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Immunoreactive Opioid Peptides in Human Breast Cancer

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Opioid peptides have a variety of actions on inter alia pituitary hormone secretion and the immune system. Release of endogenous opioids has been found to stimulate growth of experimental breast cancers and opiate receptor blockers have reduced the growth of chemically induced rat breast tumors. Opioid peptides may therefore play a role in human breast cancer. Invasive ductal carcinomas from 61 premenopausal women were immunocytochemically analyzed for the presence of opioid peptide immunoreactivity. Positive staining was unambiguously identified in 34 of the tumors (56%). In addition, a medullary carcinoma was positive. In a smaller series of tumors, opioid peptide immunoreactive cells were detected in both primary tumors and metastases. Positive tumor cells were usually few and scattered. Therefore, underestimates of their true frequency of occurrence are likely to have occurred, making accurate correlations with clinical behavior and estrogen receptor status difficult. No correlations with estrogen receptors were established for the unambiguously opioid peptide-positive tumors. Many of the positive tumors also stained with antibodies to γ-endorphin and alpha-melanocyte-stimulating hormone, suggesting the presence of proopiomelanocortin-derived peptides in them. However, peptides derived from other opioid precursors also may be present in breast cancer. (Am J Pathol 1989, 134:473–479)

Peptides with the ability to specifically bind to opiate receptors have been isolated from both brain and peripheral tissues. In mammals, these peptides can be derived from at least three distinct precursors. Thus, the proopiomelanocortin precursor yields βendorphin together with a host of other bioactive peptides, including adrenocorticotropin (ACTH) and alpha-melanocyte-stimulating hormone (αMSH). Processing of the preproenkephalin A precursor yields met-enkephalin and leu-enkephalin together with certain C-terminally elongated enkephalin congeners, whereas the preproenkephalin B precursor contains, inter alia, the sequences of dynorphin and α-neoendorphin. This large variety of opioid peptides is matched by the existence of different opiate receptors, including mu, kappa, and delta sites, which show different degrees of selectivity for the different peptides.[1,2]

Opioid peptides are important for the control of many bodily functions, including pain perception, mood, and psychic sensations of reward.[3,4] In addition, these peptides have striking effects on the humoral and cellular immune system,[5,6] and evidence for production of proopiomelanocortin by human lymphocytes and murine macrophages has been presented.[7–12]

Many studies have attempted to define the roles of opioid peptides and of opiates in neoplasia.[13] Early studies on chemically-induced rat mammary carcinomas showed that opiate receptor blockers inhibited growth of these tumors.[14] Moreover, studies in rats with transplantable mammary carcinomas have revealed that stress-induced release of endogenous opioids decreases the survival time and the natural killer (NK) cell activity of the hosts. This effect could be prevented by administration of opiate receptor blockers.[15] Subsequent studies indicated that the detrimental effects of opioid peptides on the tumor-bearing rats most likely was mediated by effects on the central nervous system.[16]

Much attention has been paid to effects of stress-induced opioid peptide release and of therapeutically ad-

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ministered opiates on tumor growth. Recently, however, we have found that a transplantable mouse ascites mammary tumor cell line contains abundant opioid peptide immunoreactivity.[17] In addition, preliminary studies revealed the existence of similar opioid peptide immunoreactivity in human breast cancers and in breast cancer explants from nude mice (Larsson L-I, Brünner N, unpublished data). We therefore decided to screen a series of human breast carcinomas for the presence of opioid peptides. This screening formed part of the tumor screening program at the Unit of Histochemistry and initially made use of a pan-specific opioid peptide antibody (KA_3),[18,19] which recognizes the biologically active sequence Tyr-Gly-Gly-Phe, which is common to all three opioid peptide precursor forms. Subsequently, sequence-specific antisera were employed for defining in more detail what precursor type was responsible for the KA_3 staining of tumor cells.

Material and Methods

Patients and Tissue Material

A total of 62 premenopausal female patients, obtained from the files of the Danish Breast Cancer Cooperative Group (DBCG)[20] were studied. The material encompassed paraffin blocks from mastectomy specimens of primary lesions treated between 1978 and 1979, with a diagnosis of invasive ductal carcinoma (stage I and II). None of the patients had evidence of clinically apparent hormonal syndromes or major metabolic abnormalities in the preoperative period or during the 5-year period of postoperative follow-up. Paraffin blocks were obtained from three different centres: 1) The Pathology Department, Herlev Hospital; 2) The Cancer Research Institute, Kommunehospitalet, Aarhus; and 3) The Pathology Department, Bispebjerg Hospital, Copenhagen.

Tumors were excised and immersion-fixed for 24–48 hours in either 4% buffered formaldehyde, pH 7.0 (1 and 2) or in 10% formaldehyde (3). The tumors were then cut into 3–4 mm slices and again immersed in the fixative for an additional 4–8 hours. The slices were subsequently dehydrated in ascending ethanols, cleared in xylene, and embedded in paraffin (Paraplast Monoject Scientific Inc., Kildare, Ireland). Tissue specimens that had been subject to frozen section procedures before fixation were excluded from the study because these procedures were found to destroy most peptide hormone immunoreactivity in control tissues.

Immunocytochemistry

The paraffin-embedded specimens were collected at the Unit of Histochemistry and processed further for routine hematoxylin and eosin (H & E) staining and immunocytochemistry.

Three to five micron sections were deparaffinized and hydrated, soaked in 0.05 M TRIS buffer, pH 7.4, containing 0.15 M NaCl (TRIS-buffered saline, TBS), fortified with 1% Triton X-100, and then covered with 1% human serum albumin in TBS for 1 hour at room temperature. The sections were then reacted with primary polyclonal antibodies as indicated in Table 1 for 20 hours at 4 C, followed by reequilibration for 1 hour at room temperature. The site of antigen-antibody reaction was revealed using the peroxidase-antiperoxidase (PAP) procedure[21] or the silver-enhanced immunogold method.[22,23] Peroxidase activity was demonstrated by standard diaminobenzidine (DAB) development.[24] Selected tumor sections also were submitted to DAB development in the presence of 0.1 M imidazole or were exposed to $HAuCl_4$ followed by silver intensification as detailed elsewhere.[25] Colloidal gold particles were silver-enhanced using a physical developer containing gum arabic,[26] as detailed previously.[23] Counterstaining was performed with hematoxylin or nuclear fast red in case of brown and black reaction deposits, respectively.

Immunoreagents

Primary antibodies are detailed in Table 1. In the PAP method, sheep anti-rabbit IgG (SBL, Stockholm, Sweden) was used in dilution 1:30 and PAP complexes (Dakopatts A/S, Copenhagen, Denmark) were used diluted 1:75. Unlabeled sheep anti-rabbit IgG (SBL) was coupled to 5 nm colloidal gold particles, fabricated by the tannic acid method,[27] by the procedure of Wang et al.[28] The gold conjugate was used at a dilution corresponding to an optical density of 0.3 at 520 nm and applied for 1 hour at room temperature. Dilutions of unlabeled IgG and PAP complexes were made in 0.25% bovine serum albumin (BSA) in TBS, while colloidal gold probes were diluted in 0.02 M TRIS buffer containing 0.15 M NaCl, 1% BSA and 0.02% NaN_3.

Control Procedures

Control sections were incubated with primary antibodies preabsorbed against different peptides (Table 2) either in liquid phase or after coupling of the peptides to cyanogen bromide-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden) as described previously.[29] In addition, all antibodies were liquid-phase absorbed against 2 mg per ml of low molecular weight poly-L-lysine (PLL) as described.[30] Staining controls, involving sequential deletions of the various antibody layers as well as positive control tissue sections (pituitary, adrenal medulla and antropy-

Table 1. *Primary Antibodies**

Code	Raised against	Produced in	Working dilution	Source
KA_3	met-enkephalin	Rabbit	1:500	All from the Department of Molecular Cell Biology, Copenhagen, Denmark
γ-V-4	γ-endorphin	Rabbit	1:400	
Albert V-3	αMSH	Rabbit	1:500	

* Detailed characterizations of the KA_3 antiserum are given in references 18 and 19 and of the Albert antiserum in reference 36. The γ-V-4 antiserum was produced by immunizations against BSA-glutaraldehyde-conjugated synthetic γ-endorphin. As shown by absorption controls and tissue staining experiments the antiserum recognizes γ- and β-endorphin but not met- or leu-enkephalin (Larsson L-I, Stengaard-Pedersen K, unpublished observation).

loric mucosal specimens) also were included in the staining procedure.

Determinations of Estrogen Receptors

The method is detailed elsewhere.[31,32] We used a three-layer indirect immunoperoxidase technique, which is briefly described below.

The formalin-fixed, paraffin-embedded biopsies were cut into 6 μ sections, conventionally deparaffinized, and rehydrated. After washing they were trypsinized with 0.1% trypsin in 0.1% $CaCl_2$ followed by incubation with 10% normal goat serum for 20 minutes. Without washing, they were covered with primary monoclonal estrogen receptor (ER) antibody (H222, minimal concentration 0.1 μg/ml, obtained from the Abbott kit) and incubated at 4 C for 16 hours. The ER-anti-ER binding was visualized by incubation with biotinylated goat-anti-rat IgG (0.05 mg/ml) for 60 minutes, followed by Vectastain avidin-biotin complex for 60 minutes according to the manufacturer's instructions, and developed in 3-amino-9-ethylcarbazole. In each specimen, controls were run by replacing the monoclonal antibody with the corresponding immunoglobulin to rule out nonspecific staining.

The monoclonal antibody used in this study has been shown to be specific for ER by nitrocellulose blotting, distribution within normal tissue, and inhibition of staining by adsorption with excess ER.[33,34]

Histologic Evaluation

Tumor specimens were reviewed before entering the project. Histologic classification and malignancy grading were performed in accordance with the recommendations of the World Health Organization.[35]

Results

Histologic examination of the breast carcinomas showed that 61 were of invasive ductal type, whereas one represented a medullary carcinoma. Seventeen of the carcinomas contained additional foci of ductal carcinoma *in situ*, 11 cases of which were comedo type.

Immunocytochemical staining (PAP procedure and standard development [PAP-sDAB]) revealed that the majority of the carcinomas contained cells staining with the pan-specific opioid peptide antiserum KA_3 (Figure 1). The single medullary carcinoma and 34 of the 61 (56%) invasive ductal carcinomas contained sufficiently numerous and intense immunoreactive cells to make interpretation of staining and specificity controls unambiguous. Absorption of the KA_3 antibody against met-enkephalin-Sepharose abolished all staining, whereas absorption against poly-L-lysine was without effects on the staining. Absorption against leu-enkephalin-Sepharose failed to affect staining in 31 of the positive carcinomas. In the three remaining carcinomas, however, leu-enkephalin-Sepharose preabsorption also abolished staining. Conventional staining controls were negative. The immunoreactive cells were usually few and characteristically occurred together in small foci. This focal distribution results in a significant sampling error. All 62 carcinomas were independently screened by three observers (LS, EB, and L-IL). There was agreement between the observers that the incidence of positive tumors given above represents a minimum estimate due to sampling errors and the wish to evaluate adjacent sections by controls, necessitating selection of tumors containing a significant number of cells of sufficient staining intensity.

In 10 of the 17 specimens that contained foci of ductal carcinoma *in situ*, single, scattered immunoreactive tumor cells also were found in the *in situ* areas. In two of these cases, only the carcinoma *in situ* areas and not

Table 2. *Absorption Controls**

Antibody	Antigen	Amount of added antigen (μg/ml of diluted antibody)	Phase
KA_3	met-enkephalin	100 (twice)	Solid
	leu-enkephalin	100 (twice)	Solid
γ-V-4	γ-endorphin	100–200	Solid
	met-enkephalin	100–200	Solid
Albert V-3	αMSH	100	Liquid
	ACTH[1–24]	100	Solid

* All peptides used for absorption controls were synthetic and obtained from Peninsula Labs.

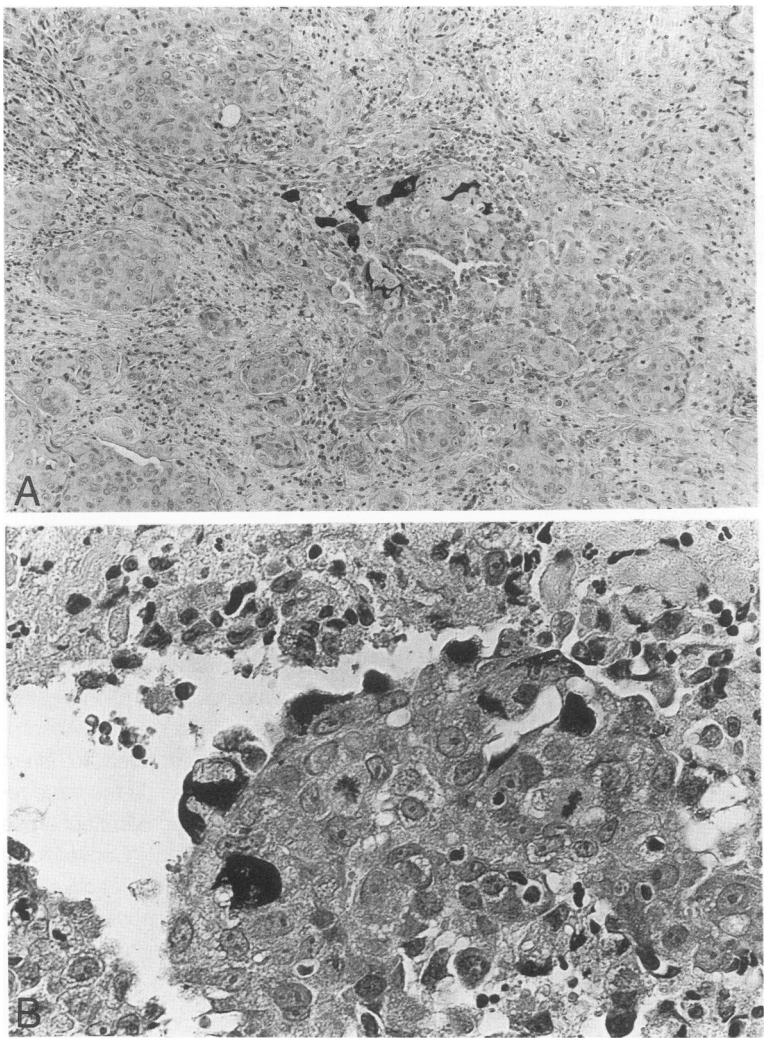

Figure 1A. *Focal area of KA_3-positive cells in an infiltrating duct carcinoma. PAP-sDAB staining, ×250.* **B:** *Infiltrating duct carcinoma. Note the single KA_3 positive cells. PAP-sDAB staining, ×400.*

the invasive carcinoma areas contained immunoreactive cells. Of the 11 cases of comedo carcinomas, 4 contained immunoreactive cells in the tumor-infiltrated ducts.

Twenty-six of the tumors that stained with antiserum KA_3 in the PAP-sDAB procedure were subsequently stained by the silver-enhanced immunogold (SIG) procedure,[23] again using KA_3 as primary antibody. All of these tumors also were positive by the SIG procedure and in seven of the tumors a higher number of positive cells was recorded after SIG staining than after PAP-sDAB staining. In addition, the six most weakly positive tumors in the PAP-sDAB procedure also were stained using DAB development, $HAuCl_4$ impregnation, and silver intensification.[25] In four of these tumors a higher number of cells was recorded together with an increased intensity of staining. Of the 28 tumors that were negative in the PAP-sDAB procedure, 6 were stained by the DAB-$HAuCl_4$-silver intensification procedure. One of these tumors revealed positive cells after staining with antiserum KA_3. Absorption against met-enkephalin-Sepharose eliminated this staining.

In a small additional series of nine invasive ductal carcinomas, tissue specimens from both the primary breast lesions and from subsequent distant metastases (N = 6) or from local recurrences (N = 3) were studied. Five of the nine primaries contained KA_3 immunoreactive tumor cells (PAP-sDAB procedure). Four of the six primaries associated with distant metastases stained with the KA_3 antiserum and the metastases (bone marrow, axillary lymph nodes, and skin) also contained KA_3-positive cells. The two KA_3-negative tumors were associated with KA_3-negative distant metastases (skin and ovary). Of the three mammary recurrences, one primary was negative for KA_3, one was positive, and one was questionable. A similar pattern of reactivity was noted in the recurrences. One patient of this series had a KA_3-negative tumor in the left breast and, 1 year later, developed a KA_3 positive tumor in the right breast.

Finally, a limited number of tumors were subjected to staining with an αMSH antiserum (Albert)[36] (N = 27) or a γ-endorphin antiserum (γ-V-4, N = 23). The γ-V-4 antise-

rum was routinely used after preabsorption against poly-L-lysine to diminish background staining. Seventeen of the 27 tumors stained with the αMSH antiserum were found to contain positive cells. Notably, most of the positive tumors were obtained from one of the centers (Herlev) included in our study. All 23 tumors tested with the γ-V-4 antiserum contained positive cells. Absorption controls were performed in four of the positive tumors and documented that αMSH (but not ACTH[1–24]) preabsorption of antiserum Albert and γ-endorphin-Sepharose (but not met-enkephalin-Sepharose) preabsorption of antiserum γ-V-4 removed all staining. Comparisons between the staining patterns obtained by these latter antisera and with the KA_3 antiserum documented that, in many tumors, the KA_3-positive cells were more numerous and more intensely stained.

Of the 62 cases, 58 specimens were evaluable for immunohistochemical ER determination. Forty-one were ER positive and the rest ER negative. Of the positive cases 25 had been scored as KA_3 positive and 16 as KA_3 negative. Of the ER negative cases, eight were KA_3 positive and nine KA_3 negative.

Discussion

Our results show that a high percentage of primary ductal carcinomas of the breast contain scattered tumor cells with opioid peptide immunoreactivity. The frequency found (56%) most likely underestimates the true incidence. Thus, first, retrospective material that had not been optimally fixed and preserved for immunocytochemistry was analyzed. As previously detailed,[18,19,36] the antisera used work optimally on cryostat sections of aldehyde-fixed material and react less well with formalin-fixed paraffin-embedded material. Second, positive tumor cells were often few and occurred clustered in certain areas of the tumors, raising the possibility for distributional artifacts in our study. Third, to interpret the staining and specificity controls a certain number of cells of a certain staining intensity need to be present in the sections. All of these factors lead to an underestimate of the true frequency, so that on microscopy we often raised the question of whether or not all ductal carcinomas in fact contained some opioid peptide immunoreactive cells. This uncertainty about the true number of positive tumors makes correlations with clinical course and ER status impossible. However, no correlation between unquestionably KA_3 positive tumors and ER positive status was found in the present study.

One way to pursue this question would be to examine a prospective material adequately processed for immunocytochemistry. Because this currently is not possible we undertook studies using different immunocytochemical procedures that have been documented previously to possess a detection efficiency and sensitivity higher than that of the standard PAP-DAB procedure.[23,25] Our results show that the more sensitive silver-enhanced immunogold staining and silver-intensified peroxidase methods demonstrate larger numbers of specifically immunoreactive cells in the carcinomas. Moreover, one tumor, which was reported initially as negative in the standard PAP-DAB procedure, was found to be positive when stained by a silver-intensified PAP-DAB procedure. Thus, in agreement with previous data, we find that use of these highly sensitive procedures results in improved detection of immunoreactive cells.

Opioid activity can be derived from three distinct precursors: proopiomelanocortin and preproenkephalin A and B. In addition, limited proteolysis of the milk protein casein may yield peptides with opioid activity (casomorphins).[37–39] The KA_3 antiserum used in our study[18,19] contains at least two antibody subpopulations. One subpopulation is specific for met-enkephalin and C-terminally elongated congeners thereof (such as α-, γ-, and β-endorphin), whereas the second subpopulation is a panopioid peptide specific antibody, recognizing the Tyr-Gly-Gly-Phe sequence, common to all opioid peptide precursors but not shared with the casomorphins. In the vast majority of positive tumors, absorption against leu-enkephalin (Tyr-Gly-Gly-Phe-Leu), which removes the panspecific antibody subpopulation, but does not affect the met-enkephalin/endorphin-specific population was without effect on the staining. This finding indicates that a met-enkephalin/endorphin congener is being detected and this is further corroborated by the absence of staining after absorption with met-enkephalin-Sepharose and the positive staining obtained by an antiserum (γ-V-4), directed against the non-met-enkephalin region of γ-endorphin.

Notably, however, the number of cells reactive with antisera recognizing the γ-endorphin and αMSH regions of the proopiomelanocortin precursor were, in many tumors, lower than the number of overall opioid peptide immunoreactive cells. Importantly, when the tumors were analyzed with γendorphin and αMSH antisera it was found that the αMSH antiserum reacted with a large percentage of tumors derived from only one of the three centers included in our study. This could be related to different routines of fixation and embedding at the three centers, including a shorter fixation time at the center with the highest frequency of positive tumors.

These data lend support to the notion that at least part of the opioid peptide immunoreactivity in ductal carcinomas represents endorphinlike peptides derived from the proopiomelanocortin precursor. Ongoing radioimmunoassay studies performed on high performance liquid chromatographic fractions of tumor extracts support this notion and indicate that the three tumors examined so far contain both αMSH and CLIPlike peptides. In addition to

proopiomelanocortin, it is probable that part of the tumor cells also contain opioid peptides derived from other precursors, particular in the three cases in which leu-enkephalin-Sepharose preabsorption removed staining with the KA_3 antiserum. While this study was in progress, Zagon et al[40] published radioimmunoassay data on the presence of both met-enkephalin and βendorphin in a breast adenocarcinoma.

The occurrence of immunoreactive neurohormonal peptides in cancer cells is often ascribed to a so-called neuroendocrine differentiation of these cells.[41–44] We are reluctant to employ this term because many cells known to produce POMC are not neuroectodermal derivatives (eg, lymphocytes, macrophages, and testicular cells). A vast number of bioregulatory peptides, including transforming growth factors α and β, insulinlike growth factors, and platelet-derived growth factor have been shown to be produced by breast cancer cells.[45–50] All of these factors are believed to be important for tumor growth and tumor cell interactions with host tissues. It is therefore possible that expression of opioid peptides by mammary tumor cells also is important in this respect.

There are many putative tumor-related roles for opioid peptides. In the past, most studies focussed on their interactions with the immune system and on effects of treatment with opiate receptor antagonists and agonists on the immune response. In addition, opioid peptides affect the secretion of several hypothalamic and pituitary factors that may be important regulators of normal and neoplastic mammary growth.[51] Finally, it has been noted that the expression of opioid receptors in the brain and in the uterus is strongly regulated by oestrogens[52] and that ACTH may regulate aromatase activity.[53] Further studies are needed to establish which, if any, of these functions may be important in breast cancer.

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