Characterization of Progesterone Receptor A and B Expression in Human Breast Cancer

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ABSTRACT

The human progesterone receptor (PR) is a ligand-activated nuclear transcription factor that mediates progesterone action in target tissues. Two PR proteins, PR-A (Mr 81,000–83,000) and PR-B (Mr 116,000–120,000), have been described and different physiological activities ascribed to each on the basis of in vitro studies, suggesting that their ratio of expression may control progesterone responsiveness in target cells. Presence of PR in breast tumors is an important indicator of likely responsiveness to endocrine agents. However, the relative expression of PR-A and B in breast cancer has not been described, and its clinical significance has not been addressed. Expression of PR-A and B was measured by immunoblot analysis of 202 PR-positive human breast tumor cytosols. The ratio of expression of the two PR proteins (PR-A/B) ranged from 0.04 to 179.3. The median PR-A/B ratio was 1.26, and 61.4% of samples had PR-A/B ratios between 0 and 2. PR-A/B ratios deviated significantly from a normal log distribution; tumors containing a PR-A/B ratio greater than 4 were overrepresented in the group. Linear regression analysis revealed that high PR-A/B ratios, in general, derived from a low concentration of PR-B rather than high expression of PR-A. PR-A/B protein ratios were not correlated with the age of the patient or with total PR concentration. A third PR protein band (PR78kDa) was detected in a number of samples and comprised greater than 20% of total PR protein in 52 (25.7%) of the 202 tumor samples examined. The range or frequency distribution of PR-A/B ratios in samples containing PR78kDa was different to that of the overall group. In summary, PR-B-positive breast tumors, the ratio of expression of PR-A and B proteins is close to unity, as is seen in a number of other progestin target tissues. However, a significant proportion of tumors expressed very low levels of PR-B and a consequently high PR-A/B ratio. Although the clinical consequence of this observation is not known, the in vitro findings that PR-A may act as a repressor of PR-B suggest that tumors containing primarily PR-A may identify a subset of patients with low or aberrant response to endocrine agents.

INTRODUCTION

The PR4 is a specific and effective mediator of progesterin action in target tissues such as the breast and endometrium and is essential in the complex control of proliferation and differentiation at various stages of development (1). PR is a member of a superfamily of ligand-activated nuclear transcription factors and is composed of specific domains involved in DNA and hormone binding and transcriptional activation (2). Progestin activation of PR in target tissues is mediated via dimerization and phosphorylation of the receptor, resulting in binding to cis-acting progestin-responsive elements on DNA and modulation of the activities of target genes (2, 3). PR is detected in the chick and human as two distinct proteins of different molecular weights (4, 5); in humans the PR-A protein is Mr 81,000–83,000 and PR-B is Mr 116,000–120,000 in size (6–9). The two proteins, which differ only in that PR-A lacks the first 164 amino acids contained in PR-B, are translated from distinct mRNA subgroups transcribed from a single gene under the control of separate A and B promoters (10). Although both PR-A and PR-B bind progestins and interact with progestin-responsive elements, there is increasing evidence that they are functionally different. In transfection studies, the two proteins have different abilities to activate progestin-responsive promoters, and these differences are promoter and cell specific (11–14), suggesting that cellular responsiveness to progestins may be modulated via alterations in the ratio of PR-A and B expression. Whereas PR-B tends to be a stronger activator of target genes in many settings, PR-A has been shown to act as a dominant repressor of PR-B (13, 14), suggesting that high PR-A expression may result in reduced progesterin responsivity. Furthermore, PR-A has been shown to diminish the response of other hormone receptors such as the androgen, glucocorticoid, mineralocorticoid, and estrogen receptors to their cognate ligands (15–17). It has also been reported that PR-A but not B can confer antiestrogenic actions to progestin antagonists (16), possibly via noncompetitive binding of PR-A to specific transcription factors required for ER and PR action.

Approximately equimolar expression of PR-A and B is observed in chick oviduct (4) and human uterus (6), and a similar ratio of expression is seen in human breast cancer cells in culture (8). In contrast, PR in the rabbit uterus is expressed as a single form homologous to human PR-B (18). In the rodent, PR-A expression predominates over PR-B in a 3:1 ratio (19, 20). The biological importance of these different ratios of PR expression has not been explored extensively. Little is known of whether relative PR-A and B expression is modulated in vivo, except in the chick oviduct, where alterations in the ratio of PR forms during late winter or in aged, nonlaying animals result in a measurable decrease in PR functional activity (21, 22).

The functional differences between PR-A and B that have been described in vitro imply that response to progesterone may be modulated by differential expression of the two PR forms. Clearly, if these observations are borne out in vivo, the balance of expression of the two receptor forms may be critical in the control of progesterone responsiveness in normal and malignant tissue. PR is an important marker of responsiveness in breast cancer, and the presence of PR in ER-positive breast tumors indicates likely responsiveness to endocrine agents (23, 24). Absence of PR is associated with markedly reduced response to endocrine agents (25). Given that the two PR proteins may be functionally different, the ratio of expression of PR-A and B may be as important as the absolute PR level in determining endocrine response. The relative expression and regulation of PR-A and B proteins in breast tumors is not known, and the purpose of this study was to measure PR expression in PR-positive human breast tumor biopsies, and to determine the extent of variability in the relative expression of the two PR proteins.
MATERIALS AND METHODS

Materials. Chemicals used were of analytical reagent grade and were obtained from the sources listed previously (26). The anti-PR mAbs, hPRa6 and hPRa7, which have been described previously (27), were concentrated by ammonium sulfate precipitation of hybridoma supernatants and dialysis in PBS containing 0.02% sodium azide. The dilutions used for saturating detection of PR on immunoblots were determined for each batch. Abbott PgR-EIA monoclonal and ER-EIA monoclonal reagents for EIAs were from Abbott Laboratories (Diagnetics Division).

Preparation of Tumor Cytosols. Biopsy samples were collected at the time of breast lump excision and frozen at —70°C. The tissues were pulverized in a precooled vessel, then homogenized at 4°C in a buffer containing 10 mM Tris pH 7.4–7.5, 1.5 mM EDTA, 5 mM sodium molybdate, and 1 mM monothioglycerol. Homogenates were centrifuged at 436,000 × g for 20 min at 4°C, and then cytosols were transferred to fresh tubes. Protein concentrations were determined by a method based on that of Lowry et al. (28), as recommended in notes accompanying the Abbott EIA kits. PR and ER concentrations were determined by EIA. The cytosols were stored up to 18 months at —80°C.

Protein Electrophoresis and Immunoblot Analysis. Tumor cytosol proteins (300 µg/lane) were separated by electrophoresis through 7.5% polyacrylamide gels containing SDS and were electroblotted overnight onto nitrocellulose as described previously (26). PR proteins were detected using anti-PR mAbs; hPRa7 recognizes both PR-A and PR-B proteins, and hPRa6 detects PR-B protein but not PR-A (27). Both hPRa6 and hPRa7 were used in combination (each at 1:1000) for detection of tumor PR proteins. Specific PR protein bands were visualized on autoradiographic film using a horseradish peroxidase-conjugated goat antimouse secondary antibody (Dako, Australia) at a 1:5000 dilution and enhanced chemiluminescence detection substrates (Amersham, Australia). A cytosol made from the T-47D breast cancer cell line (29), which is highly PR positive, was included on each gel as a positive control. Band intensities were measured densitometrically using a Molecular Dynamics densitometer and Imagequant software. The linear range of detection of PR on immunoblot was established by a standard curve constructed using increasing concentrations of T-47D cytosol PR. This was analyzed by densitometry and established the relationship between PR concentration and linearity of detection by this method. Multiple exposures were always made from each immunoblot of tumor cytosols, and results were taken only from those that fell within this linear range. More than one exposure of an immunoblot was generally analyzed, and the results were compared and found to be close to identical in most cases. Results that were outside the linear range were omitted from the analysis. PR-A/B ratios were calculated by dividing PR-A AU by PR-B AU. In a small number of samples (n = 6) where PR-A or B expression was visible by eye, but so low as to be abolished by the background correction protocol of the densitometry software, this correction was switched off to allow estimation of the PR-A/B ratio. However, for consistency with the total sample population, the correction for background in these samples was taken into account when calculating absolute expression levels (see below).

To allow correlation of PR concentration with other parameters, such as PR and ER EIA and age, tumor protein levels were normalized to total PR concentration in the T-47D-positive control cytosol. This was done by dividing the total immunoreactive PR protein AU in the tumor sample by the total immunoreactive PR AU in the T-47D cytosol from the same immunoblot. The normalized PR values represent the amount of PR per 300 µg cytosol protein as a percentage of PR in 10 µg T-47D cytosol protein. Linear regression and Spearman’s rank correlation analyses were carried out using SPIDA and Statview (Abacus Concepts, Inc.) statistical analysis software.

RESULTS

Cytosols from 202 PR-positive primary tumors were analyzed by protein immunoblot. Ninety % (182) of these tumors were also ER positive by EIA. Fig. 1 shows a representative immunoblot of tumor cytosols probed for PR. The PR-B protein band was visualized as a group of two or three closely migrating bands with a mobility of Mr, 115,000–120,000, as reported previously in other tissues (6, 8, 9, 27, 30). An immunoreactive band representing PR-A protein was also detected in most tumor cytosols (Fig. 1A, Lanes 1–7). This band migrated slightly ahead of the Mr, 81,000–83,000 PR-A band seen in the positive control breast cancer cell line T-47D (Fig. 1A, Lane 8) (Ref. 8). This difference in migration is likely to be due to the difference in protein loading rather than representing a significant difference in size between tumor and T-47D cell PR-A. Alternatively, the difference in migration may reflect a difference in the level or number of sites of phosphorylation. A third immunoreactive band (PR78kDa) migrating ahead of PR-A at approximately Mr, 78,000 was detected in a proportion of tumors (Fig. 1A, Lanes 2, 6, and 7). Some faint, low molecular weight, immunoreactive bands were seen occasionally in individual cytosols (see Fig. 1A, Lanes 5–7, for example). However, these bands had different mobilities in different samples and were seen in only a small proportion of cases.

All tumor samples used in the study were PR positive by both immunoblot and EIA and represented 78% of the total number that were PR positive by EIA. To correlate levels of PR detected on
This was done by dividing PR-A AU in tumors by total PR protein AU in T-47D and breast tumor cytosols were quantitated densitometrically from immunoblots and normalized to total PR in concurrently run T-47D-positive control cytosol to correct for loading. PR-B AU in tumors by total PR protein AU in T-47D. O and bars, median value and interquartile range, respectively, representing AU (n = 140).

PR-A/B ratios were calculated by dividing PR-A AU by PR-B AU. The median PR-A/B ratio was 1.26, minimum = 0.04, and maximum = 179.3. The numbers of tumors with PR-A and B protein levels are shown

PR-A/B ratios were calculated by dividing PR A AU by PR B AU. The median PR A/B ratio was 1.26, minimum = 0.04, and maximum = 179.3. The numbers of tumors with A/B ratios in the indicated ranges are shown.

The distribution of PR-A/B ratios in breast tumors is shown in Table 1. There was no correlation between PR-A/B ratios and age (Fig. 3A). The ratio of PR protein expression was not related to age (Fig. 3A), suggesting that the PR-A/B ratio is unlikely to be related to menopausal status.

PR-A/B ratios ranged between 0.04 and 179.3, with a median ratio of 1.26 and an interquartile range of 0.57–3.88. The majority of tumors (61.4%) had a PR-A/B ratio between 0 and 2, and 75.2% of tumors had a PR-A/B ratio between 0 and 4 (Table 1). Tumors with PR-A/B ratios greater than 4, above the upper quartile range of 3.88, comprised 24.8% of the total (Table 1). Immunoblots revealed that these tumors generally contained low levels of PR-B (see Fig. 1A, Lanes 4–7, for example), and this was borne out by statistical analysis. There was a negative correlation between PR-A/B ratio and PR-B concentration, which was statistically significant by Spearman's rank correlation analysis (Fig. 4). This negative correlation between PR-B concentration and A/B ratio was nonlinear at low levels of PR-B, which suggests that high PR-A/B ratios were explained by low levels of PR-B expression rather than high PR-A. This relationship between PR-A and B protein expression is described by the fitted model calculated from the linear regression line of best fit; PR-A concentration = e^{1.431 \times (PR-B concentration)^{0.556}} (shown in Fig. 4, inset).

A faster migrating M, 78,000 PR protein was detected in 52 of 202 (25.7%) tumors at levels greater than 20% of total PR concentration (Fig. 5). This band was detected only by the hPRa7 antibody, which detects both PR-A and B, and not by hPRa6, which recognizes PR-B alone (data not shown), suggesting that this protein may represent an NH2-terminally truncated form of PR. The distribution of PR-A/B
ratios in samples containing PR78kDa, which had a median value of 1.93, did not differ significantly from that of the entire tumor cytosol group. Tumors containing PR78kDa did not represent a unique sub-group of the overall population in regard to the other parameters examined, since expression of the protein was not correlated with total PR concentration (Fig. 5B) or PR-A/B ratio (P = 0.76), and no relationship was seen between the presence of PR78kDa and age (not shown).

DISCUSSION

The relative expression of PR proteins has not been examined previously in human breast tumors. Expression of PR-A and B was measured on immunoblots of PR-positive primary breast tumor cytosols. The median PR-A/B ratio in a group of 202 breast tumors was close to unity. This result agrees with the equal expression of PR-A and B observed in chick oviduct (4) and human uterus (6), and a similar ratio of expression is seen in human breast cancer cells in culture (8). In contrast, PR in the rabbit uterus is expressed as a single form homologous to human PR-B (18). In the rodent, PR-A expression predominates over PR-B in a 3:1 ratio (19, 20). These different tissues have differing functional requirements for PR in terms of progestin responsiveness. Possibly differential PR-A and B expression is one mechanism by which their distinct physiological requirements are fulfilled by this relatively well conserved but differently expressed protein. Indeed, even in a particular tissue type such as the breast, control over relative PR-A and B expression may be important in modulating progestin responsiveness as required at different times in the normal ovarian cycle.

Whether the ratio of PR-A and B proteins in target cells is fixed or whether it can be altered under physiological conditions is poorly understood. In mammalian target tissues PR expression is under dual control by estrogen and progesterone (24, 31), with their coordinate regulation of PR concentration resulting in modulation of responsiveness to progestins. In the normal human endometrium and in breast cancer cells, progestin exposure leads to a similar decrease in the concentration of both PR-A and B (5, 6) resulting in no change to PR-A/B. Retinoic acid, which decreases PR mRNA and protein levels in breast cancer cells, resulting in diminished cellular responsiveness to progestins, is equally effective on both PR-A and B (26, 32). However, there is some evidence that estrogen, which is the major physiological modulator of PR levels in vivo, can alter the relative expression of PR-A and B. Estrogen induces PR mRNA and protein in most target cells (20, 33–37). In breast cancer cells this effect is mediated primarily through PR-B, resulting in a decrease in PR-A/B with increased PR expression.5 In the rat uterus (where PR-A predominates), estrogen withdrawal results in a decrease in PR-A/B from 3.2 to 1.9 (19).

Although the median PR-A/B ratio in the breast tumor cytosols was close to 1, a number of tumors had PR-A/B ratios that differed quite dramatically from this value, resulting in a nonnormal distribution of PR-A/B. In particular, samples with high PR-A/B ratios were over-

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Footnote:

5 J. D. Graham, S. D. Roman, E. McGowan, and C. L. Clarke, unpublished observations.
represented in the tumor population. Analysis by Spearman’s rank correlation revealed a significant negative relationship between PR-B expression and PR-A/B. Further linear regression analysis of the natural log-transformed data showed that, whereas the inverse relationship observed between ln PR-A/B and ln PR-B remained linear for most values, this relationship was lost at low levels of PR-B. Furthermore, it was these samples that gave rise to the high PR-A/B ratios, which were overrepresented in the tumor population.

The consequences of high PR-A/B ratios on progesterone responsiveness in human tissues are not known. However, there is increasing evidence that PR-A and B are functionally different. In the chick oviduct, relative expression of PR-A and B is under seasonal and developmental control. Alterations in the ratio between the PR forms during late winter or in aged, nonlaying animals results in a measurable decrease in PR functional activity (21, 22). Although both PR proteins are able to mediate progesterin-activated transcription in transfection studies, they differ in their efficacy and specificity for target genes. Whereas PR-B is able to activate mouse mammary tumor virus-chloramphenicol acetyltransferase more effectively (12), PR-A is known to stimulate a tyrosine aminotransferase progesterone-responsive reporter to a greater extent than is PR-B (14). PR-A also acts synergistically with estradiol to induce the ovalbumin promoter, whereas PR-B is inhibitory under the same conditions (11). Furthermore, there is emerging evidence that, whereas PR-B is, in most contexts, a transcriptional activator, the ability of PR-A to function as an activator is more restricted; in settings where both forms activate transcription, PR-B is more effective than PR-A (13, 17, 38). The observed differences in PR-A and B activity have focused attention on the NH2-terminal region of PR, which is unique to PR-B. PR mediates progesterin regulation through specific AF domains contained in the amino-terminal (AF1) and ligand-binding (AF2) domains (39). It has been postulated that the PR-B-unique NH2-terminal portion of PR binds an intermediary factor involved in activation by these domains (12) or, itself, a third activation function domain (AF3), and that this accounts for the functional differences between PR-A and B (40).

PR-A has been shown to act as a dominant repressor of PR-B function. Cotransfection of increasing amounts of PR-A with PR-B resulted in suppression of PR-B activation of a progesterin responsive reporter, mouse mammary tumor virus-chloramphenicol acetyltransferase (13, 14). In the presence of progestins or the progesterin antagonist RU486, PR-A represses transcriptional activation by cotransfected glucocorticoid receptors, mineralocorticoid receptors, ARs, and ERs (15–17). Furthermore, PR-A mediates antiestrogenic effects of progestins and RU486 on endogenous ER in MCF-7 breast cancer cells cotransfected with a vitellogenin ERE reporter construct (17). Contrary to this, PR-B and not PR-A inhibited ER activation of pS2 and cathepsin D promoter constructs in the same cell line when treated with the progesterone R5020 but not RU486 (41). Clearly, the effect is complex and depends strongly on the cellular and promoter context. The nature and level of sensitivity of this effect in breast tumors is not known; however, if these findings are true in vivo, then relative expression of PR-A and B in breast tumors may strongly influence cellular responsiveness to endocrine agents. Clearly, a tumor with the maximum observed PR-A/B ratio of 179.3, containing almost exclusively PR-A, will respond in a different way to endocrine agents than a tumor in which PR-B predominates. If PR-B is the more transcriptionally active of the two PR forms, it could be anticipated that tumors with a high PR-A/B ratio may be the least likely to respond to endocrine agents. Work is currently under way to address whether any correlation is seen between PR-A/B and other known markers of responsiveness.

The expression of multiple transcripts from a single gene encoding two receptor proteins is not unique to PR among the steroid hormone receptors and is observed also for AR expression. Two androgen-regulated promoters give rise to distinct mRNA species that are differentially expressed in different cell lines and tissues (42). A second NH2-terminally truncated human AR protein has been described, and the two AR isoforms have been postulated to be functionally different (43). Furthermore, the suggestion that the transcriptional activity of PR-B may be negatively regulated by PR-A is not unprecedented. A number of reports have suggested that an activator and repressor can be encoded by the same gene (44). The transcriptional regulation of the TR gene by its isoforms is a case in point. Three TR isoforms (α1, α2, and β) are generated as the result of alternative splicing. The α2 form is transcriptionally inactive alone but blocks activation by the α1 or β forms of TR, even when these activators are in 2-fold molar excess (45). The murine transcription activator, mTFE3, which binds to the intronic enhancer of the immunoglobulin heavy chain gene, acts in a similar manner. A natural splicing variant, mTFE3-S, acts as a dominant negative repressor of mTFE3 activation in a highly ratio-dependent manner (46).

A significant proportion of the tumor samples examined contained a M, 78,000 PR protein. The presence of PR78kDa was not correlated with PR-A/B ratio, and the source of this PR species is not yet clear. The band was not detected by hPRα6, which recognizes only PR-B, suggesting that it may represent an NH2-terminally truncated form of the receptor. There are no possible sites of alternative initiation of translation contained in the NH2-terminal region of the PR gene, which would predict a protein of this size. A methionine at amino acid position 595 has been suggested as a possible site of alternative initiation, and this would give rise to a M, 45,000–50,000 protein termed PR-C (47). However, no immunoreactive band of that size was detected in the tumor cytosols examined. It is possible that the presence of PR78kDa reflects expression of a PR variant. There is evidence from breast tumor mRNA studies that several ER variants may exist (25, 48). In particular these comprise whole exon deletions ascribed to splicing errors. A number of the exons of PR are between 100 and 150 bp in length (49), so deletion of any of these could potentially predict a size difference in the order of M, 5000–6000, as seen between PR78kDa and PR-A, allowing speculation that this protein may represent a variant PR-A. Further characterization of the origin of PR78kDa in breast tumors is currently under way.

In summary, whereas the majority of PR-positive breast tumors contain similar levels of PR-A and B, a significant proportion demonstrates low PR-B and, consequently, a high ratio of PR-A/B. Although the functional consequences of unequal expression of PR-A and B are not known, in vitro evidence suggests that an abnormal ratio of PR expression may be indicative of an impaired or aberrant progesterin response. The implications of altering PR-A/B ratio on cellular responsiveness to progesterin remains to be determined.

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