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Y_1-Mediated Effect of Neuropeptide Y in Cancer: Breast Carcinomas as Targets

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ABSTRACT

Overexpression of selected peptide receptors in human tumors has been shown to represent clinically relevant targets for cancer diagnosis and therapy. Neuropeptide Y (NPY) is a peptide neurotransmitter mediating feeding behavior and vasoconstriction. It has never been shown to be involved in human cancer. We show here, using *in vitro* receptor autoradiography, a NPY receptor incidence of 85% in primary human breast carcinomas ($n = 95$) and of 100% in lymph node metastases of receptor-positive primaries ($n = 27$), predominantly as Y_1 subtype, whereas non-neoplastic human breast expressed Y_2 preferentially. Y_1 mRNA was detected in Y_1-expressing tumors by *in situ* hybridization, whereas Y_2 mRNA was found in normal breast tissue. The strong predominance of Y_1 in breast carcinomas compared with Y_2 in normal breast suggests that neoplastic transformation can switch the NPY receptor expression from Y_2 to Y_1 subtype. Moreover, in Y_1-expressing human SK-N-MC tumor cells, an NPY-induced, dose-dependent inhibition of tumor cell growth of >40% was observed, suggesting a functional role of NPY in cancer, mediated by Y_1. NPY should therefore be added to the list of small regulatory peptides related to cancer. The high incidence of Y_1 in *in situ*, invasive, and metastatic breast cancers allows for the possibility to target them for diagnosis and therapy with NPY analogues.

INTRODUCTION

Regulatory peptides can be of clinical relevance, diagnostically and therapeutically, in tumors that express their respective receptors in high amounts (1–3). Indeed, it has been shown recently that selective tumors and their metastases can be precisely localized in patients by means of *in vivo* peptide receptor scintigraphy. This strategy was first developed for the diagnosis of somatostatin receptor-positive tumors using radiolabeled somatostatin analogues (4) with subsequent use of ^{123}I-labeled VIP[2] to localize VIP receptor-expressing tumors (5–7) and radiolabeled cholecystokinin/gastrin analogues to detect cholecystokinin-B-expressing medullary thyroid carcinomas (8, 9). Another clinical application has been the radiotherapeutic targeting of receptor-expressing tumors by using high doses of these radiolabeled peptides (10, 11).

The regulatory peptides with established or prospective clinical implications based on their overexpressed receptors in cancers, such as somatostatin, VIP, gastrin-releasing peptide, cholecystokinin, gastrin, and neurotensin, belong to a group of brain-gut peptides with a predominant neurotransmitter function as well as gastrointestinal and endocrine actions (12, 13). In addition to their physiological action, these peptides have been shown to play a specific role in cancer, inasmuch as they have marked effects on tumor cell growth in animal models (14). The high amount of receptors in tumors may be indicative of their pathophysiological relevance in tumor growth regulation.

NPY is a member of a family of 36 amino acid long peptides, including NPY, PYY, and PP. Human NPY has the following amino acid sequence: H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Met-Ala-Arg-Tyr-Tyr-Ser-Ala-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH_2. Its main function is not that of an endocrine or gut hormone, but that of a neurotransmitter; its best-known actions are at the level of the central nervous system and include stimulation of feeding behavior and inhibition of anxiety (15–17). Actions mediated by the peripheral nervous system include vasoconstriction, effects on gastrointestinal motility and secretion, insulin release, and renal secretion (18–21). The effect of NPY can be mediated by several NPY receptor subtypes, named Y_1-Y_6, of which Y_1, Y_2, Y_4, and Y_5 have been extensively characterized (22). Several NPY analogues, in particular Y_1 and Y_2 antagonists, are being developed for potential clinical use to treat feeding disturbances and anxiety (23–25).

Compared with other regulatory peptides, NPY has never been associated with human cancer. In the present study, we have investigated whether there is a molecular basis for a putative NPY role in tumors and/or for the development of NPY drugs for tumor targeting. We have determined NPY receptors in one of the most frequent and harmful cancers, breast carcinoma. In more than 100 human breast tumor and metastasis samples, we have evaluated the expression at the mRNA and protein levels of the two best-investigated NPY receptor subtypes, Y_1 and Y_2, using *in vitro* receptor autoradiography and *in situ* hybridization. Moreover, we have evaluated the effect of NPY on the growth of NPY receptor-expressing SK-N-MC tumor cells in culture.

MATERIALS AND METHODS

Patient Tissues. Breast tissue samples with primary breast neoplasias were obtained from 95 patients, 36 to 91 years of age, who were operated on in several institutions. Tissue samples were kept frozen at −80°C. The diagnosis was reviewed and formulated by use of cryostat sections according to the WHO guidelines stated by Tavassoli (26). In the main group of 89 patients, 64 (72%) showed an invasive ductal carcinoma. Histological evaluation identified 49 cases with intermediate (G2), 12 cases with low (G1), and 3 cases with high (G3) grade, according to a modified Bloom-Richardson grading method (26). There were 12 invasive lobular carcinomas and 5 ductal carcinomas *in situ*, as well as 3 mucinous, 2 medullary, 2 apocrine, and 1 tubular carcinomas. In an additional group of six patients (four ductal and two lobular breast carcinomas), we could investigate tissue samples obtained from the primary tumor and from all of the axillary metastases. Moreover, we investigated the non-neoplastic breast tissue adjacent to the carcinoma tissue in 44 tissue samples and the breast tissue sample of one patient who had been operated on for suspicion of carcinoma but received a final diagnosis of breast fibrosis. Those breast tissues were all found to be histopathologically inconspicuous.

NPY Receptor Autoradiography. Twenty-μm-thick cryostat sections of the tissue samples were processed for NPY receptor autoradiography as described in detail previously for other peptide receptors (27). One radioligand used was ^{125}I-labeled PYY (2000 Ci/mmol; Anawa, Wangen, Switzerland), known to label NPY receptors specifically. For autoradiography, tissue sections were mounted on precleaned microscope slides and stored at −20°C for at least 3 days to improve adhesion of the tissue to the slide. Sections were then processed according to Dumont *et al.* (28). They were first preincubated in 119 mM NaCl, 3.2 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25 mM $NaHCO_3$, 2.53 mM $CaCl_2 \times 2H_2O$, and 10 mM D-glucose (pH 7.4) preincubation solution for 60 min at room temperature. The slides were then incubated in a solution containing the same medium as the preincubation solution in which the

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[2] The abbreviations used are: VIP, vasoactive intestinal peptide; NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide.

following compounds were added: 0.1% BSA, 0.05% bacitracin (pH 7.4), and the radioligand at an approximate concentration of 22 pM ^{125}I-labeled PYY. The slides were incubated at room temperature with the radioligand for 120 min. To estimate nonspecific binding, paired serial sections were incubated as described above, except that 25 nM PYY were added to the medium. To be able to distinguish Y_1 from Y_2 subtypes, displacement experiments were performed with 22 pM ^{125}I-labeled PYY and increasing amounts of nonradioactive NPY, the Y_1-selective ligand [Leu^{31}, Pro^{34}]-NPY, and the Y_2-selective ligand PYY(3–36) to generate competitive inhibition curves on successive sections using the same incubation medium as above. Additional analogues used as competitors in these displacement experiments included PP and PYY(13–36). Displacement curves were performed for all compounds in a series of neoplastic and non-neoplastic tissues and led us to conclude that a 25-nM concentration of [Leu^{31}, Pro^{34}]-NPY or PYY(3–36) was adequate to evaluate their rank order of potencies in a given tumor and, therefore, to distinguish Y_1 from Y_2 subtype expression in that tumor tissue. On completion of the incubation, the slides were washed four times for 5 min each in ice-cold preincubation solution (pH 7.4). The slides were rinsed twice in ice-cold distilled water, and then dried under a stream of cold air at 4°C, apposed to ^{3}H-Hyperfilms, and exposed for 7 days in X-ray cassettes.

To further distinguish Y_1 from Y_2 receptors, all cases demonstrating binding with ^{125}I-labeled PYY were evaluated with the Y_1-selective radioligand ^{125}I-labeled [Leu^{31}, Pro^{34}]-PYY and with the Y_2-selective ^{125}I-labeled PYY(3–36) (29, 30). The experiments were performed with ^{125}I-labeled [Leu^{31}, Pro^{34}]-PYY as radioligand and unlabeled PYY or [Leu^{31}, Pro^{34}]-PYY as competitors or with ^{125}I-labeled PYY(3–36) as radioligand and PYY or PYY(3–36) as competitors. Identical experimental conditions as mentioned for ^{125}I-labeled PYY were used. The autoradiograms were quantified using a computer-assisted image processing system, as described previously (27, 31). Tissue standards for iodinated compounds (Amersham, Aylesbury, UK) were used for this purpose. A tissue was defined as receptor-positive when the absorbance measured in the total-binding section was at least twice that of the nonspecific-binding section (in the presence of 25 nM PYY). In each experiments, we have included as positive controls Y_1-expressing tissue (rat cortex) and Y_2-expressing tissues (stratum oriens and radiatum of the rat hippocampus; Ref. 28).

***In Situ* Hybridization Histochemistry.** Y_1 and Y_2 receptor mRNAs were identified in selected normal and tumoral breast tissue samples with *in situ* hybridization histochemistry on cryostat sections, as described previously in detail (32). Oligonucleotide probes complementary to nucleotides 493–529 or 850–879 (33) of the human Y_1 receptor gene and to nucleotides 223–252 (33) of the human Y_2 receptor gene, or 1008–1052 (34) of the rat Y_2 receptor gene (that sequence having 96% homology to the corresponding human one), were synthesized and purified on a 20% polyacrylamide-8 M urea sequencing gel (Microsynth, Balgach, Switzerland). They were labeled at the 3′-end by using [α-^{32}P]dATP (>3000 Ci/mmol; NEN, Life Science Products, Boston, MA) and terminal deoxynucleotidyl transferase (Boehringer, Mannheim, Germany) to specific activities of 0.9–2.0 × 10^4 Ci/mmol. Control experiments were carried out as reported previously (32) with the probes used in the present study to determine the specificity of the hybridization signal obtained.

Growth of SK-N-MC Tumor Cells in Culture. Human neuroblastoma SK-N-MC cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Eagle's minimal essential medium supplemented with 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified 5% CO_2 atmosphere at 37°C. All culture reagents were supplied by Life Technologies, Inc. (Grand Island, NY).

For cell growth assay, cells were plated in 24-well dishes at 2 × 10^4 cells per well and cultured for 3 days to allow cell attachment and growth. The medium was then replaced by fresh medium containing peptides [NPY or NPY(3–36)] at various concentrations (10^{-7}–10^{-9} M), and cells were incubated for 48 h. Media were refreshed after 24 h and peptides added again after 12 and 36 h. Cell growth was measured by cell counting using a Coulter Counter (Model ZB; Coulter Electronics, Hialeah, FL) and a hemocytometer after the trypsinization and the dispersion of cells.

RESULTS

Table 1 summarizes the NPY receptor incidence in breast tissue in the main group of 89 patients. They were expressed in a total of 76 of 89 breast carcinomas tested. These were found in 61 of 69 ductal carcinomas (58 of 64 invasive and 3 of 5 *in situ*) and 10 of 12 lobular carcinomas. Within the group of NPY receptor-positive invasive ductal carcinomas, we identified 15 cases with concomitant *in situ* ductal carcinoma lesions sufficiently large to evaluate their NPY receptor status: 14 of these *in situ* carcinomas expressed NPY receptors. Of the special types of invasive carcinomas, two of three mucinous carcinomas, two of two medullary and one of one tubular carcinoma were NPY receptor-positive. The 13 NPY receptor-negative cases consisted of 8 ductal carcinomas (6 invasive and 2 *in situ*), 2 lobular carcinomas, 1 mucinous, and 2 apocrine carcinomas. For Y_1 and Y_2 subtype characterization, the two approaches used in the present study, *i.e.*, the use of the universal radioligand ^{125}I-labeled PYY and its displacement by unlabeled Y_1 and Y_2 subtype-selective analogues (28) or the use of the two Y_1- and Y_2-selective radioligands (30), gave congruent results: Y_1 was the predominantly expressed receptor subtype in NPY receptor-positive tumors, with 100% incidence of this subtype in receptor-positive tumors, whereas Y_2 was found only in 24% of cases (Table 1). In 58 of 76 (76%) of the receptor-positive tumors, Y_1 was found to be the only (46 cases) or the predominant (12 cases containing more than 90% of Y_1 compared with Y_2) receptor subtype expressed; whereas in 24%, both Y_1 and Y_2 were highly expressed. In none of the tumors was Y_2 found alone. In general, a comparable pattern of Y_1 and Y_2 expression was seen in the *in situ* and invasive part of a ductal carcinoma from a single patient. Another important observation was that Y_1 was much more often distributed homogeneously within the tumor than was the Y_2 receptor, which was expressed focally, *i.e.*, in restricted areas of the tumor only, in 55% of the cases. Even in tumors concomitantly expressing Y_1 and Y_2, we found no tumor area that expressed Y_2 alone; Y_2 was only found in areas where Y_1 was also expressed. In the majority of receptor-positive tumors, the density of NPY receptors was high. The mean density value for the 58 Y_1-expressing tumors was 4946 ± 485 dpm/mg tissue (mean ± SE; $n = 58$). The mean density values for the 18 cases expressing Y_1 and Y_2 were the following: (*a*) 9754 ± 684 dpm/mg tissue (mean ± SE; $n = 18$) for Y_1; and (*b*) 5681 ± 782 dpm/mg tissue (mean ± SE; $n = 18$) for Y_2. It should be noted that, in the latter group, the values for Y_2 were based, in the majority of the cases, on measurements in a very restricted area of the tumor where

Table 1 *Incidence of NPY receptors Y_1 and Y_2 in human breast tissues*

Tissue	NPY-R incidence[a]	Differentiation by Y_1 and/or Y_2 expression[b]		Cases with focal R distribution	
Breast carcinomas	76/89 (85%)	Y_1 tumor type:[c]	58/76 (76%)		21/58 (36%)
		Mixed Y_1/Y_2 tumor type:	18/76 (24%)	Y_1:	3/18 (17%)
				Y_2:	10/18 (55%)
		Y_2 tumor type:	0/76 (0%)		
Non-neoplastic breast (ducts and lobules)	45/45 (100%)	Y_2-only breast type:	19/45 (42%)		
		Y_2/Y_1 breast type:	26/45 (58%)		
		Y_1-only breast type:	0/45 (0%)		

[a] Detected with "universal" ligand ^{125}I-labeled PYY.

[b] Detected with Y_1-selective (^{125}I-labeled [Leu^{31},Pro^{34}]-PYY) or Y_2-selective (^{125}I-labeled PYY(3–36)) radioligands.

[c] Y_1-type tumors were defined as those tumors expressing predominantly Y_1 but not Y_2. Forty-six cases had only Y_1; the remaining 12 cases had a density of Y_2 amounting to <10% of the Y_1 density.

Table 2 *Incidence and density of Y_1 and Y_2 subtypes in the primary tumor and in all lymph node metastases of six patients with breast cancer*[a]

Case no.	Primary tumor: Receptor density Y_1	Primary tumor: Receptor density Y_2	Metastases: No. of metastases	Metastases: Receptor incidence/Mean density Y_1		Metastases: Receptor incidence/Mean density Y_2	
Case 1 (ductal Ca)	7200	0	5	5/5	3091 ± 617	0/5	
Case 2 (lobular Ca)	1398	0	3	3/3	5429 ± 27	0/3	
Case 3 (ductal Ca)	12262	0	9	9/9	10906 ± 368	0/9	
Case 4 (ductal Ca)	12542	6535	6	6/6	11446 ± 373	3/6	7359 ± 431
Case 5 (lobular Ca)	9787	2879	3	3/3	7854 ± 659	3/3	3773 ± 333
Case 6 (ductal Ca)	9445	0	1	1/1	8627	1/1	4561

[a] Density is expressed as dpm/mg tissue (mean ± SE where $n > 2$).

the receptors were focally expressed. Conversely, the Y_1 values were based, in most cases, on measurements in the whole tumor sample characterized by a homogenous receptor distribution. In an additional group of six patients listed in Table 2, from whom primary breast tumors could be obtained together with all lymph node metastases, we found that the six primaries as well as all of the metastases were expressing NPY receptors; Y_1 was present in all cases, Y_2 in a few cases only, both in primaries as well as in metastases (Table 2).

As reported in Table 1, NPY receptors can be detected in all tested normal breast tissues as well. In 42% of the cases, the Y_2 receptor is expressed alone, whereas in none of the tested breast tissues is the Y_1 receptor expressed alone. However, in the remaining breast tissues, Y_1 and Y_2 can be expressed concomitantly (58%). The density of Y_1 and Y_2 receptors in all investigated individual breast tissue samples is high and comparable with the density found in breast cancers. The mean density values in non-neoplastic breast are higher for Y_2 than for Y_1, with 7377 ± 497 dpm/mg tissue (mean ± SE; $n = 45$) for Y_2 and 3793 ± 500 dpm/mg tissue (mean ± SE; $n = 26$) for Y_1.

Fig. 1*a* shows a typical and representative example of the NPY receptor expression in a sample containing a breast carcinoma surrounded by normal breast tissue. The breast carcinoma expresses Y_1 receptors only, as shown by the labeling of the tumor by ^{125}I-labeled PYY and its displacement with [Leu^{31}, Pro^{34}]-NPY but not by PYY(3–36). These results are confirmed further by the additional experiments using two other radioligands: the tumor is labeled by the Y_1-selective ^{125}I-labeled [Leu^{31}, Pro^{34}]-PYY but not by the Y_2-selective ^{125}I-labeled PYY(3–36) (Fig. 1*a*). Conversely, in the same tissue sections, the surrounding breast expresses predominantly Y_2 receptors, as shown by the opposite rank order of potency of NPY analogues, *i.e.,* the high affinity of labeled and unlabeled PYY(3–36) but the low affinity of [Leu^{31}, Pro^{34}]- NPY and [Leu^{31}, Pro^{34}]-PYY.

Fig. 1*b* shows representative displacement curves using the universal ^{125}I-labeled PYY radioligand and increasing concentrations of Y_1- and Y_2-selective analogues. Whereas, in a typical Y_1-expressing breast carcinoma, the Y_1-selective [Leu^{31}, Pro^{34}]-NPY and [Leu^{31}, Pro^{34}]-PYY completely displaced the radiotracer with high affinity, the Y_2-selective PYY(3–36) displaced it with low affinity only. The contrary is observed in the Y_2-only-expressing normal breast tissue, whereas surrounding vessels preferentially express Y_1. Another Y_2-ligand, PYY(13–36), also displaced ^{125}I-labeled PYY with high af-

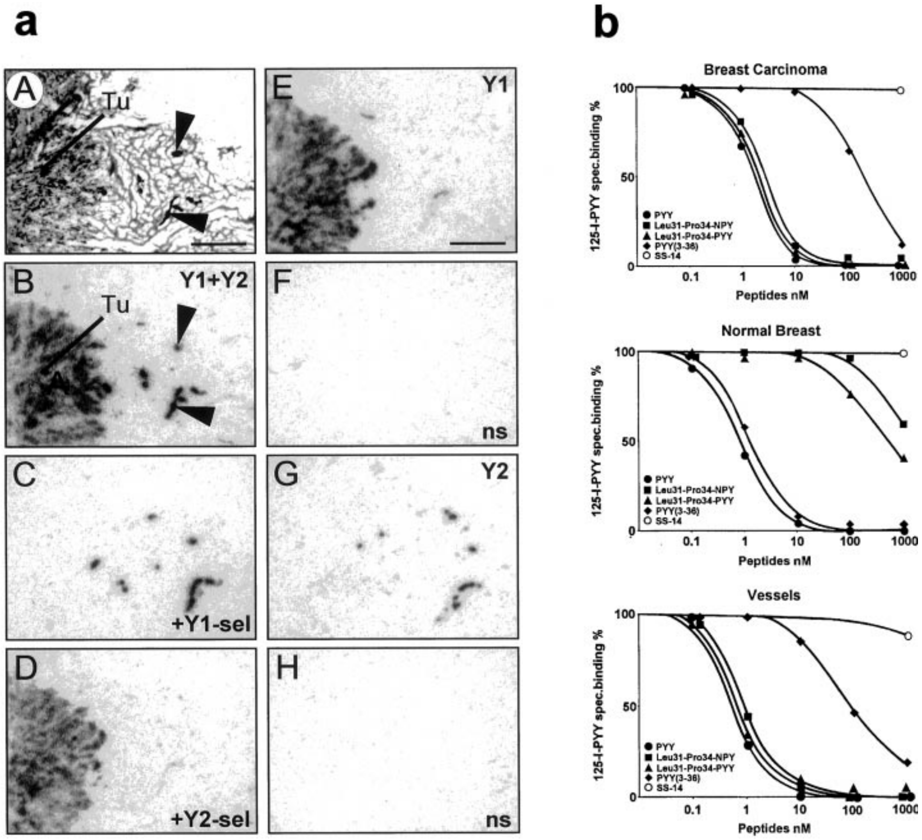

Fig. 1. *a,* NPY receptors in breast carcinoma and adjacent normal breast. *A,* H&E-stained section showing tumor (*Tu*) and normal breast (*arrowheads*). *Bar* = 1 mm. *B,* autoradiogram showing total binding of the universal ligand ^{125}I-labeled PYY with strong labeling of tumor and breast representing Y_1 and Y_2. *C,* autoradiogram showing ^{125}I-labeled PYY binding in the presence of 25 nM of the Y_1-selective [Leu^{31}, Pro^{34}]-NPY. Complete displacement of the radioligand is seen in tumor but not in breast, suggesting Y_1 in the tumor. *D,* autoradiogram showing ^{125}I-labeled PYY binding in the presence of 25 nM of the Y_2-selective PYY(3–36). Complete displacement is seen in breast but not in tumor, suggesting Y_2 in the breast. *E,* autoradiogram showing total binding of the Y_1-selective ligand ^{125}I-labeled [Leu^{31}, Pro^{34}]-PYY. The tumor strongly expresses Y_1; the breast tissue is not or only very weakly labeled. *F,* autoradiogram showing nonspecific binding of ^{125}I-labeled [Leu^{31}, Pro^{34}]-PYY (in the presence of 25 nM [Leu^{31}, Pro^{34}]-NPY). *G,* autoradiogram showing total binding of the Y_2-selective ligand ^{125}I-labeled PYY(3–36). The tumor is not labeled, but the adjacent breast tissue expresses Y_2. *H,* autoradiogram showing nonspecific binding of ^{125}I-labeled PYY(3–36) [in the presence of 25 nM of PYY(3–36)]. *b,* Competition curves showing Y_1 in human breast carcinoma (*top*), Y_2 in normal breast (*middle*), and Y_1 in vessels (*bottom*). *Top* and *bottom graphs,* high-affinity displacement of ^{125}I-labeled PYY by PYY, [Leu^{31}, Pro^{34}]-NPY, and [Leu^{31}, Pro^{34}]-PYY, but not by PYY(3–36). Somatostatin (*SS-14*) is inactive. *Middle graph,* high affinity displacement of ^{125}I-labeled PYY by PYY and PYY(3–36) but not by [Leu^{31}, Pro^{34}]- PYY, and [Leu^{31}, Pro^{34}]-NPY. Somatostatin (*SS-14*) is inactive.

finity in Y_2-expressing normal breast and was inactive in Y_1 tumors. Furthermore, PP, known to have high-affinity binding for Y_4 (22), displaced ^{125}I-labeled PYY with low affinity in breast and in breast tumors. In selected cases, ionic manipulations of the incubation solution were performed as an additional way to differentiate between Y_1 and Y_2 (35). In the presence of 5 mM Mn^{2+}, the Y_1 could not be detected in the tumors anymore, whereas Y_2 remained present in the adjacent breast.

Fig. 2 shows a high magnification of Y_2-expressing lobules and one duct from normal breast tissue labeled with the Y_2-selective ^{125}I-labeled PYY(3–36). Note that not all elements are homogeneously labeled. In normal breast, we have often found few lobules and ducts that were either nonhomogeneously labeled or unlabeled next to a majority of labeled elements.

In situ hybridization for Y_1 and Y_2 mRNAs was performed in cases selected for their high expression of the respective receptor proteins. We could consistently show Y_1 mRNA in the 12 investigated Y_1-type of tumors. Furthermore, it was possible to detect Y_2 mRNA in isolated Y_2-expressing tubules of the normal breast. Fig. 3 illustrates Y_1 mRNA in a breast tumor and Y_2 mRNA in a tubule of the normal breast and compares it with Y_1 and Y_2 receptor autoradiography using Y_1- and Y_2-selective radioligands, respectively.

To search for a potential function of NPY in cancer, we have tested the effect of NPY in the Y_1-expressing SK-N-MC tumor cells. NPY (10^{-7} M) for 24 h was able to inhibit tumor cell growth by >40%, whereas the Y_2-selective NPY(3–36) was inactive (Fig. 4). This growth-inhibitory effect was dose-dependent. Moreover, it was particularly pronounced during the first 24 h of NPY treatment (Fig. 4).

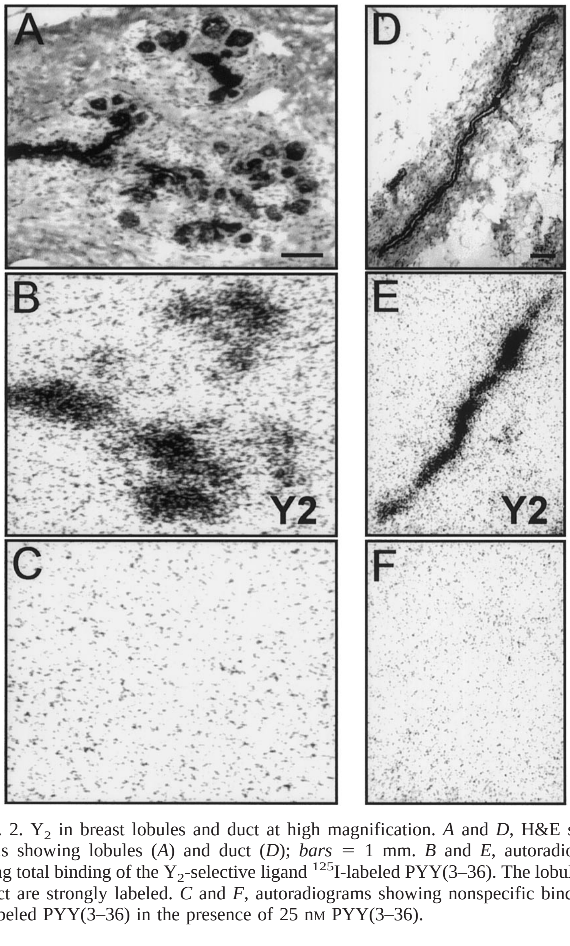

Fig. 2. Y_2 in breast lobules and duct at high magnification. *A* and *D*, H&E stained sections showing lobules (*A*) and duct (*D*); *bars* = 1 mm. *B* and *E*, autoradiograms showing total binding of the Y_2-selective ligand ^{125}I-labeled PYY(3–36). The lobules and the duct are strongly labeled. *C* and *F*, autoradiograms showing nonspecific binding of ^{125}I-labeled PYY(3–36) in the presence of 25 nM PYY(3–36).

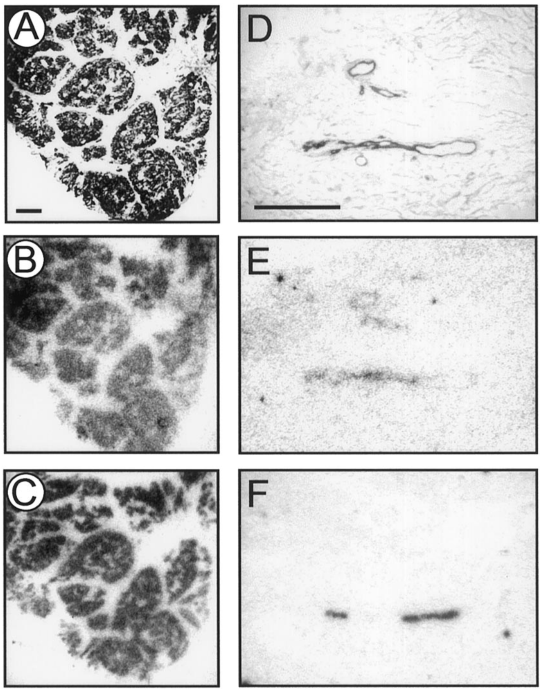

Fig. 3. Y_1 and Y_2 mRNAs detected by *in situ* hybridization. *A–C*, Y_1-expressing breast tumor. *A*, H&E-stained section showing breast carcinoma; *bar* = 1 mm. *B*, autoradiogram showing Y_1 mRNA in the tumor tissue. Nonspecific labeling (in the presence of a 20-fold excess of the corresponding probe) is negligible. *C*, autoradiogram showing binding of ^{125}I-labeled [Leu^{31}, Pro^{34}]-PYY in the same tumor tissue. *D–F*, Y_2-expressing ducts. *D*, H&E-stained sections showing several ducts; *bar* = 1mm. *E*, autoradiogram showing Y_2 mRNA in the ducts. Nonspecific labeling (in the presence of a 20-fold excess of the corresponding probe) is negligible. *F*, autoradiogram showing binding of ^{125}I-labeled PYY(3–36) in a normal duct in the same patient.

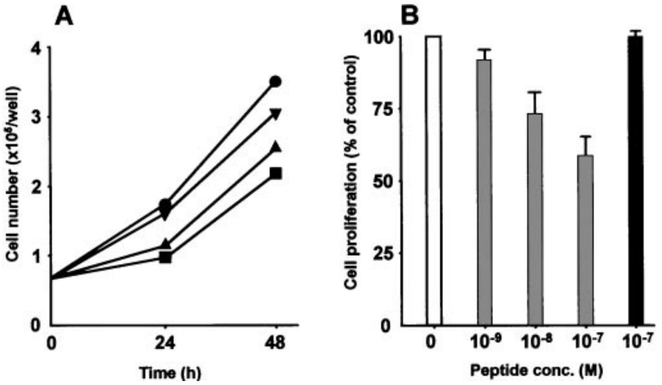

Fig. 4. Effect of NPY on proliferation of Y_1-expressing SK-N-MC tumor cells. *A*, cells were incubated for 48 h in the absence (●) or the presence of 10^{-9} (▼), 10^{-8} (▲), or 10^{-7} M NPY (■). Values represent the mean cell number determined in triplicate from a typical experiment representative of three others. *B*, cells were untreated (☐) or treated with increasing concentrations of NPY (▨) or 10^{-7} M of the Y_2-selective NPY(3–36) (■) for 24 h. Results are expressed as the percentage of control values obtained with untreated cells (mean ± SE of three to six separate experiments done in triplicate). NPY induced a concentration-dependent inhibition of growth of Y_1-expressing SK-N-MC tumor cells, whereas the Y_2-selective NPY(3–36) had no effect.

DISCUSSION

This study is the first evidence that the neuropeptide NPY may play a role in human cancer. It is remarkable that a great majority, *i.e.,* 85% of the patients with breast cancers, often have an high expression of NPY receptors in their cancers. In all cases, the NPY receptor subtype Y_1 is expressed, whereas Y_2 is only expressed in 24% of the cases, and, when it is expressed, it never represents the predominant subtype of the tumor and is never found alone. In the 24% of the cases with a mixed expression of Y_1 and Y_2, a much more focal, topographically restricted distribution can be recognized for Y_2 than for Y_1, emphasizing once more the predominance of Y_1 in tumors. Both ductal and lobular breast cancers, of the *in situ* and invasive types, as well as all lymph node metastases, can express NPY receptors.

We also give here the first evidence that Y_1 receptors may be of functional relevance in tumor cell proliferation. Indeed, the addition of 10^{-7} M NPY for 24 h to cultures of Y_1-expressing human SK-N-MC cells in the growing phase was able to inhibit the growth of these cells by >40%. This NPY effect seems to be a specifically Y_1-mediated process for the following reasons: (*a*) the SK-N-MC cells express Y_1 receptors selectively (36, 37); and (*b*) the Y_2-selective NPY(3–36) was unable to induce this antiproliferative effect under the same conditions. We conclude that NPY has antiproliferative properties at Y_1 receptors; the degree of NPY-induced antiproliferation seen in SK-N-MC cells is remarkable, and compares favorably with antiproliferative effects reported for other peptide hormones such as somatostatin (38), which has an established role in clinical oncology.

Among the numerous cloned NPY receptors, the Y_1, Y_2, Y_4, and Y_5 currently represent the only fully defined subtypes (22). There are several arguments showing that the subtypes detected in the tumors of the present study correspond to Y_1 and Y_2. Pharmacological evidence for Y_1 expression in tumors are: (*a*) specific binding of ^{125}I-labeled PYY that is fully displaced in the high affinity range by the Y_1-selective [Leu^{31}, Pro^{34}]-NPY but not by PYY(3–36), PYY(13–36), or PP, compounds known to have high affinity for Y_2 or Y_4 (22, 28); (*b*) selective binding of ^{125}I-labeled [Leu^{31}, Pro^{34}]-PYY in the same tissues (30); (*c*) subtype-selective ionic dependence, *i.e.*, the inability to detect Y_1 in the presence of Mn^{2+}, although Y_2 is identified under these conditions (35); and (*d*) Y_1 mRNA detected by *in situ* hybridization in the tumor tissues. Furthermore, with those techniques, we could confirm in humans the data from previous reports in animals showing Y_1-expression in vessels (39, 40).

Evidence for Y_2 expression in normal breast include: (*a*) specific binding of ^{125}I-labeled PYY displaced by nanomolar concentrations of the Y_2-selective PYY(3–36) or PYY(13–36), whereas PP and [Leu^{31}, Pro^{34}]-NPY were not active; (*b*) selective labeling of the breast tissues by ^{125}I-labeled PYY(3–36); (*c*) Mn^{2+}-dependence characteristic for Y_2; and (*d*) Y_2 mRNA detected in single ducts, albeit in low amounts.

Another novel observation is that the ducts and lobules of the non-neoplastic breast tissue, representing the tissue from which breast tumors originate, also express NPY receptors. Although all investigated breast tissues express NPY receptors, it is remarkable, however, that in all tested cases, the subtype Y_2, rather than Y_1, is consistently and predominantly detected in both lobules and ducts; the subtype Y_1 is only found in a minority of cases and only concomitantly with Y_2. Y_1 was never identified as the unique subtype present in a normal breast tissue.

The observation that neoplastic human breast tissue can express a different NPY receptor subtype than non-neoplastic breast strongly suggests an alteration of the NPY receptor subtype expression during the process of neoplastic transformation of the breast. The normal condition appears to be a predominant Y_2 expression by the non-neoplastic breast. However, in addition, Y_1 may be present in some of the histologically inconspicuous breast tissues; it is yet unknown whether this Y_1 expression is found preferentially in breast tissues surrounding a growing tumor and whether it may reflect an early sign of neoplastic transformation. In the frankly neoplastic tissue of both lobular and ductal cancers, the NPY receptor found predominantly in all cases is the Y_1 type, although Y_2 may be present concomitantly in some of the cases in a focal distribution. Therefore, neoplastic transformation of breast tissue may induce a switch of expression from receptor subtype Y_2 to Y_1. This switch in receptor expression possibly may be related to the increasing dedifferentiation of the tissue. Indeed, it was reported recently that, in rat pheochromocytoma PC12 cells, the NGF-differentiated cells were expressing Y_2 preferentially, whereas the undifferentiated PC12 cells contained Y_1 (41). This differentiation-specific expression of subtypes may be a general characteristic of NPY receptors.

The present study points toward a potential functional role of Y_1 receptors in cancer, because NPY can inhibit the growth of the Y_1-expressing SK-N-MC cells in culture; the high density and high incidence of Y_1 in breast cancers suggest that these neoplasms may represent an important target for NPY-related drugs. First, long-term treatment with Y_1-selective analogues (23, 24) may be used to inhibit tumor proliferation. Second, one may think of targeting breast tumors and metastases with radiolabeled Y_1 analogues for diagnostic *in vivo* scintigraphic tumor detection or for receptor-mediated radiotherapeutic treatment of these tumors, in analogy to *in vivo* targeting of other peptide receptors (1, 10, 11). One may argue that the Y_1 receptors present in approximately one-half of the normal breast could interfere *in vivo* with the tumor-Y_1 targeting; however, the labeling of a low number of Y_1 ducts and lobules disseminated within the whole breast is likely to be negligible compared with the focal labeling of the whole tumor mass. Moreover, for radiotherapy, it may not necessarily be a disadvantage to target the Y_1-expressing breast tissue, in the event that the Y_1 expression in this tissue turns out to reflect an early stage of neoplastic transformation. Y_1 targeting of breast tumors may be superior to somatostatin, VIP, or gastrin-releasing peptide receptor targeting, as these three receptors are either expressed in lower incidence and/or heterogeneously in breast tumors (somatostatin receptors; Ref. 42), or concomitantly in the normal and tumoral breast tissue (VIP and gastrin-releasing peptide receptors; Refs. 43 and 44). NPY should therefore be added to the list of small regulatory peptides relevant to cancer, and to breast cancer in particular. It may be attractive to combine Y_1 radioligands with radiolabeled VIP, gastrin-releasing peptide, and somatostatin analogues, as all four peptide families can have their receptors overexpressed in breast cancers (42–44). Such a cocktail of four peptide radioligands, given concomitantly, may substantially increase the diagnostic sensitivity of the scintigraphic procedure as well as the radiotherapeutic efficacy.

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