Advances in Brief

Immunocytochemical Detection of Somatostatin Receptors sst₁, sst₂A, sst₂B, and sst₃ in Paraffin-embedded Breast Cancer Tissue Using Subtype-specific Antibodies

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Abstract

The long-acting somatostatin analogue octreotide (SMS 201–995) inhibits growth of certain breast cancer cell lines in vivo and in vitro. Because the antiproliferative action of octreotide depends on at least the presence of somatostatin receptors, it is crucial to determine the pattern of somatostatin receptor protein expression on the tumor cells. In the present study, we have raised polyclonal antibodies to somatostatin receptor subtypes (sst) sst₁, sst₂A, sst₂B, and sst₃ using peptides corresponding to their COOH-terminal sequences. These antisera were used for immunocytochemical staining of paraffin sections of 33 primary breast cancers. Somatostatin receptor-like immunoreactivity (Li) was predominantly localized to the plasma membrane of the tumor cells. In the vast majority of positively stained tumors, somatostatin receptor-Li was uniformly present on nearly all tumor cells. Both the level and the pattern of expression of sst varied greatly between individual carcinomas. sst₂A-Li and/or sst₂B-Li was detectable in 28 tumors (85%); among these, 14 tumors (42%) showed particularly high levels of sst₂-Li, sst₁-Li was found in 17 (52%) cases and sst₂-Li in 16 (48%) cases. The expression of sst was independent of patient age, menopausal status, diagnosis, histological grade, and levels of estrogen and progesterone receptors. The immunocytochemical determination of somatostatin receptor status allows direct detection of receptor protein on the tumor cells and, hence, may provide more precise information than reverse transcription-PCR for predicting response to octreotide therapy in breast cancer.

Introduction

Octreotide (SMS 201–995, sandostatin) is a synthetic peptide derivative of natural somatostatin with markedly increased metabolic stability; it has been used successfully in the treatment of some neuroendocrine malignancies (1, 2). Somatostatin analogues are also effective in inhibiting growth of a number of human breast cancer cell lines including MCF-7 and T47D in vivo and in vitro (3–7). In addition, octreotide has been shown to enhance the antineoplastic effects of tamoxifen and ovarietomy on 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinomas (8). The responsiveness of tumors to octreotide is critically dependent on the presence of somatostatin receptors.

Recently, five subtypes of somatostatin receptors, designated sst₁–sst₅, have been identified (9). Two isoforms of sst₂, sst₂A and sst₂B, have been isolated that differ in size and sequence of their intracellular COOH-terminal domain (10, 11). All receptors bind natural somatostatin with high affinity but differ in the characteristics of their binding to various long-acting somatostatin analogues (12). There is also evidence for different, although not mutually exclusive, pathways of intracellular signaling of sst, e.g., the antiproliferative action of octreotide, which binds with high affinity to sst₂ and sst₅, is thought to be linked to stimulation of sst₂-associated tyrosine phosphatases (13, 14). Furthermore, the antiproliferative effects of somatostatin analogues seem to require high numbers of somatostatin receptors, whereas the antimetabolic effects occur in the presence of a relatively low number of receptors. Therefore, to select one or more somatostatin analogues for optimal therapeutic effect, it is crucial to determine the pattern of sst expression for a specific tumor.

Somatostatin receptors have been detected in 75% of primary breast cancers by [¹¹¹In-DTPA-o-Phe₁]-octreotide scintigraphy (15). By using RT-PCR, sst₂ transcripts were found in all of the breast carcinomas studied as well as in the adjacent normal tissue (16). However, this method is based on total RNA isolation from a fresh tumor sample and, therefore, would detect somatostatin receptor transcripts originating not only from tumor cells but also from fibrocytes, lymphocytes, or other non-malignant cells. In the present study, we have raised polyclonal antibodies that specifically detect the COOH-terminal tails of sst₁, sst₂A, sst₂B, or sst₃. These antisera were used for immunocytochemical determination of the somatostatin receptor status of 33 formalin-fixed, paraffin-embedded breast cancers.
Materials and Methods

Patients, Tumors, and Tissue Preparation. Tumors from 33 patients with primary carcinoma of the breast were studied. All of the patients were initially treated by surgical tumor resection between 1995 and 1996 at the Department of Obstetrics and Gynecology, Otto-von-Guericke University, Magdeburg, Germany. Pertinent data from patient histories (age, menstrual status, diagnosis, histological grade, and estrogen and progesterone receptor status) are given (see Table 2). Histological grading was based on the method of Bloom and Richardson. Estrogen and progesterone receptor-Li was scored according to Remmele and Stegner (17). Tumor specimens were fixed in phosphate-buffered 4% formalin for a minimum of 24 h. After dehydration through graded percentages of ethanol and then xylene, the tissue was embedded in paraffin wax.

Generation of Antipeptide Antisera. Polyclonal antisera were generated against the COOH-terminal tails of sst1, sst2A, sst2B, and sst3. The identity of the peptides is given in Table 1. Peptides were custom-synthesized by Gramsch Laboratories (Schwabhausen, Germany), purified by high-performance liquid chromatography, and coupled via an amino-terminally added cysteine and a succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate linker to keyhole limpet hemocyanin. The conjugates were mixed 1:1 with Freund’s adjuvant and injected into groups of two rabbits 4818-4819, one group of four guinea pigs GP1-GP4 were mixed 1:1 with Freund’s adjuvant and injected into groups of two rabbits 4818-4819 for anti-sst1, 4820-4821 for anti-sst2A, 4820-4821 for anti-sst2B, and 4822-4823 for anti-sst3 antisera production. In addition, one group of four guinea pigs GP1-GP4 was injected for anti-sst2A antisera production. Animals were injected at 4-week intervals, and serum was obtained 2 weeks after immunizations beginning with the second injection.

Immunodot-blot Analysis. The specificity of the antisera was tested in dot-blot assays using the immunizing peptides. Serial dilutions of the unconjugated peptides corresponding to the COOH-terminal sequences of sst1, sst2A, sst2B, and sst3 were blotted onto nitrocellulose membranes and then incubated with the antibodies at a dilution of 1:2000. Blots were developed using the enhanced chemiluminescence method (Amersham, Braunschweig, Germany).

Immunocytochemistry. Seven-μm sections were cut and floated onto positively charged slides (SuperFrost/Plus, Menzel, Braunschweig, Germany) for immunocytochemical staining. Sections were dewaxed three times in xylene and rehydrated in a graded series of ethanol. After rinsing in TPBS (10 mM Tris, 10 mM phosphate buffer, 137 mM NaCl, and 0.05% thimerosal [pH 7.4]), sections were incubated in methanol containing 0.3% H2O2 for 30 min at room temperature. Sections were transferred into TPBS and subsequently microwaved in 10 mM citric acid (pH 6.0) for 20 min at 600 W. Specimens were then allowed to cool to room temperature, washed in TPBS, and preincubated in TPBS containing 3% normal goat serum for 1 h at room temperature. Sections were then incubated with anti-sst1, anti-sst2A, anti-sst2B, or anti-sst3 antibodies at dilutions ranging from 1:1000 to 1:5000 in TPBS containing 1% normal goat serum at room temperature overnight. Staining of primary antibody was detected using the biotin amplification procedure as described (18, 19). Briefly, tissue sections were transferred to biotinylated goat anti-rabbit IgG or biotinylated goat anti-guinea pig IgG (1:200, Vector, Burlingame, CA) for 1 h, then incubated in AB solution (reagents from Vector ABC “Elite” kit, 25 μl A and 25 μl B) for 60 min, followed by a final incubation in AB solution (12.5 μl A and 12.5 μl B). Tissue was rinsed and stained with 3,3′-diaminobenzidine-glucose oxidase for 30 min. All of the incubation steps were carried out at room temperature. The cell nuclei were lightly counterstained with hematoxylin. Sections were then dehydrated through several concentrations of alcohol, cleared in xylol, and coverslipped with DPX. For immunohistochemical controls, the primary antibody was either omitted, replaced by preimmune sera, or adsorbed with several concentrations ranging from 1–10 μg/ml homologous or heterologous peptides for 2 h at room temperature. A tumor known to stain positively was included in each batch of staining as a positive control.

Assessment of Staining Patterns. The presence or absence of staining and the depth of color were noted as well as the number of cells showing a positive reaction and whether the staining was localized to the plasma membrane. The depth of color was recorded as pale, medium, or dark according to how easily it was seen. The tumors were then categorized as weak, moderate, or strong stained according to the following criteria: (a) strong, dark staining at the plasma membrane that is easily visible with a low-power objective; (b) moderate, medium staining still seen with a low-power objective; (c) weak, pale staining not easily seen under a low-power objective; and (d) negative, tumors that show none of the above staining.

Statistical Evaluation. Data were analyzed by the use of statistical program package SAS (SAS Institute, Cary, NC). Data grouped into categories were analyzed for correlations with the χ2 test, and continuous variables were analyzed with the Kruskal-Wallis test.

Results

Characterization of Antibodies. Specificity of the antisera was monitored using immunodot-blot analysis. After four booster injections, one anti-sst1, one rabbit anti-sst2A, three guinea pig anti-sst2A, two anti-sst2B, and one anti-sst3 antiseraum developed a titer against their immunizing peptides. As shown in Fig. 1, the antisera 4819 (anti-sst1), GP3 (anti-sst2A), 4820 (anti-sst2B), and 4823 (anti-sst3) specifically detected quantities as low as 500 ng (4819, GP3) and 25 ng (4820, 4823) of their cognate peptide but not the peptides corresponding to other ssts. Antibodies were then subjected to immunocytochemical staining of a panel of human breast cancer tissues. The antisera 4819 (anti-sst1), GP3 (anti-sst2A), 6291 (anti-sst2B), 4820 (anti-sst2B), and 4823 (anti-sst3) yielded prominent staining that was localized to the plasma membr-
brane of the tumor cells and were used throughout this study. As shown in Fig. 2, immunostaining was abolished by preabsorption of the antisera with 10 μg of the immunizing peptides/ml. The staining intensity for each antibody varied greatly between individual tumors and gave consistently different sample-specific patterns of sst expression under otherwise identical conditions. The anti-sst2A antibodies GP3 and 6291 were further characterized using transfected HEK-293 cells as well as using Zamboni-perfused rat brain tissue (100% homology of the COOH-terminal regions of human, rat, and mouse sst2A). Both antisera selectively stained HEK-293 cells transfected with mouse sst2A but not mouse sst2B. On Western blots from rat brain tissue, these antisera detected a broad band of approximately M, 80,000, the presumed molecular weight of the sst2A receptor. In fixed rat brain tissue, these antisera yielded a staining pattern that was essentially identical to that previously published by others (20, 21) with prominent sst2A-Li in the deep layers of the cortex, CA1 hippocampal region, medial habenula, amygdala, and locus coeruleus and in the superficial layers of the spinal cord (22).

Immunocytochemical Staining of Paraffin Sections: Methodological Considerations. Extensive cross-linking during formalin fixation often limits the accessibility of antigenic epitopes. In preliminary experiments, several antigen-unmasking steps were individually tested to determine how effectively they improved somatostatin receptor-Li staining intensity. Microwave pretreatment in citric acid provided retrieval of antigenicity of all of the somatostatin subtypes along with good preservation of morphology and was used, therefore, throughout this study. The staining of paraffin-embedded breast cancer tissue was further improved by means of an amplification step comprising peroxidase-catalyzed deposition of biotinylated tyramine, which provided a strong specific enhancement of signal. Optimal results were obtained when biotinylated tyramine was used at a dilution of 1:250 from a stock solution prepared as described by Adams in 1992 (18).

Somatostatin Receptor Immunocytochemical Staining in Breast Cancer. A series of 33 primary breast tumors was stained immunocytochemically with polyclonal anti-sst1, anti-sst2A, anti-sst2B, and anti-sst3 antibodies. The staining pattern of somatostatin receptor-Li is shown in Table 2. Unequivocal staining for sst1 was present in 17 (52%) of the tumors, for sst2A in 16 (48%) of the tumors, for sst2B in 22 (67%) of the tumors, and for sst3 in 16 (48%) of the tumors. The total number of sst2A-positive (sst2A and sst2B) tumors was 28 (85%). There was a significant correlation (P < 0.05, χ² test) between sst2A-Li and sst2B-Li. However, it is important to note that some tumors expressed either sst2A or sst2B independently. No correlations were observed among other somatostatin subtypes. In the vast majority of positively stained tumors, somatostatin receptor immunoreactivity was uniformly present on nearly all tumor cells. Both the level and the pattern of expression of ssts varied greatly between individual tumors. Somatostatin receptor staining patterns were analyzed for correlation of each subtype with patient age, menopausal status, diagnosis, histological grade, and estrogen and progesterone receptor immunoreactivity. No correlations among these data groups were found. The lack of correlation of sst expression and patient age, menopausal status, diagnosis, histopathological grade, and estrogen and progesterone receptor status indicates that sst expression was regulated independently of these variables. Furthermore, in a number of tumors, non-malignant cells, e.g., fibrocytes and a subset of lymphocytes, also showed somatostatin receptor-Li most frequently sst2A-Li or sst2B-Li.

Discussion

In an effort to study the pattern of somatostatin receptor protein expression in primary breast cancer, we generated antibodies that exert selective specificity for the ssts, sst1, sst2A, sst2B, and sst3, hitherto most frequently detected in human nonpituitary tumor tissue (16, 23–25). We show that the cytoplasmic tails of these receptors can serve as an epitope for the generation of antisera that
Fig. 2  Somatostatin receptor immunocytochemical staining of primary breast cancer. *Left panel*, staining patterns for sst1, sst2A, sst2B, and sst3 in typical breast carcinomas. *Right panel*, corresponding peptide adsorption controls (10 μg/ml). Sections were dewaxed, treated with methanol-H2O2, microwaved in citric acid, and incubated with anti-sst1 (4819), anti-sst2A (GP3), anti-sst2B (4820), or anti-sst3 (4923) antisera at a dilution of 1:2000. Sections were then sequentially treated with biotinylated anti-rabbit IgG or biotinylated anti-guinea pig IgG, AB solution (reagents from Vector ABC “Elite” kit), biotinylated tyramine, and AB solution. Sections were then developed in 3,3'-diaminobenzidine-glucose oxidase and lightly counterstained with hematoxylin. For adsorption controls, primary antibodies were preincubated with the peptides (10 μg/ml) used for immunizations. Bar, 25 μm.
effectively stain formalin-fixed, paraffin-embedded breast cancer tissues. Several lines of evidence indicate that these antisera specifically detect their targeted receptor and do not cross-react: (a) in immunodot-blot assays the antisomatostatin receptor antisera specifically detected their cognate peptides but not the peptides corresponding to the COOH-terminal region of other sst's; (b) in immunohistochemical adsorption controls, somatostatin receptor-Li was completely blocked by pretreatment with the peptides used for immunization; (c) somatostatin receptor-Li was seen as punctuate staining (presumably representing clusters of receptors) localized to the plasma membrane, and the staining intensity for each antibody varied greatly between individual tumors giving consistently different sample-specific patterns of signal expression; and (d) the COOH-terminal peptides are likely to have served as somatostatin receptor-specific immunogen inasmuch as these peptides were found to have minimal homologies (no greater than 66%) to other peptide sequences when aligned to current entries in the European Molecular Biology Laboratory databases using BLASTp or FASTa.

In our series of 33 nonselected malignant breast tissues, sst2-Li was detected in 28 (85%) of the tumors. This parallels the report by van Eijck et al. (15) who detected somatostatin analogue-binding sites in situ in 75% of primary breast carcinomas by $[^{111}]$In-DTPA-D-Phe$^1$-octreotide scintigraphy. Reubi et al. (25) examined six breast cancers selected for good binding of somatostatin analogues; sst2 transcripts were found in all six tumors using in situ hybridization. Similarly, sst3 transcripts were detected in each of 46 breast carcinomas by Vikic-Topic et al. (16) using RT-PCR. The apparent discrepancies between these findings and our results may be explained in two ways: (a) with RT-PCR, one cannot distinguish between somatostatin receptor transcripts in malignant and non-malignant cells (in our analysis fibrocytes and lymphocytes were frequently sst2-positive); (b) somatostatin receptor expression in breast cancer cells may also be regulated by factors beyond transcriptional control.

The 33 primary breast cancers that we examined immunocytochemically differed greatly in their level of sst2 expression; for example, 3 (9%) of the tumors showed strong staining, 11 (33%) showed moderate staining, 14 (43%) showed weak staining, and 5 (15%) showed no staining. The clinical utility of octreotide depends on the number of sst2 receptors on the tumor cells (tumors with high sst2 levels would be expected to specifically retain $[^{111}]$In-DTPA-D-Phe$^1$-octreotide and to respond to octreotide therapy). It is thus conceivable that the immunocytochemical de-
tection of sst₂ may provide predictive information for octreotide therapy and scintigraphy. Octreotide has also been shown to strongly enhance the antineoplastic effects of tamoxifen in a rat mammary tumor model (8). However, among the 33 untreated breast cancers that we analyzed, there were only 9 cases with an estrogen receptor immunoreactivity score of 3 or more (precondition for tamoxifen treatment) and detectable immunostaining for sst. It would be interesting to see what changes in the estrogen and/or somatostatin receptor status might occur after combined treatment with tamoxifen and octreotide.

Generating antibodies against the COOH-terminal domains of ssts also enabled us to distinguish between sst₂A and sst₂B. Although there was a significant correlation between sst₂A-Li and sst₂B-Li, our data demonstrate that some tumors expressed either sst₂A or sst₂B independently. Overall, sst₂B was more frequently detected than sst₂A. These differences may hold important clues for the responsiveness of some breast cancers to octreotide. sst₂A and sst₂B bind SS-14 with similar high affinity but differ in certain aspects of the intracellular signaling: e.g., sst₂B is more resistant to agonist-induced desensitization of coupling to adenylate cyclase (11).

sst₁ and sst₃ were sporadically expressed in breast cancers. Although the precise role of these ssts for octreotide response remains unclear, it is important to establish patterns of sst expression. With the development of novel agonists/antagonists, it may be possible to select one or more somatostatin receptor ligands for optimal therapeutic effect.

In conclusion, we have generated anti-sst₁, anti-sst₂A, anti-sst₂B, and anti-sst₃ antibodies. Using these antibodies, we have provided the first demonstration of ssts in formalin-fixed, paraffin-embedded human tissues. It is now possible to carry out large retrospective studies of somatostatin receptor protein expression in a variety of human tumors. It is hoped that future clinical trials will correlate receptor pattern and response to somatostatin analogues to determine the predictive value of immunocytochemical determination of somatostatin receptor status in primary breast cancer.

Acknowledgments

The authors thank M. Albrecht for skillful technical assistance and Dr. S. Kropf for help with statistical analysis.

References

Immunocytochemical detection of somatostatin receptors sst1, sst2A, sst2B, and sst3 in paraffin-embedded breast cancer tissue using subtype-specific antibodies.

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