Peripheral-Type Benzodiazepine Receptor (PBR) in Human Breast Cancer: Correlation of Breast Cancer Cell Aggressive Phenotype with PBR Expression, Nuclear Localization, and PBR-mediated Cell Proliferation and Nuclear Transport of Cholesterol

Matthew Hardwick, Djamil Fertikh, Martine Culty, Hua Li, Branislav Vidic, and Vassilios Papadopoulos

Departments of Cell Biology [M. H., D. F., M. C., H. L., B. V., V. P.] and Pharmacology [V. P.], and the Lombardi Cancer Center [M. C., V. P.], Georgetown University Medical Center, Washington, DC 20007

ABSTRACT

Aberrant cell proliferation and increased invasive and metastatic behavior are hallmarks of the advancement of breast cancer. Numerous studies implicate a role for cholesterol in the mechanisms underlying cell proliferation and cancer progression. The peripheral-type benzodiazepine receptor (PBR) is an Mr 18,000 protein primarily localized to the mitochondria. PBR mediates cholesterol transport across the mitochondrial membranes in steroidogenic cells. A role for PBR in the regulation of tumor cell proliferation has also been shown. In this study, we examined the expression, characteristics, localization, and function of PBR in a battery of human breast cancer cell lines differing in their invasive and chemotactic potential as well as in several human tissue biopsies. Expression of PBR ligand binding and mRNA was dramatically increased in the highly aggressive cell lines, such as MDA-231, relative to nonaggressive cell lines, such as MCF-7. PBR was also found to be expressed at high levels in aggressive metastatic human breast tumor biopsies compared with normal breast tissues. Subcellular localization with both antibodies and a fluorescent PBR drug ligand revealed that PBR from the MDA-231 cell line as well as from aggressive metastatic human breast tumor biopsies localized primarily in and around the nucleus. This localization is in direct contrast to the largely cytoplasmic localization seen in MCF-7 cells, normal breast tissue, and to the typical mitochondrial localization seen in mouse tumor Leydig cells. Pharmacological characterization of the receptor and partial nucleotide sequencing of PBR cDNA revealed that the MDA-231 PBR is similar, although not identical, to previously described PBR. Addition of high affinity PBR drug ligands to MDA-231 cells increased the incorporation of bromodeoxyuridine into the cells in a dose-dependent manner, suggesting a role for PBR in the regulation of MDA-231 cell proliferation. Cholesterol uptake into isolated MDA-231 nuclei was found to be 30% greater than into MCF-7 nuclei. High-affinity PBR drug ligands regulated the levels of cholesterol present in MDA-231 nuclei but not in MCF-7. In addition, the PBR-dependent MDA-231 cell proliferation was found to highly correlate (r = −0.99) with the PBR-mediated changes in nuclear membrane cholesterol levels. In conclusion, these data suggest that PBR expression, nuclear localization, and PBR-mediated cholesterol transport into the nucleus are involved in human breast cancer cell proliferation and aggressive phenotype expression, thus participating in the advancement of the disease.

INTRODUCTION

The PBR4 was originally discovered as an alternative binding site for the benzodiazepine diazepam (Valium; Ref. 1). PBR is a multimeric receptor composed of the Mr 18,000 receptor protein and the Mr 34,000 voltage-dependent anion channel protein. The Mr 18,000 subunit is thought to be responsible for the binding of isoxquinolines, whereas both subunits are required for the binding of benzodiazepines (2). Expression of PBR has been found in every tissue examined; however, it is most abundant in steroid-producing tissues such as the adrenals, testes, ovaries, placenta, and brain, where it is primarily localized in the outer mitochondrial membrane (3). In steroid-producing tissues, PBR is known to regulate the transport of cholesterol from the outer to the inner mitochondrial membrane, the rate-determining step in steroid biosynthesis (4). Although the primary function of PBR is the regulation of steroidogenesis, its existence in nonsteroidogenic tissues as well as in other cellular compartments including the plasma membrane (3) suggests that there may be other roles for PBR.

In the early 1980s it was shown that diazepam (Valium), a ligand for both the central (GABA_A) and PBR receptors, induced murine Friend erythroleukaemia (MEL) cell differentiation and inhibited 3T3 cell proliferation at micromolar concentrations (5). A series of 15 benzodiazepines, including diazepam, also inhibited thymoma cell proliferation at micromolar concentrations (6) and further suggested that this effect was mediated by PBR rather than the GABA_A receptor. Stimulation of cell proliferation was shown to occur when glioma cells were incubated with nanomolar concentrations of Ro-4864 and PK 11195, both high-affinity PBR ligands (7). In this same study, it was shown that Ro-4864 and PK 11195 inhibited glioma cell proliferation at micromolar concentrations. Similar results were obtained in testicular Leydig tumor cells (8). The effect of PK 11195, an exclusive ligand of PBR, provided unequivocal evidence that the effects seen were mediated exclusively by PBR. Inhibition of cell proliferation by micromolar concentrations of Ro-4864 and PK 11195 have also been reported in astrocytes and V79 Chinese Hamster lung cells (9, 10). In addition, micromolar concentrations of PBR ligands have also been shown to inhibit growth factor-induced cell proliferation in both astrocytes and lymphoma cells (11, 12). Considering the abundance of PBR in tumors (6, 7, 9–12) and the proposed correlation between PBR expression and human brain tumor aggression (13, 14), we examined PBR expression and, if present, its function in breast cancer.

Breast cancer is the most common neoplasm and the leading cause of cancer-related deaths for women in most developing countries (15). The American Cancer Society estimates that in 1998 nearly 180,000 new cases of invasive breast cancer will be diagnosed among women, with over 43,000 deaths, in the United States alone. Two fundamental...
questions in breast cancer research must be answered: (a) what are the changes in cellular and molecular functions that account for the development and progression of breast cancer? and (b) how can investigators use what is known about the genetic and cellular changes in breast cancer patients to improve detection, diagnosis, prevention, treatment and follow-up care?

Tumor progression is a multistep process by which normal cells gradually acquire more malignant phenotypes, including the ability to invade tissues and form distal metastases, the primary cause of mortality in breast cancer. During this process, the “aberrant” expression of a number of gene products may be the cause or the result of tumorigenesis. Considering that the first step of tumor progression is cell proliferation, a fundamental biological phenomenon common to normal and “abnormal” development, it can be proposed that tumorigenesis and malignancy are related to the proliferative potential of tumoral cells.

In this report, we tested the hypothesis that the PBR is: (a) part of the changes in cellular and molecular functions that account for the development and progression of breast cancer; and (b) could be used in the future as a tool to improve detection, diagnosis, prevention, and treatment in breast cancer patients. Using a battery of well-characterized breast cancer cell lines differing in their invasive and metastatic abilities (16, 17), we demonstrate that expression of PBR correlates with the expression of breast cancer cell aggressive phenotype. We confirmed our findings in human biopsies from normal breast tissue and aggressive metastatic breast tumors. In agreement with the well-documented function of PBR in steroid synthesizing tissues, control of cholesterol transport into mitochondria (4), we identified that PBR in the mitotic breast cancer cells regulates cell proliferation and cholesterol transport into the nucleus. Statistical analysis reveals that these two functions are highly correlated in aggressive breast tumor cells.

MATERIALS AND METHODS

Cell Culture. Human breast cancer cell lines (BT549, HS-578-T, MCF-7, ADR, MDA-231, MDA-435, MDA-468, T47D, and ZR-75-1) were obtained from the Lombardi Cancer Center, Georgetown University Medical Center. MA-10 mouse Leydig tumor cells were a gift from Dr. Mario Ascoli (University of Iowa) and were maintained in Waymouth’s MB752/1 medium supplemented with 15% horse serum as described previously (18). All cell lines were cultured on polystyrene culture dishes (Corning, Corning, NY) and grown in DMEM supplemented with 10% FBS.

Radioligand Binding Assays. Cells were scraped from 150-mm culture dishes into 5 ml PBS, dispersed by trituration, and centrifuged at 500 × g for 15 min. Cell pellets were resuspended in PBS and assayed for protein concentration. [3H]PK 11195 binding studies on 50 μg of protein from cell suspensions were performed as described previously (2). Scatchard plots were analyzed by the LIGAND program (19). Specific binding of [3H]PK 11195 suspensions were performed as described previously (2). A 0.2-μg hPBR cDNA fragment (derived from the pCMV5-PBR plasmid vector containing the full-length hPBR kindly given by Dr. Jerome Strauss, University of Pennsylvania, Philadelphia, PA) was radio-labeled with [α-32P]dCTP using a random primers DNA labeling system (Life Technologies, Inc., Gaithersburg, MD). The filter was first prehybridized overnight at 68°C in 6× SSC, 0.5% SDS, and 100 μg/ml denatured, fragmented, salmon sperm DNA. After hybridization, the membrane was washed twice with 2× SSC, 0.5% SDS for 10 min, once with 0.2× SSC, 0.5% SDS for 30–60 min at room temperature, and once with 0.2× SSC, 0.5% SDS for 30 min at 60°C. Autoradiography was performed by exposing the blots to X-Omat AR film (Kodak, Rochester, NY) at −70°C for 4–48 h. Quantification of PBR mRNA was carried out using the SigmaGel software (Jandel Scientific, San Rafael, CA).

Partial cDNA Sequencing. PBR cDNAs were prepared from total MDA-231 and MCF-7 RNA using the Perkin-Elmer RT-PCR kit (Branchburg, NJ). PCR was performed on cDNAs using primers designed from the known human sequence (22). Labeling of PCR products was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). Labeled PCR products were sequenced by the Lombardi Sequencing Core Facility (Georgetown University Medical Center, Washington, DC).

Fluorescent Microscopy with the Fluorescent PBR Ligand Compound 4. MA-10, MDA-231, MCF-7, and ADR cells were grown on glass coverslips as described previously (23). Cells were then washed twice with sterile PBS and incubated for 45 min with 1 μm compound 4, a fluorescent derivative of the PBR ligand FGIN-27, with or without a competing PBR ligand, FGIN-27, at a concentration of 100 μM. After the incubation period, the cells were washed with PBS and examined by fluorescent microscopy using an Olympus BH-2 fluorescence microscope.

Immunocytochemistry of MDA-231 Cells. MDA-231 cells were cultured overnight on eight-chambered SuperCell Culture Slides (Fisher Scientific, Pittsburgh, PA) at a concentration of ~50,000 cells/chamber. Cells were then fixed in 70% ethanol for 15 min at 4°C. After washing three times in distilled H2O, for 2 min each, the fixed cells were incubated overnight at 4°C with either anti-PBR (24) or anti-DBI, a generous gift from Dr. A. S. Brown (Prince of Wales Hospital, Randwick, Australia); polyclonal antisera concentrations were 1:100, 1:200, 1:500, or 1:1000. The slides were washed three times in PBS for 2 min each. Slides were then incubated at room temperature for 1 h with HRP-coupled goat anti-rabbit secondary antibody diluted 1:2000 in PBS supplemented with 10% calf serum. After washing the slides three times in PBS for 2 min each, fresh H2O2 diluted 1:1000 with 3-aminio-9-ethyl carbazole was added, and the slides were incubated for 1 h at 37°C. The slides were then rinsed in distilled H2O before mounting with CrystalMount.

Nuclear Transport of [3H]Cholesterol. Nuclei were isolated from MDA-231 and MCF-7 as described by Elango et al. (25). Isolated nuclei were resuspended in 1 ml of ice-cold PBS. [3H]Cholesterol uptake in MDA-231 and MCF-7 nuclei was examined in the presence of 6.7 nM [3H]cholesterol (50.0 Ci/mmol) and 3 μg of nuclear protein for 60 min at 37°C (26). Samples were then centrifuged at 500 × g for 30 min, and pellets were washed in 500 μl of ice-cold PBS. After a second centrifugation at 500 × g for 30 min, 200 μl of 1.0 N NaOH was then added to the pellets and incubated overnight at 37°C. After incubation, 200 μl of 1.0 N HCl was added, and samples were mixed vigorously. Three ml of scintillation cocktail were then added, and radioactivity was measured by liquid scintillation spectrometry using a Wallac 1409 Liquid Scintillation Counter.

Brdu/Proliferation Assays. MDA-231 cells were plated on 96-well plates at a concentration of ~10,000 cells/well (24-h incubation) or 5,000 cells/well (48-h incubation) in DMEM supplemented with 0.1% FBS. The cells were then incubated in either 0.1% or 0.01% PBS with various concentrations of PK 11195 for either 24 or 48 h. Differences in cell proliferation were analyzed by measuring the amount of Brdu incorporation determined by the Brdu/ELISA kit (Boehringer Mannheim, Indianapolis, IN).
**RESULTS**

Increased Expression of the PBR Correlates with Increased Aggressive Phenotype in Human Breast Cancer Cell Lines. Initial studies performed in MCF-7 human breast cancer cells indicated extremely limited expression of PBR (Fig. 1). The data generated were insufficient for further Scatchard analysis. Before dismissing our original hypothesis, we decided to examine expression of the receptor in eight other human breast cancer cell lines using the PBR-specific, high-affinity ligand PK 11195. The results from these experiments indicated that those cell lines with a more invasive and chemotactic potential, such as ZR-75-1, T47D, and MCF-7 (Fig. 1). Furthermore, the MCF-7 Adriamycin-resistant derivative cell line, ADR, which expresses medium invasive and chemotactic potential as well as intermediate levels of CD44, was the most invasive cell line (Table 1). Scatchard analysis of PBR binding in the MDA-231 and ADR cell lines further shows that a high level of expression is required for significant binding to be detected. In contrast, the MCF-7 cell line, which expresses medium invasive and chemotactic potential as well as intermediate levels of CD44, was the most invasive cell line (Table 1).

**Table 1** Comparison of invasive characteristics of human breast cancer cell lines to PBR expression

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Estrogen receptor</th>
<th>Vimentin</th>
<th>Invasion</th>
<th>Chemotaxis</th>
<th>CD44</th>
<th>PBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZR-75-1</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T47D</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MCF-7</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MDA-435</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ADR</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BS549</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MDA-468</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HS-578-T</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MDA-231</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

The various characteristics of the human breast cancer cell lines described above are from Azam et al. (16) and Culity et al. (17). The invasive and chemotaxis assays were performed by quantifying the migration of cells in Boyden chamber assays using fibroblast-conditioned medium as the chemotactic target. Chemoinvasion was studied using polycarbonate filters coated with a uniform layer of Matrigel constituting a barrier that the cells had to degrade to reach the filters and migrate through them. To determine the chemotactic behavior of the cells, the filters were coated with a thin layer of collagen IV that promotes cell attachment and allows the free migration of the cells toward the gradient of fibroblast-conditioned medium. The relative amounts of PBR were determined with binding assays in which increasing concentrations of cellular protein were incubated with a constant level of [3H]PK 11195 (6 nM). Non-specific binding was determined in the presence of cold PK 11195. CD44 levels are relative among the cell lines listed.

**Fig. 1**. The specific PBR binding characteristics of various human breast cancer cell lines. PK 11195-specific binding was determined using increasing concentrations of cellular protein for 50 μg of protein from each of the indicated cell lines described in Table 1. Each value is the mean ± SE of triplicate determinations. **Significance:** **P < 0.05,** **P < 0.01,** **P < 0.001.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Ks (nm)</th>
<th>Bmax (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-231</td>
<td>7.8 ± 1.8b</td>
<td>8.7 ± 1.4b</td>
</tr>
<tr>
<td>ADR</td>
<td>1.9 ± 0.47b</td>
<td>1.3 ± 0.23b</td>
</tr>
<tr>
<td>MCF-7</td>
<td>NDb</td>
<td>NDb</td>
</tr>
</tbody>
</table>

**Table 2** PBR-binding characteristics of MDA-231, ADR, and MCF-7 cells

Scatchard analysis of MDA-231, ADR, and MCF-7 cells (50 μg) was performed as described previously (2). Unpaired t test comparison of the mean Bmax for MDA-321 and ADR PBRs gave P = 0.0062, suggesting that the differences between the two Bmax are significant. Similar comparison of the mean Ks of these cell lines also shows significant differences in their binding affinity for PK 11195 (P = 0.017).

**Table 3** Determination of DNA content with Hoechst 33258. MDA-231 cells were plated on six-well plates at a concentration of ~150,000 cells/well in DMEM supplemented with 10% FBS. After 24 hr, the cells were washed twice with PBS, and the medium was changed to DMEM supplemented with 0.1% FBS. After an additional 24 hr, cells were treated with either 0, 10⁻¹⁰, 10⁻⁹, 10⁻⁸, and 10⁻⁷ M PK 11195 for 48 hr. After incubation, cells were again washed twice with PBS and then solubilized with 200 μl of 0.2% SDS in ETN buffer (10 mM EDTA, 10 mM Tris-HCl, and 100 mM NaCl, pH 7.0) for 15 min at 37°C. Determination of DNA content was accomplished with the Hoechst 33258 dye as described previously (27).
differential expression of PBR mRNA. As shown in Fig. 2, MDA-231 cells express ~20-fold more PBR mRNA than MCF-7 cells. This result fits with the correlation between PBR expression and increased aggressive behavior between these cell lines. The amount of PBR mRNA expressed in the ADR cell line does not conform to this, however. In fact, ADR cells express as much PBR mRNA as MDA-231 cells (Fig. 2). This seemingly anomalous result will be discussed later.

Previous studies demonstrated that, in most tissues, PBR is primarily localized to the mitochondria (3). To rule out the possibility that the differences between aggressive and nonaggressive human breast cancer cell lines are not due to differences in mitochondrial content, morphometry analysis was performed on transmission electron micrographs of two of the extreme representative cell lines, MDA-231 and MCF-7 (data not shown). Numerous morphological differences were found between the two cell lines, including differences in vacuole content and the presence of mysterious dark bodies, which may reflect their differences in metabolic activity. Upon first observation, the micrographs appear to reveal that MDA-231 cells contain more mitochondria than MCF-7 cells. Morphometric analysis, however, indicates that the larger MCF-7 mitochondria cover the same surface area/cell in the micrographs as do the MDA-231 mitochondria.

To further characterize the differences between these human breast cancer cell lines, subcellular localization was carried out using compound 4, the fluorescent derivative of FGIN-27, a specific PBR ligand (23). PBR has been shown previously to localize primarily to the outer mitochondrial membrane in MA-10 mouse tumor Leydig cells, the cell line used to characterize the only known function of PBR (3). In MA-10 cells, compound 4 fluorescent labeling is localized to the cytoplasm, presumably to the mitochondria (Fig. 3A). Similar to MA-10 cells, PBR is localized almost exclusively to the cytoplasm in MCF-7 cells (Fig. 3B). Strikingly however, PBR localizes primarily to the nucleus in MDA-231 cells (Fig. 3, C and D). This fluorescence indicates localization to either the nucleus (Fig. 3C) or the perinuclear envelope (Fig. 3D). Although not shown, in ADR cells PBR localizes chiefly to the cytoplasm, although nuclear fluorescence is also seen. Displacement of fluorescent labeling by 100 μM of FGIN-27 indicates that compound 4 binding is specific for PBR (Fig. 3, E and F). Scatchard analysis of [3H]PK 11195 binding to nuclei isolated from MDA-231 cells revealed a Kᵦ of 10.3 ± 8.4 nm and a Bᵦᵦ of 6.9 ± 4.8 pmol/mg nuclear protein. Furthermore, anti-PBR immunostaining of MDA-231 cells supports the nuclear localization of the receptor seen with compound 4 (Fig. 4, A and B).

**PBR Expression and Localization Correlates with Increased Aggressive Phenotype in Human Breast Tumor Biopsies.** Before characterization of the receptor in the MDA-231 cells, we examined whether the differences seen in the expression and localization of PBR in human breast cancer cell lines reflects real differences found in human breast cancer or an artifact of the cell culture system. To this end, we examined the expression and localization of PBR in normal breast tissue procured by reductive mammoplasty and metastatic aggressive breast tumor biopsies. Fig. 5A shows HRP staining of the PBR antiserum used to detect the Mr 18,000 molecular weight protein in normal breast tissue. This section was counterstained with hematoxylin. A light immunostaining for PBR can be seen. The low staining is better seen in Fig. 5B in which the hematoxylin counterstain was omitted. These data were confirmed using a FITC-labeled secondary antibody (Fig. 5C). It should be noted that PBR appears to be localized in the ductal epithelium. Fig. 5D is the phase contrast image of the Fig. 5C. The level of expression and localization of the Mr 18,000 molecular weight PBR protein was further examined in normal human breast tissue using compound 4 (Fig. 5E). Incubation of the sections with unlabeled PK 11195 displaced the fluorescent labeling seen with compound 4 (Fig. 5F). Similarly, low levels of cytoplasmic expression were obtained in specimens from in situ and noninvasive breast tumors (data not shown). These data were replicated in multiple sections taken from specimens from three separate individuals.

PBR expression and localization was then examined in a series of biopsies taken from aggressive metastatic breast tumors. Fig. 5G shows HRP detection of the PBR protein in tumor cells, where staining was more pronounced in the nucleus. Omission of the hematoxylin counterstain further enhanced PBR nuclear staining (Fig. 5H). These data were further confirmed using the FITC labeled secondary antibody (Fig. 5I), where robust perinuclear and nuclear staining is evident. Fig. 5J is the phase contrast image of the Fig. 5I. The level of expression and localization of the PBR...
protein was further examined in the tumor biopsies using compound 4 (Fig. 5). A high level of perinuclear and nuclear staining was seen, thus confirming the data obtained using the FITC and HRP antibody labeling procedures. Incubation of the sections with unlabeled PK 11195 displaced the fluorescent labeling seen with compound 4 (Fig. 5L). These data were replicated in multiple sections of specimens taken from three separate individuals.

**PBR Found in the MDA-231 Human Breast Cancer Cell Line Is Similar to PBR Found in Other Human Tissues.** Given the numerous differences between both the expression and localization of PBR in MDA-231 cells and the other human breast cancer cell lines studied, as well as reports published previously, it became important to determine whether we were dealing with the same receptor. The first step toward this end was to establish a pharmacological profile for the MDA-231 PBR. Displacement of [3H]PK 11195 with increasing concentrations of the indicated PBR ligands is similar, although not identical, to the pharmacological profiles reported previously for human PBR (Table 3; Refs. 28–31). Specifically, whereas the affinities for the benzodiazepines Ro5-4864 and diazepam are similar to findings reported previously, the affinities for the isoquinoline enantiomers, (-) PK 14067 and (+) PK 14068, were roughly 100-fold lower, suggesting that this receptor has distinct binding characteristics relative to the previous studies. It should be noted, however, that these previous studies are rather incomplete in their assessment of the ligand binding characteristics of PBR and, therefore, should be used only to a limited extent in the comparison of human PBR characteristics from tissue to tissue.

Next we obtained partial PBR cDNA sequences for both MDA-231 and MCF-7 PBR. The obtained nucleotide sequences revealed two point mutations resulting in the replacement of alanine 147
with a threonine and histidine 162 with an arginine in both MDA-231 and MCF-7 (Fig. 6). Given that these mutations occur in both cell lines, it is unclear what role they play in cancer pathogenesis. Despite many efforts, sequences could not be obtained for the region immediately surrounding the translation start sight. The region 3’ to the start sight may provide key evidence for the differential localization (cytoplasmic versus nuclear) of PBR between these two cell lines.

Fig. 4. PBR and DBI immunocytochemistry in MDA-231 and MCF-7 cells. In A and B, cultured MDA-231 cells were probed with anti-PBR primary antiserum at a concentration of 1:500; A, ×300; B, ×600. C, MDA-231 cells incubated in the absence of primary antibody; ×300. In D and E, cultured MDA-231 cells were probed with anti-DBI primary antiserum at a concentration of 1:500; ×400. In F, cultured MCF-7 cells were probed with anti-PBR primary antiserum at a concentration of 1:500; ×400. In G, cultured MCF-7 cells were probed with anti-DBI primary antiserum at a concentration of 1:500; ×400. H, MCF-7 cells incubated in the absence of primary antibody; ×400. PBR and DBI antiserum localization was revealed using HRP-coupled secondary antibody at a concentration of 1:2000.
A Functional Role for PBR in Human Breast Cancer. Previous studies from this laboratory have shown that PBR plays a key role in steroidogenesis by mediating the translocation of cholesterol from the outer to the inner mitochondrial membrane (18). More recently, we have shown that PBR mediates cholesterol uptake even in nonmitochondrial membranes (26). To test whether PBR plays a similar role in MDA-231 nuclear membranes, intact nuclei were isolated from both MDA-231 and MCF-7 cells. Isolated nuclei were incubated with 10 nM $[^3H]$cholesterol in the absence or presence of increasing concentrations of PK 11195 (Fig. 7A). In the absence of PK 11195, MDA-231 nuclear membranes demonstrated the ability to take up 30% more cholesterol relative to MCF-7 nuclei. In MDA-231 nuclei, addition of $10^{-8}$ to $10^{-6}$ M PK 11195 resulted in a decrease (25–30%; $P = 0.012$ and 0.006, respectively) in the amount of cholesterol retained by the nuclear membrane to levels relative to control treatment. This change corresponds to 0.52 pmol of cholesterol/mg of nuclear protein. MCF-7 nuclei failed to respond to the PK 11195 dose response ($F = 2.204, P = 0.0625$; Fig. 7A). This change in cholesterol levels may reflect either reduced cholesterol uptake or increased release of nuclear membrane cholesterol. Considering our previous studies showing that perturbation of PBR by specific ligands induces the transport of cholesterol through bacterial membranes (26, 32), we favor the second hypothesis.

Numerous studies performed in the early 1980s showed that Ro5-4864 and PK 11195, specific PBR ligands, regulate cell proliferation in a number of cancer models (5–12). Using the Bromodeoxyuridine Cell Proliferation ELISA (Boehringer Mannheim), we examined the effects of PK 11195 on MDA-231 cell proliferation (Fig. 7B). After 24 h, low nanomolar PK 11195 ($10^{-10}$ and $10^{-9}$ m) showed no effect on MDA-231 cell proliferation. However, $10^{-8} \text{ m}$ PK 11195 stimulated MDA-231 cell proliferation between 20 and 25%, an increase similar to earlier reports (7). Stimulation of MDA-231 cell proliferation was maximal (40%) at $10^{-7} \text{ m}$ PK 11195. After 48 h, the dose-response curve shifted to the left (data not shown). Cell proliferation was stimulated 40% by $10^{-8} \text{ m}$ PK 11195, although no stimulation was seen at any of the micromolar concentrations. In MCF-7 cells, on the other hand, after 48 h there was no stimulation of cell proliferation at any of the PK 11195 concentrations tested (data not shown). PK 11195 ($10^{-4} \text{ m}$), however, did result in a 40% decrease in MCF-7 cell proliferation relative to no treatment at this time point (data not shown). We believe the 40% decrease in MCF-7 cell proliferation seen with $10^{-4} \text{ m}$ PK 11195 is meaningless given the obvious nonspecific effects of any drug at this concentration.

Although it is generally assumed that the incorporation of BrdUrd into DNA is the result of cell division, it is also possible that the results presented in this study are due to repair of damaged DNA. However, using the Hoechst 33258 DNA assay, we saw increases in total MDA-231 DNA content in response to PK 11195, similar to the results presented above (data not shown). These data strongly suggest that the increases in BrdUrd incorporation into MDA-231 cells reflect increases in MDA-231 cell division and not DNA damage.

Change in MDA-231 Nuclear Cholesterol Levels Correlates with an Increase in Cell Proliferation. We have shown that PK 11195 reduced the amount of radiolabeled cholesterol retained in MDA-231 nuclear membranes at nanomolar and low micromolar concentrations. We have also shown that PK 11195 also stimulates MDA-231 cell proliferation at these concentrations. We were then interested in ascertaining whether the regulation of nuclear cholesterol transport correlates with the PBR-mediated regulation of cell proliferation in these cells. To determine such a relationship, all of the MDA-231 cholesterol data for $0$, $10^{-10}$, $10^{-8}$, and $10^{-6} \text{ m}$ PK 11195 was plotted against the proliferation data at the same PK 11195 concentrations. A regression line for all points gave a coefficient of correlation ($r$) of $-0.99$ (Fig. 7C). The data corresponding to $10^{-4} \text{ m}$ PK 11195 was omitted from regression analysis due to nonphysiologic and potentially toxic effects of this concentration. It should be noted, however, that the inclusion of this data still resulted in an $r$ of $-0.75$.

MDA-231 Cells Express DBI, the Endogenous PBR Ligand. Given the ability of exogenous PBR ligands to regulate nuclear cholesterol transport and cell proliferation in MDA-231 cells, we then examined whether MDA-231 cells express the polypeptide DBI, the endogenous PBR ligand. DBI has been shown to stimulate synthesis of steroids in adrenocortical, Leydig, and glial cells through specific activation of PBR (3). DBI expression in cells has been reported at levels comparable with those needed for PBR activation, further supporting its role as an endogenous PBR ligand (33). The presence of a PBR-specific ligand such as DBI in an aggressive human breast cancer cell line would support the hypothesis that PBR is involved in the advancement of human breast cancer. Indeed, immunocytochemistry of MDA-231 cells with anti-DBI antisera reveals that this cell line possesses DBI (Fig. 4, D and E). Interestingly, the pattern of DBI immunostaining was shown to be similar (nuclear and perinuclear) to that of PBR fluorescence and immunostaining. MCF-7 cells displayed DBI immunostaining in a similar pattern to that seen in MDA-231 (Fig. 4G). However, DBI immunostaining appears to be less robust in MCF-7 relative to MDA-231.

DISCUSSION

In this report, we examined the role of PBR in human breast using a series of human breast cancer cell lines and human breast tumor biopsies. Through the course of this study, we describe a strong correlation between the expression of PBR ligand binding activity and the invasive and chemotactic potential, as well as the expression of the breast cancer marker CD44, among the cell lines. In addition, we show that PBR is expressed at much higher levels in aggressive metastatic human breast tumor biopsies compared with normal breast tissue and to noninvasive breast tumors. Furthermore, we show that PBR is differentially localized between highly aggressive and nonaggressive cell lines and between normal and aggressive metastatic human breast tissue. Characterization of breast cancer PBR reveals that it is similar, although not identical, to PBR studied in other human tissues. Two point mutations were found that result in the replacement of an alanine residue at position 147 with a threonine residue and a histidine residue at position 162 with an arginine residue.

Functionally, we find that PBR is responsible for the increased cholesterol transport into the nuclei of a highly aggressive cell line, MDA-231, relative to a nonaggressive cell line, MCF-7 ($P = 0.0009$ and 0.0625, respectively). Furthermore, we find that PBR regulates cell proliferation of MDA-231 ($P < 0.0001$) and, moreover, that this regulation is strongly linked to the ability of PBR to regulate cholesterol transport into MDA-231 nuclei ($r = -0.99$). The fact that nanomolar and low micromolar concentrations, and not high micromolar concentrations, of PK 11195 are responsible for both of these actions indicates that these events are the result of specific interactions between the drug ligands used and PBR. The lack of significant effects of the PBR ligand PK 11195 on MCF-7 nuclear cholesterol transport and cell proliferation suggests that PBR’s regulation of these events occurs only in more aggressive breast cancer cells, further supporting a role for PBR in breast tumor progression. Although the functional data presented in this paper were elicited by an exogenous PBR ligand, the identification of the endogenous PBR ligand, DBI, in MDA-231 cells around the nucleus further supports a physiological role for the function of PBR described herein.

The expression of PBR protein levels in the cell lines and human...
Fig. 5. PBR expression in normal human breast and aggressive metastatic tumor tissues. In A, paraffin-embedded sections of normal breast tissue were immunostained with an anti-PBR antiserum at 1:500 dilution and counterstained with hematoxylin as described in “Materials and Methods”. In B, the hematoxylin counterstain was omitted to better determine whether the nucleus of the cells contained immunoreactive PBR protein. C, localization of immunoreactive PBR protein using a FITC-coupled secondary antibody. D, phase contrast microscopy of the same tissue area. E, detection of PBR ligand binding activity using the fluorescent PBR ligand compound 4. A filter was used to enhance the detection of low.
Fluorescence levels. F, displacement of the fluorescence with 1000-fold excess of the competitive ligand PK 11195. In G, paraffin-embedded sections of aggressive metastatic human breast carcinoma tissue were immunostained with an anti-PBR antiserum at 1:500 dilution and counterstained with hematoxylin as described previously (HRP staining). In H, the hematoxylin counterstain was omitted to examine whether the nucleus of the cells contained immunoreactive PBR protein. I, localization of immunoreactive PBR protein in aggressive metastatic human breast carcinoma tissue using the FITC-coupled secondary antibody. J, phase contrast microscopy of the same tissue area. K, detection of PBR ligand binding activity in aggressive metastatic human breast carcinoma tissue using the fluorescent compound 4. A filter was used to enhance the detection of low fluorescence levels. L, displacement of the fluorescence with 1000-fold excess of the competitive ligand PK 11195. A–H, I and L, ×75; J and K, ×150.
tissues studied in this paper mirrors that seen in other human cancer studies. Cornu et al. (13) have shown that PBR site densities are as much as 12-fold higher in high-grade astrocytomas and glioblastomas relative to normal brain tissue. A study by Miettinen et al. (14) also indicates that PBR is highly up-regulated in high-grade human astrocytic tumors relative to low-grade tumors. Furthermore, a positron emission tomography study by Pappata et al. (34) revealed that binding of PK 11195, the PBR-specific ligand used throughout the present study, is 2-fold greater in glioblastomas than in normal human gray matter. Our data support these previous studies by showing that PBR binding in MDA-231 cells is 7-fold higher than the mildly aggressive ADR cell line and immeasurably greater than in the non-aggressive MCF-7 cell line. At the mRNA level, however, this correlation does not appear to be as tight. Whereas MDA-231 cells express 17–20-fold higher PBR mRNA than MCF-7 cells, PBR mRNA expression is as much in the ADR cell line as in MDA-231 cells. This result appears to be anomalous and is difficult to explain because little is known about the regulation of PBR expression. Considering that ADR cells apparently localize PBR to the cytoplasm and the nuclear envelope, increased transcription of PBR mRNA may represent a transitional phase between the nonaggressive state and a more aggressive state in the context of the human breast cancer cell lines examined in this paper. Alternatively, the low PBR binding levels seen in ADR cells may implicate differences in translational regulation of PBR expression between MDA-231 and ADR cells. Neither possibility can be ruled out until further characterization of PBR expression is performed.

Partial sequence analysis revealed two point mutations in both MDA-231 and MCF-7 cells that result in the replacement of alanine 147 with a threonine residue and histidine 162 with an arginine residue. Molecular modeling of the receptor indicates that the first residue lies within the cholesterol entry region of the receptor (28, 32).

Table 3 Pharmacological characterization of MDA-231 PBR: Comparison of various human PBR pharmacological profiles

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Brain IC50 (nM)</th>
<th>Brain KI (nM)</th>
<th>Renal cortex IC50 (nM)</th>
<th>Renal cortex KI (nM)</th>
<th>MDA-231 IC50 (nM)</th>
<th>MDA-231 KI (nM)</th>
<th>Lymphocytes IC50 (nM)</th>
<th>Lymphocytes KI (nM)</th>
<th>Glioma IC50 (nM)</th>
<th>Glioma KI (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGIN-27</td>
<td>1.94</td>
<td>1.54</td>
<td>138</td>
<td>822</td>
<td>528</td>
<td>419</td>
<td>6.7</td>
<td>84.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ro5-4864</td>
<td>1,780</td>
<td>360</td>
<td>3.94</td>
<td>3,130</td>
<td>17.80</td>
<td>58.80</td>
<td>46.700</td>
<td>588.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-) PK 14067</td>
<td>23.3</td>
<td>3,130</td>
<td>149.00</td>
<td>1,480</td>
<td>19.30</td>
<td>55.20</td>
<td>10,000</td>
<td>1,840</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+) PK 14068</td>
<td>1,422</td>
<td>&gt;10,000</td>
<td>186,000</td>
<td>148,000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* From Chang et al. (28).
* From Broaddus and Bennett (29).
* From Alexander et al. (30).
* From Olson et al. (31).

Fig. 6. Partial cDNA sequence for MDA-231 and MCF-7 human breast cancer PBR. The partial PBR nucleotide sequences obtained for both MDA-231 and MCF-7 cells were compared with the published human PBR sequence. The sequences reveal three point mutations common to both MDA-231 and MCF-7 PBR cDNA and a single point mutation unique to MDA-231. Deduction of the amino acid sequence revealed that only two of the common point mutations alter the amino acid sequence (Ala 147→Thr and His 162→Arg), whereas the others were silent.
Presently, it is not apparent whether this mutation has a resulting phenotype. It appears that it does not alter the ability of cholesterol to move through PBR because cholesterol is incorporated into MDA-231 nuclei. The fact that it is present in both the MDA-231, a highly aggressive breast cancer cell line, and in MCF-7, a nonaggressive cell line, suggests that this mutation may represent an early event in the progression of this disease. The second mutation (His 162 → Arg) is a conserved mutation, given that both histidine and arginine are basic amino acids, and likely has no significant effect on PBR structure and function.

PBR is primarily targeted to the outer mitochondrial membrane in tissues in which it is expressed in great abundance (3). However, it has also been found in other cellular organelles such as the plasma membrane as well as the peroxisome (3, 35). The lack of a distinct mitochondrial target sequence and the largely hydrophobic nature of PBR make it feasible that PBR could exist in a variety of membranes. Differential localization of PBR may also be possible through the existence of chaperone proteins and PBR-associated proteins that may direct PBR to the membranes of specific organelles and may influence the functioning of PBR (36). The significance of such differential localization, however, has not been investigated and is presently unknown. It will be necessary to distinguish whether the nuclear localization of PBR in MDA-231 cells is the result of a specific amino acid sequence present in the yet undetermined NH2 terminus of the protein or the shuttling of PBR to the nucleus via association with another protein.

The data presented in this paper suggest that nuclear PBR is responsible for regulating movement of cholesterol into the nuclear membrane and that this regulation is related to its modulation of cell proliferation. Cholesterol is a major lipid component of every membrane and influences the degree of membrane fluidity (37). Cholesterol has also been shown to play a certain but controversial role in the advancement of a variety of pathologies including breast cancer (38–40). Furthermore, reports on animal dietary, cellular, and enzyme-specific studies implicate a role for cellular cholesterol in the regulation of cell proliferation (38). Cholesterol has been shown to tightly regulate the activity of the SREBPs found in the nuclear membrane and the endoplasmic reticulum (41). In the presence of excessive cholesterol, premature SREBP is not fully cleaved, and therefore, the mature form is not released and cannot enter the nucleus to carry out transcriptional activation (41). SREBPs are responsible for the transcriptional regulation of the enzymes involved in the cholesterol biosynthetic pathway as well as the enzymes involved in fatty acid synthesis and uptake (41). One possible outcome of concentrating cellular cholesterol to the nuclear membrane may be to inhibit the activation of nuclear membrane SREBPs. Conversely, we show that PBR ligands reduce the presence of cholesterol in MDA-231 nuclear membranes. As mentioned before, we have reason to believe that these data are due to release of cholesterol into the nucleus. What role cholesterol might play in the nucleus and whether the effect we show on cell proliferation is direct or indirect is completely open to debate at this point. With the tight correlation between nuclear transport of cholesterol in MDA-231 and the regulation of MDA-231 cell proliferation by PBR, the SREBP pathway may shed some light as to how PBR is regulating cell proliferation in these cells and should be the target of future research in this area. However, other possibilities such as changes in nuclear membrane fluidity induced by alterations of membrane cholesterol content and the direct effects of cholesterol on gene transcription must also be pursued to definitively answer these questions.

It is possible that the relationship between increased expression and nuclear localization of PBR with both aggressive breast cancer cell lines and malignant breast biopsies may be due to differences in metabolism and cellular activity between the cell lines and tissues studied. However, the functionality of PBR in the MDA-231 cell line, i.e., the ability to regulate both nuclear cholesterol uptake and cell proliferation, as well as the strong correlation between these two seemingly separate events, however, suggests that PBR is indeed playing a role in the progression of breast malignancies. The presence and localization of the putative endogenous PBR ligand, DBI, in MDA-231 cells further suggests the likelihood that PBR is fully functional in these cells.

---

**Fig. 7.** PK 11195 regulated MDA-231 nuclear cholesterol transport and cell proliferation are highly correlated. In A, transport of [3H]cholesterol into nuclei isolated from MDA-231 and MCF-7 cells was measured in response to varying doses of PK 11195. Data are expressed as percentage of cholesterol uptake into MCF-7 nuclei in the absence of any PK 11195. One hundred % cholesterol uptake corresponds to 1.75 pmol/mg protein. Data points represent the means of five (MDA-231) or four (MCF-7) independent experiments carried out in quadruplicate; bars, SE. One-way ANOVA indicates that treatment of isolated MDA-231 nuclei but not MCF-7 nuclei with PK 11195 induces significant changes in cholesterol content (F = 4.74, P = 0.0009; F = 2.204, P = 0.0625, respectively; GraphPad, Inc.). In B, MDA-231 cells grown in 96-well plates were washed with PBS and cultured in media supplemented with 0.1% FBS 24 h before any treatment. The indicated concentrations of PK 11195 were added to the cells cultured in DMEM supplemented with 0.1% FBS and incubated for 24 h at 37°C. Four h before the end of incubation, BrdUrd was added to each well. Incorporation of BrdUrd was measured at 450 nm (reference, 700 nm). Data points represent the means of three independent experiments carried out in quadruplicate; bars, SE. One-way ANOVA indicates that MDA-231 cell proliferation was significantly altered by treatment with PK 11195 at both the 24- and 48-h time points (F = 20.339, P < 0.0001; F = 23.126, P < 0.0001, respectively; GraphPad, Inc.). Comparison of each PK 11195 treatment to control was performed with the unpaired t test provided by the SigmaPlot software (***, P < 0.05; Jandel Scientific). C, the means of all data points for 0, −10, −8, and −6 μM PK 11195 from the previously described cell proliferation assay were plotted against the corresponding means from the cholesterol uptake assay described previously. A regression line drawn for all plotted data gives a coefficient of correlation of −0.99. Numeric values in parentheses indicate the number data points taken for each mean; bars, SE.
Malignant breast tumors are primarily characterized by aberrant cell proliferation, tumor invasion, and metastasis. Several molecular and cellular mechanisms have been proposed to account for these phenomena, and a number of prognostic indicators have been identified. Although these markers have been useful in helping clinicians develop prognoses, they have failed to provide adequate information about the mechanisms responsible for tumor malignancy so that effective anticancer therapies may be developed. Given the data presented in this report, we believe that PBR is a major component of the progression of breast cancer. Although a great deal more needs to be learned about PBR and its ability to regulate cell proliferation and cholesterol movement, we believe this work is a major step in understanding this disease. Our present work with both human breast cancer cell lines and human breast tissue, combined with the availability of radiolabeled and fluorescent PBR ligands, may be useful in the diagnosis and prognosis of the disease. Moreover, a great number of PBR ligands are known, including benzodiazepines and isoquinoline carboxamides, the PBR-binding and pharmacological characteristics of which are well documented. Some of these ligands may have potential as future anticancer therapies.

REFERENCES


Peripheral-Type Benzodiazepine Receptor (PBR) in Human Breast Cancer: Correlation of Breast Cancer Cell Aggressive Phenotype with PBR Expression, Nuclear Localization, and PBR-mediated Cell Proliferation and Nuclear Transport of Cholesterol

Matthew Hardwick, Djamil Fertikh, Martine Culty, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/4/831

Cited articles  This article cites 38 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/4/831.full#ref-list-1

Citing articles  This article has been cited by 27 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/4/831.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.