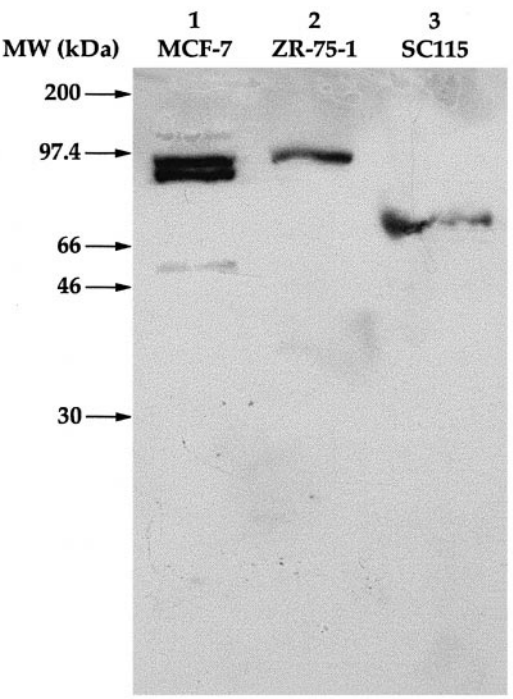

Figure 5 Immunoblots of the TCA-precipitated proteins from the cell lysate extracts of the MCF-7 (lane 1), ZR-1–75 (lane 2) and SC115 (lane 3) breast tumour cells were probed with a 1:1000 dilution of rabbit anti-PC1 amino terminus antiserum. The bands were visualised by luminescence using a 1:1000 dilution of HRP-conjugated secondary antibody. PC1 expression was detected in all three cell lines. The molecular masses of the PC1 varied in each of the cell lines. The major form of PC1 in the SC115 cells is of a lower molecular mass (68 kDa) than the predominant form of PC1 detected within the ZR-75–1 cell (~90 kDa). The major forms of PC1 in the MCF-7 cells are present in two high molecular mass forms of ~90 kDa and ~86 kDa.

Both PC1 and PC2 were detected within the cell protein extracts of the SC115 cells (Fig. 6). It is possible that the co-localisation of both PC1 (~68 kDa) and PC2 (~67 kDa) in the SC115 cell line may be responsible for the high level of processed PE measured in this cell line in comparison with the ZR-75–1 and MCF-7 cell lines (Fig. 3).

To demonstrate that PC2 is more active than PC1 in processing PE, the cDNA of hPC2 was stably transfected into the ZR-75–1 cell line, which does not express this enzyme endogenously (Fig. 6). Immunoblot analysis confirmed the expression of hPC2 in this cell line (Fig. 6) and the expression of PC2 was accompanied by a significant increase in the intracellular processing of PE to met-enkephalin immunoreactive peptides from 22 ± 5% in the ZR-75–1 wild type cell line to 76 ± 8% ($P<0{\cdot}01$) in the PC2-expressing ZR-75–1 cells (Fig. 3).

Discussion

In this study we have detected PE and met-enkephalin immunoreactive peptides co-localised with the prohormone-processing enzyme PC1 in two human breast tumour cell lines (MCF-7 and ZR-75–1) and in the mouse androgen-responsive SC115 metastatic breast tumour cell line. In addition, PC2 has been detected in the mouse SC115 cell line. The variant forms of PC1 located within the cell lysates of the MCF-7 cells may represent different post-translationally modified forms of PC1 or carboxy-terminal truncated variants. The large 100 kDa form of PC1 detected in the MCF-7 cells may represent the precursor form of the enzyme. A pro-form of PC1 has been previously characterised in AtT-20 cells (a rat anterior pituitary cell line) (Vindrola & Lindberg 1993), where it exhibited a molecular mass of 87 kDa. The 87 kDa form of PC1 is thought to be processed further to a 66 kDa form of the enzyme. Both the 66 and 87 kDa forms of PC1 are active in the processing of prohormone precursors (Benjannet *et al.* 1993, Vindrola & Lindberg 1993). These higher molecular mass forms of PC1 were not detected in the SC115 cells where only the 69 kDa

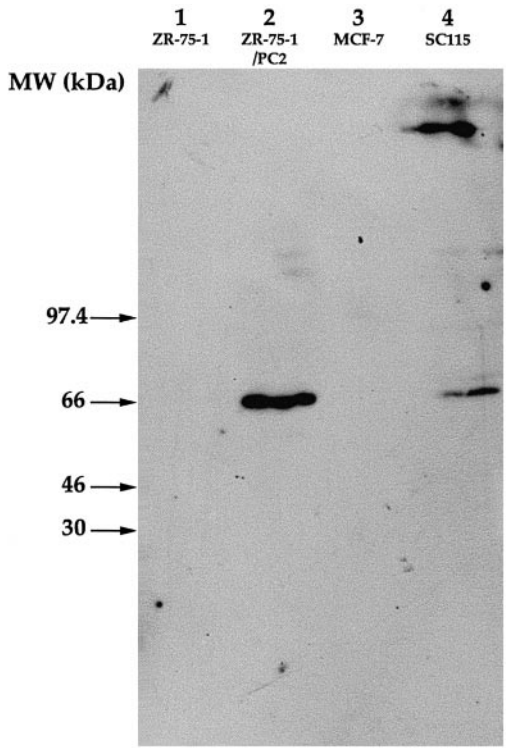

Figure 6 Immunoblots of TCA-precipitated proteins from the ZR-75–1 cell lysate extract (lane 1), ZR-75–1/PC2 cells stably transfected with the cDNA encoding hPC2 (lane 2), MCF-7 cells (lane 3) and SC115 cells (lane 4) were probed with a 1:1000 dilution of anti-PC2 antiserum. PC2 (66 kDa) was visualised only in the SC115 cell lysates (lane 4) and the ZR-75–1 cells that were stably transfected with the cDNA encoding PC2 (lane 2).

processed form of PC1 was present, co-expressed with the 67 kDa form of PC2 (Benjannet *et al.* 1993).

It was interesting to observe the 46 kDa protein in the cell lysates of the human and mouse breast tumour cells (Fig. 2), in contrast to the 32 kDa recombinant rat PE which immunoreacted with antisera to PE. Human PE has been shown to exhibit a molecular mass of 36·5 kDa in a human pheochromocytoma (Comb *et al.* 1982). As there is only a difference of two in the number of amino acids in the rat and human PE, it is possible that the larger form of PE in the breast tumour cells has been subjected to differential post-translational modifications such as glycosylation; however, initial deglycosylation and dephosphorylation experiments failed to reduce the molecular mass of the tumour PE (data not shown). The PE secreted by SK-N-MC cells (a human neuroblastoma cell line) has also been shown to be appreciably glycosylated and phosphorylated (Lindberg & Shaw 1992); however, the molecular mass for the higher molecular weight form of PE in this cell line was only 34 kDa. Met-enkephalin immunoreactivity eluting with the authentic peptide and higher molecular weight material have also been detected in other human tumour tissues (Clement-Jones *et al.* 1982) including adrenal medullary tumours, tumours of the lung, pancreas, thymus and the gall bladder; however, an explanation for these higher molecular weight forms of PE was not given.

The detection of substantially more processed PE in the SC115 cell lysates in comparison with the human breast tumour cells (Figs 3 and 4) may be due to the co-localisation of both PC1 and PC2 with PE in this cell line (Figs 5 and 6). PE processed by both PC1 and PC2 would theoretically result in four free met-enkephalins in addition to met-enkephalin-Arg^6-Phe^7 and met-encephalin-Arg^6-Gly^7-Leu^8. The latter two peptides would not be detected by our MEO assay. Therefore the theoretical percentage of PE processed by PC1 and PC2 measured by the MEO assay would be 67% similar to the 79% that we observed in the SC115 cells. The theoretical partial processing of PE by PC1 would only give two amino-terminally extended peptides with detectable enkephalin (by the immunoassay) at their carboxy terminus and therefore a much lower theoretical value in the MEO assay of 33%, similar to the 29% of processed PE that we observed in the human breast tumour cell lines.

Such differential PE processing patterns are apparent in the tissues of the central nervous system and in the nuclei of the brain (Giraud *et al.* 1984), which may be due to the differential tissue distribution of PC1 and PC2. The distribution of PE-derived peptides varies greatly between discrete regions of the brain, but is particularly apparent between the striatum and the hypothalamus (Schafer *et al.* 1993), where there is evidence for differential PE processing. For example, the level of PC1 expression is high in the supraoptic and the paraventricular nuclei of the hypothalamus (Seidah *et al.* 1991*b*), where more selectively cleaved PE-derived peptides have been found. PC2 mRNA is also observed in these areas with moderate abundance. However, high levels of PC2 mRNA have been detected in the preoptic area and the mamilliary bodies and in other regions of the CNS including the thalamic nuclei in the hippocampus, the deep superficial layers of the cortex, the amygdala, and in the striatum where more complete cleavage of PE is noticeable. Therefore, PC2 is thought to be less selective in the cleavage of PE and where PC1 and PC2 are co-localised the processing of PE is enhanced.

The less selective proteolytic action of PC2 on PE was observed in the cell lysates of both the ZR-75–1 breast tumour cell line that had been transfected with the cDNA of hPC2 and the SC115 cells. Both PC1 and PC2 would appear to be co-localised in these cells with more completely processed PE-derived peptides. The high percentage of PE processed to met-enkephalin in the SC115 and the ZR-75–1/PC2 cells is similar to the data published by Seidah and coworkers in which PC1, PC2 and PE were transiently expressed by a vaccinia virus expression system in a rat somatotroph cell line (GH_4Cl) (Breslin *et al.* 1993).

The data produced from their study revealed that the major immunoreactive enkephalins formed from the activity of PC2 are met-enkephalin-Arg^6-Phe^7, free met-enkephalin-Arg^6-Gly^7-Leu^8, leu-enkephalin and met-enkephalin (see Fig. 1). In contrast PC1 is shown to produce intermediate-sized processing products (3–10 kDa); the major immunoreactive ECP eluted at the position of peptide B (5·3 kDa) and free leu-enkephalin (Cullinan *et al.* 1991).

This work differed from the present study as they co-expressed PC1, PC2 and PE by the transient transfection procedure in a foreign cell line. The disadvantage in expressing proteins transiently is that the cells can only be used for short-term experiments and not for long-term cell manipulation studies. We have, however, stably transfected hPC2 into the ZR-75–1 human breast tumour cell line, which processes endogenous PE and PC1. These breast tumour cell lines additionally possess opiate receptors (Maneckjee *et al.* 1990). The distinct processing patterns of PE by PC1 and PC2, in our stably transfected human breast tumour cell lines, will need to be investigated in more detail. To our knowledge this is the first stably transfected human cell where PC2 has been shown to be active in the processing of PE.

The function of the expression of opioid peptides in breast tumour cells is yet to be determined. However, opioid peptides released from specific cancer tissues and cells have been shown to affect the growth of these cells (Zagon & McLaughlin 1981, 1983, 1984, Murgo 1985, Scholar *et al.* 1987, Maneckjee & Minna 1990, Maneckjee *et al.* 1990, Kirchimair *et al.* 1992, Schrey & Patel 1994). For example human small cell lung cancer (SCLC) cell lines express both opioid peptides and receptors; however, the growth of these cells is inhibited in response to endogenous opioid peptides (Maneckjee & Minna 1990). In contrast, the POMC peptide, β-endorphin, has been reported to stimulate the clonal growth of human SCLC cells (Murgo 1985). The chronic administration of the opioid agonist heroin has been shown to retard tumour growth and prolong survival time of mice with transplanted neuroblastoma tumours and to inhibit neuroblastoma cell growth *in vitro*. The opioid antagonist naloxone was shown to block these anti-tumour effects (Zagon & McLaughlin 1981). Met- and leu-enkephalins have been shown to produce anti-metastatic actions on B16-BL6 melanoma as well as decrease the number of metastases to the lung (Scholar *et al.* 1987). Pretreatment with naloxone and naltrexone may significantly inhibit the growth of carcinogen-induced mammary cancers in rats and cause complete regression in mice with spontaneous and transplantable mammary tumours (Zagon & McLaughlin 1983). In the human breast cancer cell line known as MCF-7, opioid peptides inhibited cell growth only in the presence of oestradiol (Maneckjee *et al.* 1990).

It is conceivable that PC1 and PC2 could additionally be involved in the activation of other prohormone precursors or growth factors that have also been detected in breast tumour cells, which could in turn affect tumour growth and development. Breast tumour cells are also known to express other prohormone precursors and POMC-derived opioid peptides. Like PE these are located in the tissues of the central and peripheral nervous system (Cullinan *et al.* 1991). POMC is completely processed in the pars intermedia, where there is a high expression of PC2 with a low expression of PC1 (Benjannet *et al.* 1991, Zhou & Mains 1994). The corticotrophs of the anterior pituitary only contain PC1, which has been shown *in vitro* and in co-expression studies to process POMC to adrenocorticotrophin (ACTH), β-lipotrophin and a 16 kDa amino-terminal fragment (pro-γ-melanocyte-stimulating hormone (MSH)). The melanotrophs of the intermediate lobe contain very little PC1 and large amounts of PC2 and hence the amino-terminal POMC fragment is processed to γ3-MSH, ACTH is processed to α-MSH and corticotrophin-like intermediate peptide and β-lipotrophin is processed to γ-lipotrophin and β-endorphin. Prosomatostatin (PSS) has also been detected in breast tumour cells (Nelson *et al.* 1989). It has been observed that PSS is proteolytically activated by furin, PC1 and PC2 in eukaryotic cell lines (Galanopoulou *et al.* 1993). Whether these enzymes are responsible for the activation of PSS in breast tumour cells, which may in turn have a direct or indirect effect on the growth and development of breast tumour cells, needs further investigation. A recent study reporting the comparative analysis of expression of furin, PACE4, PC1 and PC2 in human lung tumours suggests that these enzymes may be intimately involved in the production of many signalling molecules, when abnormally expressed in lung cells, and could lead to their neoplastic transformation (Mbikay *et al.* 1997).

In conclusion, we have established that PE is differentially processed in human and mouse breast tumour cell lines. In the mouse breast tumour cell, PE is completely processed to met-enkephalin, met-enkephalin-Arg-Phe and met-enkephalin-Arg-Gly-Leu, which may be due to the co-localisation of both PC1 and PC2 in this cell line. More limited PE processing is evident in the human breast tumour cell lines, which may be due to the expression of only PC1 and not PC2. Preliminary immunoblot analysis of total protein extracted from solid metastatic breast tumours (1·5 g) from six patients has detected PC1, PE and PC2 in all the tumours; however, the expression of these proteins varied between the subjects (data not shown). Further investigation of these tissues may reveal the function of these enzymes in the growth and development of breast tumour cells.

Acknowledgements

This work would not have been possible without the generous donation of the breast tumour cell lines

and helpful suggestions on cancer cell line culture from Dr P Darbre of the School of Animal and Microbial Sciences, Whiteknights, University of Reading. The authors would like to thank Dr N G Seidah, Dr J Hutton and Dr S Medbak for providing antibodies for this study.

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Received 21 September 1998
Revised manuscript received 13 January 1999
Accepted 29 January 1999

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