High Expression of Neuropeptide Y1 Receptors in Ewing Sarcoma Tumors

Meike Körner, Beatrice Waser, and Jean Claude Reubi

Abstract

Purpose: Peptide receptors are frequently overexpressed in human tumors, allowing receptor-targeted scintigraphic imaging and therapy with radiolabeled peptide analogues. Neuropeptide Y (NPY) receptors are new candidates for these applications, based on their high expression in specific cancers. Because NPY receptors are expressed in selected sarcoma cell lines and because novel treatment options are needed for sarcomas, this study assessed the NPY receptor in primary human sarcomas.

Experimental Design: Tumor tissues of 88 cases, including Ewing sarcoma family of tumors (ESFT), synovial sarcomas, osteosarcomas, chondrosarcomas, liposarcomas, angiosarcomas, rhabdomyosarcomas, leiomyosarcomas, and desmoid tumors, were investigated for NPY receptor protein with in vitro receptor autoradiography using 125I-labeled NPY receptor ligands and for NPY receptor mRNA expression with in situ hybridization.

Results: ESFT expressed the NPY receptor subtype Y1 on tumor cells in remarkably high incidence (84%) and density (mean, 5,314 dpm/mg tissue). Likewise, synovial sarcomas expressed Y1 on tumor cells in high density (mean, 7,497 dpm/mg; incidence, 40%). The remaining tumors expressed NPY receptor subtypes Y1 or Y2 at lower levels. Moreover, many of the sarcomas showed Y1 expression on intratumoral blood vessels. In situ hybridization for Y1 mRNA confirmed the autoradiography results.

Conclusions: NPY receptors are novel molecular markers for human sarcomas. Y1 may inhibit growth of specific sarcomas, as previously shown in an in vivo mouse model of human ESFT. The high Y1 expression on tumor cells of ESFT and synovial sarcomas and on blood vessels in many other sarcomas represents an attractive basis for an in vivo tumor targeting.
Materials and Methods

Tissues. The NPY receptor expression was studied in fresh-frozen tumor tissue samples obtained from surgical resection specimens. Eighty-eight cases were investigated, including 19 ESFT, 5 synovial sarcomas, 9 osteosarcomas, 6 chondrosarcomas, 4 angiosarcomas, 9 rhabdomyosarcomas, 7 leiomyomas, 10 leiomyosarcomas, 13 liposarcomas, and 6 desmoid tumors. The tested tumors originated either from samples investigated previously for other receptors (19–21) collected in accordance with the required international ethical guidelines or from samples collected prospectively at the Institute of Pathology, University of Berne, in agreement with the principles of the Helsinki Declaration, including informed consent and approval by the Institutional Review Board.

In vitro NPY receptor autoradiography. The NPY receptor expression in the tumor tissues was assessed on the basis of specific \textsuperscript{125}I-PYY binding to NPY receptor protein, as described before (4, 7). Briefly, tissue sections mounted on glass slides were incubated with \textsuperscript{125}I-PYY (2,000 Ci/mmol; Anawa). To assess nonspecific binding, parallel tissue sections were incubated with \textsuperscript{125}I-PYY and 25 nmol/L of nonlabeled PYY, which at this concentration completely displaces \textsuperscript{125}I-PYY at the NPY receptors. To differentiate NPY receptor subtypes, pharmacologic competition experiments were done with NPY receptor subtype–selective ligands. Serial tissue sections were incubated with \textsuperscript{125}I-PYY, which is bound by all NPY receptor subtypes, and increasing concentrations of one of the following NPY receptor subtype–selective cold ligands: the Y1-selective ligands \textsuperscript{[Leu31, Pro34]}-PYY (Bachem) or BIIB 3226 (Boehringer Ingelheim), the Y2-selective ligands PYY(3-36) (Bachem) or BIIE 0246 (Boehringer Ingelheim), and the Y4-prefering ligand PP (Bachem) or the Y5-selective ligand \textsuperscript{[cPP(1-27), PYY(19-23)]} (Promochem) were used. In each case, a hybridization signal was considered specific only if in a parallel tissue section the hybridization of the radiolabeled probe was completely blocked by 20-fold excess of unlabeled probe. Furthermore, in all experiments, Y1-expressing breast carcinomas were used as positive control (4), and Y2-expressing tumors as negative control.

Statistical analysis. The Fisher’s exact test was used for comparison of NPY receptor frequencies, and the Student’s \(t\) test for comparison of NPY receptor densities between different sarcoma types. A \(P\) value of <0.05 was considered to be statistically significant.

Image acquisition. Photographs were acquired under a Zeiss stereomicroscope with a Nikon Coolpix 990 camera and processed with the Corel Photopaint software.

Results

NPY receptor binding sites on tumor cells of soft tissue and bone tumors. Using in vitro NPY receptor autoradiography, NPY receptors were found to be variably expressed on the tumor cells of sarcomas, as summarized in the table and shown for the individual cases in Fig. 1. ESFT showed a strikingly high NPY receptor expression, with a receptor incidence of 84% in vivo and receptor density values often above 4,000 dpm/mg tissue, corresponding to high receptor numbers in the tumor tissues. Furthermore, synovial sarcomas were remarkable because the receptor positive cases displayed also very high NPY receptor densities. Regarding NPY receptor subtypes, all ESFT and synovial sarcomas expressed solely Y1, based on the complete displacement of \textsuperscript{125}I-PYY binding by Y1-selective ligands.
Table 1. NPY receptor expression in soft tissue and bone tumor cells: incidence, mean density, and receptor subtypes

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>NPY receptor incidence: positive/total cases (%)</th>
<th>NPY receptor density: mean ± SEM of receptor positive cases (dpm/mg tissue)</th>
<th>NPY receptor subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESFT</td>
<td>16/19 (84%)</td>
<td>5,314 ± 727</td>
<td>Y1</td>
</tr>
<tr>
<td>Synovial sarcomas</td>
<td>2/5 (40%)</td>
<td>7,497 ± 751</td>
<td>Y1</td>
</tr>
<tr>
<td>Osteosarcomas</td>
<td>4/9 (44%)</td>
<td>1,079 ± 393</td>
<td>Y1 + Y2</td>
</tr>
<tr>
<td>Chondrosarcomas</td>
<td>1/6 (17%)</td>
<td>2,210</td>
<td>Y2</td>
</tr>
<tr>
<td>Angiosarcomas</td>
<td>2/4 (50%)</td>
<td>1,185 ± 783</td>
<td>Y1</td>
</tr>
<tr>
<td>Rhabdomyosarcomas</td>
<td>3/10 (30%)</td>
<td>1,029 ± 319</td>
<td>Y2</td>
</tr>
<tr>
<td>Leiomyomas</td>
<td>2/7 (29%)</td>
<td>1,128 ± 252</td>
<td>Y1</td>
</tr>
<tr>
<td>Leiomyosarcomas</td>
<td>2/10 (20%)</td>
<td>499 ± 76</td>
<td>Y1</td>
</tr>
<tr>
<td>Liposarcomas</td>
<td>3/13 (23%)</td>
<td>766 ± 278</td>
<td>Y1 + Y2</td>
</tr>
<tr>
<td>Desmoid tumors</td>
<td>1/6 (17%)</td>
<td>1,346</td>
<td>Y2</td>
</tr>
<tr>
<td>Breast carcinomas</td>
<td>76/89 (85%)</td>
<td>4,946 ± 485</td>
<td>Y1</td>
</tr>
</tbody>
</table>

NOTE: For the purpose of comparison, NPY receptor expression data for breast carcinomas are included. The tumor types with an extraordinarily high NPY receptor expression are in bold.

*Data from ref. 4.

The remaining tumors showed a considerably lower NPY receptor expression on the tumor cells compared with ESFT and synovial sarcomas: The NPY receptor incidences were moderate in osteosarcomas and angiosarcomas (however, statistically not significantly lower than in ESFT: $P = 0.068$ for osteosarcomas and $P = 0.194$ for angiosarcomas) and low in the other tumors (statistically significantly lower than in ESFT: $P = 0.006$ for chondrosarcomas, $P = 0.012$ for rhabdomyosarcomas, $P = 0.002$ for leiomyosarcomas, $P = 0.001$ for liposarcomas, and $P = 0.006$ for desmoid tumors). The NPY receptor density values ranged between moderate and low (Table 1; Fig. 1A). The NPY receptor density was statistically significantly lower in osteosarcomas ($P = 0.008$), chondrosarcomas ($P = 0.007$), leiomyosarcomas ($P = 0.029$), liposarcomas ($P = 0.029$), and desmoids tumors ($P = 0.006$) than in ESFT. As for the NPY receptor subtype expression, angiomatous and leiomyomatous tumors expressed only Y1, as seen with complete displacement of $^{125}$I-PYY binding by Y1-selective ligands, whereas rhabdomyosarcomas, chondrosarcomas, and desmoid tumors expressed only Y2, based on complete displacement of $^{125}$I-PYY binding by Y2-selective ligands; osteosarcomas and liposarcomas expressed either Y1 or Y2.

These autoradiography results are illustrated in Fig. 2 with representative examples. The ESFT in the left column and the synovial sarcoma in the middle column express Y1 receptors in very high numbers in the entire tumor samples, as seen from the very strong $^{125}$I-PYY binding to the tumor tissues and complete displacement of $^{125}$I-PYY binding by the Y1-selective ligand. In comparison, the rhabdomyosarcoma in the right column expresses Y2 receptors in smaller amounts, based on the weaker $^{125}$I-PYY binding and complete displacement of $^{125}$I-PYY binding by the Y2-selective ligand.

NPY receptor binding sites in blood vessels of soft tissue and bone tumors. Y1 receptors were also identified in intratumoral small arteries. This observation was particularly often made in liposarcomas, desmoid tumors, and leiomyomas, i.e., in a subset of tumors with low NPY receptor expression on the tumor cells. In tumors with high density receptor expression on tumor cells, such as ESFT, we may consider that not all NPY receptor expressing blood vessels can be identified as they may be masked by the high Y1 levels on the surrounding tumor cells.

Typical examples of the vascular Y1 expression in tumors are given in Fig. 3. The rhabdomyosarcoma in the left column shows many small arteries expressing Y1 in moderate density. In the leiomyosarcoma in the right column, higher magnification reveals that the Y1 receptors are localized in the smooth muscle layer of the vascular wall.

Receptor subtype characterization in pharmacologic competition experiments with receptor subtype-selective ligands. To distinguish the NPY receptor subtypes in the tumor tissues, displacement experiments were done using $^{125}$I-PYY in competition with increasing concentrations of cold NPY receptor subtype-selective ligands. In Y1 expressing tumors, $^{125}$I-PYY was displaced completely in the nanomolar concentration range by the Y1-selective ligands [Leu$^{31}$, Pro$^{34}$]-PYY or BIBP 3226 but not or only at micromolar concentrations by the Y2-selective ligand BIIE 0246 or the Y4-preferring ligand PP. Conversely, in Y2-expressing tumors, $^{125}$I-PYY was displaced with high affinity and completely with the Y2-selective ligands PYY(3-36) or BIIE 0246 but with low affinity by [Leu$^{31}$, Pro$^{34}$]-PYY, BIBP 3226, or PP. Furthermore, the Y5-selective ligand [cPP(1-7)+ PYY(19-23), Ala$^{31}$, Alb$^{32}$, Pro$^{34}$]-hPP (22) was totally inactive at the tumoral receptors. Figure 4 shows typical examples of displacement curves, namely for a Y1-expressing ESFT, a Y1-expressing synovial sarcoma, and a Y2-expressing liposarcoma. We obtained the same rank orders of potencies of NPY receptor subtype–selective ligands in the control cells and tissues, i.e., in the Y1-expressing SK-N-MC cell line, Y1-expressing rat cortex, and Y2-expressing rat hippocampus (4, 12, 14, 23). These results provide strong evidence of specific identification of Y1 or Y2 on tumor cells and of Y1 on tumoral blood vessels, and rule out the presence of substantial amounts of Y4 or Y5.

Tumoral Y1 receptor transcripts. The tumoral Y1 expression was further confirmed with in situ hybridization of Y1 mRNA. In most tumors with Y1 binding sites in the autoradiography experiments, Y1 transcripts were detected. In general, the tumors with a high density of Y1 binding sites also exhibited...
high Y1 mRNA levels, as shown with a representative example in Fig. 5, whereas the tumors with a low density of Y1 binding sites had little or no Y1 transcripts detectable. No Y1 transcripts were present in tumors with Y2 binding by autoradiography.

**Discussion**

The present study investigates for the first time the expression of NPY receptors, a recently recognized tumor marker (4, 5), in a large number of primary human sarcomas. Particular

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**Fig. 2. In vitro NPY receptor autoradiography on serial tissue sections to assess $^{125}$I-PYY binding to sarcomas. A, F, and L, H&E-stained tissue sections showing an ESFT (A), a synovial sarcoma (F), and a rhabdomyosarcoma (L). Bars, 1 mm. Insets, tumor cells at high magnification. B, G, and M, autoradiograms showing total binding of $^{125}$I-PYY to the tumors. Very strong and homogeneous binding to the ESFT (B) and synovial sarcoma (G) but heterogeneous binding to the rhabdomyosarcoma revealing areas of high receptor density (arrows) and lower receptor density in the remaining tumor tissue (M). C, H, and N, autoradiograms showing nonspecific $^{125}$I-PYY binding in the presence of 25 nmol/L cold PYY. Cold PYY completely displaces $^{125}$I-PYY binding in all three tumors. D, I, and O, autoradiograms showing $^{125}$I-PYY binding in the presence of 25 nmol/L of the cold Y1-selective ligand [Leu$^{31}$, Pro$^{34}$]-PYY. Complete displacement of $^{125}$I-PYY binding in the ESFT (D) and the synovial sarcoma (I) provides proof of Y1 expression. Conversely, no displacement in the rhabdomyosarcoma rules out significant amounts of Y1 in this tumor (O). E, K, and P, autoradiograms showing $^{125}$I-PYY binding in the presence of 25 nmol/L of the cold Y2-selective ligand PYY(3-36). No displacement in the Y1 expressing ESFT (E) and Y1 expressing synovial sarcoma (K). Complete displacement in the rhabdomyosarcoma (P) provides evidence of Y2 expression in this tumor.
attention is given to the quantitative expression of receptor binding sites in tumor tissues, which represents an important basis for future investigations directed toward clinical applications. ESFT and synovial sarcomas are found to exhibit a strikingly high Y1 expression on the tumor cells. ESFT express NPY receptors in very high incidence, and both tumor types display impressively high Y1 receptor densities, which belong to the highest NPY receptor densities ever found in neoplasia, paralleling those in breast cancer (Table 1; ref. 4). In comparison, the remaining investigated sarcomas, including osteosarcomas, chondrosarcomas, angiosarcomas, rhabdomyosarcomas, and leiomyosarcomas, as well as gastrointestinal stromal tumors assessed previously for NPY receptors (24), show moderate to low expression levels of Y1 and Y2, indicating a high heterogeneity of NPY receptor expression among the various sarcoma types. Interestingly, a prominent vascular Y1 expression is observed in many of the sarcomas, in particular those with a low receptor density on tumor cells, such as liposarcomas, desmoid tumors, and leiomyomas.

Several lines of evidence of specific NPY receptor subtype identification are provided in the study. The rank orders of potencies of NPY receptor subtype-selective ligands obtained in the autoradiographic competition experiments, namely high-affinity binding of Y1- or Y2-selective ligands and low-affinity or no binding of Y4- and Y5-selective ligands to the tumor tissues, provide pharmacologic proof of Y1 and Y2 expression and rule out the presence of substantial amounts of Y4 or Y5. Moreover, the NPY receptor binding studies in original ESFT tissues in the present study yield the same results as those done previously in the ESFT-derived cell line SK-N-MC, namely exclusive Y1 expression (13, 14). In addition, expression of Y1, the most abundant NPY receptor subtype in this series, is substantiated at the transcription level with in situ hybridization.

In various sarcoma cell lines, NPY receptors were shown to be functional. Intracellular signaling was specifically activated upon NPY binding in ESFT and osteosarcoma cell lines (12, 25, 26). Moreover, NPY receptor activation was found to inhibit SK-N-MC cell proliferation in vitro by induction of apoptosis (4, 27). Importantly, this effect could also be reproduced in vitro in SK-N-MC cell–derived tumors xenografted into nude mice in the presence of abundant NPY (27). Furthermore, NPY was shown to act on the vascular system of ESFT. It significantly stimulated tumoral angiogenesis in an in vivo mouse model of a human ESFT cell line (27). Conversely, NPY may hypothetically also have an inhibitory effect on sarcoma perfusion. Activation of Y1 receptors expressed on the smooth muscle cells of intratumoral small arteries could result in vasoconstriction, as known from the human skeletal muscle (28). At present, however, it is unclear to what extent such cell line data may be extrapolated to human tumor biology.

Sarcomas with a high NPY receptor expression are candidates for a potential in vivo NPY receptor targeting analogous to the somatostatin receptor targeting of gut endocrine tumors (2, 3). Specifically, NPY analogues coupled with a chemotherapeutic agent or a radioisotope could be used for a targeted therapy or scintigraphy of sarcomas. Such NPY analogues are currently being developed by the Beck-Sickinger group, for instance an NPY compound coupled with daunorubicin suitable for therapy or an NPY compound coupled with radioactive

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**Fig. 3.** *In vitro* NPY receptor autoradiography to assess $^{125}$I-PYY binding to intratumoral blood vessels. A and D, H&E-stained tissue sections showing numerous intratumoral small arteries (arrows) in a rhabdomyosarcoma (A) and at higher magnification the muscular layer of an arterial wall (arrows) surrounded by tumor cells (asterisks) in a leiomyosarcoma (D). Bars, 0.1 mm. B and E, autoradiograms showing total binding of $^{125}$I-PYY. In both examples, labeling of the blood vessel walls, no labeling of the surrounding tumor cells; in E, high magnification reveals that $^{125}$I-PYY binding is localized in the smooth muscle layer of the vascular wall. C and F, autoradiogram showing $^{125}$I-PYY binding in the presence of 25 nmol/L of the cold Y1-selective ligand [Leu$^{31}$, Pro$^{34}$]-PYY. Complete displacement of $^{125}$I-PYY binding to the blood vessels is consistent with Y1 expression.
First-choice tumors for such applications would be ESFT and synovial sarcomas, as these tumors frequently express Y1 in high density on the tumor cells. Indeed, high density of receptors has been considered as one prerequisite for the success of somatostatin receptor targeting, where only tumors with high receptor density levels were showing significant clinical responses (31). Furthermore, sarcomas with an abundant Y1 expression on tumoral blood vessels may also be candidates for therapeutic applications. In these various tumors, NPY analogues coupled with a radioactive isotope may not only induce radionecrosis of the targeted tumor cells but possibly also of intratumoral small arteries. In the future, even tumors with a lower density of NPY receptors may become an indication for targeted radiotherapy. Indeed, the recent observation that somatostatin receptor antagonists show in vivo a considerably stronger and longer-lasting binding to tumors than receptor

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**Fig. 4.** Pharmacologic displacement experiments in a Y1 expressing ESFT (A), a Y1 expressing synovial sarcoma (B), and a Y2 expressing liposarcoma (C). A and B, in the ESFT and synovial sarcoma, high-affinity displacement of $^{125}$I-PYY by cold PYY (○) or the Y1-selective ligands [Leu$^{31}$, Pro$^{34}$]-PYY (■) or BIBP 3226 (♦), but low-affinity displacement by the Y2-selective ligand BIE 0246 (▲) or the Y4-prefering PP (●) provide strong pharmacologic evidence of Y1 expression. C, conversely, in the liposarcoma, high-affinity displacement of $^{125}$I-PYY by PYY (○) or the Y2-selective ligands PYY(3-36; ▲) or BIE 0246 (▼), but low-affinity displacement by [Leu$^{31}$, Pro$^{34}$]-PYY (■), BIBP 3226 (♦), or PP (●), provide proof of Y2 expression.

**Fig. 5.** Y1 expression in an ESFT detected with in situ hybridization at the mRNA level (left) and with in vitro receptor autoradiography at the protein level (right). A, H&E-stained tissue section showing the tumor tissue. Bar, 1 mm. B, autoradiogram showing Y1 mRNA in moderate to high amounts in the tumor tissue. C, nonspecific labeling in the presence of 20-fold excess of the corresponding cold probe is negligible. D, autoradiogram showing total binding of $^{125}$I-PYY to the tumor in high density. E, complete displacement of $^{125}$I-PYY by the cold Y1-selective ligand [Leu$^{31}$, Pro$^{34}$]-PYY provides proof of expression of Y1 binding sites. F, no displacement of $^{125}$I-PYY by the cold Y2-selective ligand BIE 0246 rules out the presence of Y2 binding sites.
agonists (32), has suggested that somatostatin receptor targeting with antagonists may be expanded to lower receptor density tumors. The same may apply to the NPY receptor system. NPY receptor–targeted therapies of sarcomas could be particularly useful in the setting of sarcoma recurrence, which remains, at present, an important therapeutic problem (17, 18). NPY receptor–targeted scintigraphy might be helpful for imaging of the primary tumor site and the search for metastases in the preoperative diagnostic work-up and follow-up of patients.

Various molecular targets have been identified in sarcomas, however, often only at low levels. Peptide receptors such as neurotensin, vasoactive intestinal peptide, and somatostatin receptors are also expressed in ESFT and synovial sarcomas but in much smaller amounts than NPY receptors (19, 20). Likewise, c-kit positivity was reported to be strong in only 29% of ESFT (33). Furthermore, epidermal growth factor receptor and HER-2/neu were expressed at only low levels in about half of synovial sarcomas (34). Therefore, NPY receptors may belong to the most promising candidates for an in vivo targeting of ESFT and synovial sarcomas identified to date, due to their high expression levels in these tumors with compared other molecular tumor markers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
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References
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