A Unique Form of Proliferating Cell Nuclear Antigen Is Present in Malignant Breast Cells


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Abstract

Despite extensive research efforts to identify unique molecular alterations in breast cancer, to date, no characteristic has emerged that correlates exclusively with malignancy. Recently, it has been shown that the multiprotein DNA replication complex (synthesome) from breast cancer cells has a significantly decreased replication fidelity compared to that of nonmalignant breast cells. Proliferating cell nuclear antigen (PCNA) functions in DNA replication and DNA repair and is a component of the synthesome. Using two-dimensional PAGE analysis, we have identified a novel form of PCNA in malignant breast cells. This unique form is not the result of a genetic alteration, as demonstrated by DNA sequence analysis of the PCNA gene from malignant and nonmalignant breast cells. This novel form is most likely the result of an alteration in the post-translational modification of PCNA in malignant breast cells. These findings are significant in that it is now possible to link changes in the fidelity of DNA replication with a specific alteration of a component of the DNA synthetic apparatus of breast cancer cells. The novel form of PCNA may prove to be a new signature for malignant breast cells.

Introduction

Breast cancer is a leading cause of death among women. In 1996, there were ~44,000 deaths attributed to breast cancer (1). Breast tumors show a strong correlation between high rates of DNA synthesis and poor overall patient prognosis. Data suggest that high levels of breast cancer cell DNA synthesis are associated with an increased probability of lymph node metastases (2–7). Extensive genetic damage in mammary cancer cells is also associated with increased levels of DNA synthesis. An evaluation of thousands of clinical cases indicated that ~65% of mammary cancer cells are aneuploid (8). Therefore, the observed high levels of DNA synthesis and extensive genetic damage in breast tumors strongly suggest that an alteration in the DNA replication machinery of these cells may contribute to uncontrolled and error-prone DNA synthesis.

We have recently found that human breast cells mediate DNA synthesis using a multiprotein replication complex, designated the DNA synthesome (9). The human breast cell DNA synthesome is fully competent to support SV40 in vitro DNA replication. Recently, it was found that the fidelity of DNA synthesis mediated by the synthesome isolated from the malignant breast cells is several-fold lower than the synthesome isolated from the nonmalignant breast cells (9, 10). The decreased replication fidelity of the malignant breast cell DNA synthesome clearly suggests a critical alteration(s) in the composition of the replication machinery of these cells.

PCNA (proliferating cell nuclear antigen) is associated with the human cell DNA synthesome (11) and is required for synthesome-mediated in vitro DNA replication (12). PCNA has been demonstrated to function in mammalian cell DNA replication (reviewed in Ref. 13) and DNA repair (14, 15). In DNA replication, PCNA serves as an accessory factor to polymerase δ. PCNA is also commonly used as a proliferation marker in breast cancers. Unfortunately, there has been no conclusive evidence that increased levels of PCNA correlate with tumor progression or patient prognosis (16–18). Here, the DNA synthesome-associated PCNA isolated from nonmalignant and malignant breast epithelial cells was analyzed by 2D PAGE. Here, we describe, for the first time, a unique form of PCNA that is found in all malignant breast cells. This is an exciting finding that may prove to be a new signature for malignant breast cells.

Materials and Methods

Cell Culture. MCF7, early-passage MCF10A, MBA-MD468, and Hs578T cells were cultured according to protocols provided by American Type Culture Collection. Primary cells were cultured in Mammary Epithelial Growth Medium (Clonetics, San Diego, CA) supplemented with 2× penicillin/streptomycin, 2.5 mg/ml amphotericin B, 50 units/ml polymixin B sulfate, 50 mg/ml gentamicin sulfate, 10 ng/ml epidermal growth factor, 5 mg/ml insulin, 0.5 mg/ml hydrocortisone, and 52 mg/ml bovine pituitary extract and grown as described previously (19). To stimulate MCF7 cells with estrogen, we grew cells in medium containing charcoal-treated serum. Cells were treated for 48 h with 1 nM 17β-estradiol or an equivalent volume of vehicle. Cells were harvested, and the DNA synthesome isolated as described previously (9).

Isolation and Purification of the DNA Synthesome from Breast Tumor Tissue and Breast Cell Lines. The mammary mouse tumor was induced using polyoma virus as described previously (20). The isolation and purification of the DNA synthesome were performed as described previously (9).

2D PAGE. DNA synthesome protein (20–40 μg) was loaded onto the first-dimension tube gel [9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3–10), and 20% Triton X-100]. The polypeptides were separated along a pH gradient created using 100 mM NaOH and 10 mM H3PO4. The tube gels were placed onto an 8% acrylamide-SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western Blot Analysis. The antibody directed against PCNA (PC 10; Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light-enhanced chemiluminescence system (Amersham).

Immunoprecipitation of PCNA. One hundred μg of isolated DNA synthesome were incubated overnight with a PC10 antibody directed against PCNA. Thirty μl of protein A-conjugated agarose beads were added to the
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reactions for 1 h. The reaction mixtures were washed twice with buffer A [0.154 M NaCl, 10 mM Tris–HCl (pH 7.4), 0.05% Triton X-100, and 0.05% SDS] and three times with buffer B [0.154 M NaCl, 50 mM Tris–HCl (pH 7.4), 2.5 M KCl, and 0.5% Triton X-100]. The protein was removed from the beads by incubation at 100°C for 30 s and analyzed by 2D PAGE.

Mutational Analysis of the PCNA Gene. The cDNA sequence encoding the entire PCNA translation unit was prepared using Superscript Preamplification System First Strand cDNA Synthesis (Life Technologies, Inc.) followed by cloning of the amplified cDNA into the vector pCR2.1 (InVitrogen) according to the manufacturer’s instructions. Total RNA was isolated using Trizol reagent (Life Technologies, Inc.). Second-strand cDNA synthesis was carried out by priming the first-strand cDNA with oligonucleotide 5’-GCCTACGGTCTTTGAGACTCCG-3’ on the 5’ end of the cDNA and 5’-GCAGTTCTAAAGAGCTTAG-3’ on the 3’ end of the cDNA and amplifying the primed first strand using reverse transcriptase PCR.

Results

Unique Form of PCNA Identified in Malignant Breast Cell Cultures. Studies were performed to determine whether DNA synthesome components are structurally altered in malignant breast cells compared to nonmalignant cells. The DNA synthesome was isolated from four established breast cell lines (MCF7, MDA-MB468, Hs578T, and early passage MCF10A), as well as from nonmalignant primary breast epithelial cells using our published procedures (9). The malignant breast cell lines (MCF7, MDA-MB468, and Hs578T) produce tumors in animal breast cancer models (21), whereas the nonmalignant breast cell line (MCF10A) does not (22). The nonmalignant primary breast cells were prepared from a human breast reduction sample as described by Stampfer (19). Thirty μg of DNA synthesome isolated from MCF10A, MCF7, MDA-MB468, Hs578T and nonmalignant primary breast cells were each subjected to individual 2D PAGES (23, 24). These gels, containing the resolved synthesome polypeptides, were transferred to nitrocellulose membranes. Western blot analyses of the membranes were performed using an antibody directed against the 36 kDa PCNA polypeptide. A comparison of the mobility of the PCNA component of the MCF10A, MCF7, MDA-MB468, Hs578T, and primary cell-derived DNA synthesome (Fig. 1) indicated a clear and significant difference in this protein’s 2D PAGE profile for the nonmalignant and malignant cells. The PCNA associated with the synthesome isolated from malignant MCF7 and MDA-MB468 cells was present in two forms, a basic form and an acidic form (Fig. 1, A and E, respectively). The PCNA isolated from the malignant Hs578T cells exhibited PCNA with an acidic pi (Fig. 1C) and barely detectable levels of PCNA with a basic pi. PCNA in nonmalignant MCF10A and primary breast cells was present in a

Fig. 1. Protein migration of PCNA from human breast cell lines. Thirty μg of DNA synthesome protein isolated from four human breast cell lines (MCF7, MDA-MB468, Hs578T, and MCF10A) and nonmalignant primary breast cells were subjected to 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and analyzed by Western analysis with an antibody directed against PCNA. The protein migration patterns shown are: A, MCF7; B, MCF10A; C, Hs578T; D, nonmalignant primary breast cells; and E, MDA-MB468. Arrow, form of PCNA that is unique to malignant breast cells.
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**Fig. 2.** Protein migration of PCNA from malignant human and mouse breast tissue and nonmalignant human breast tissue. Thirty µg of isolated DNA synthesome from malignant human and mouse breast tumors and nonmalignant human breast tissue were subjected to 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and analyzed by Western analysis using an antibody directed against PCNA. The resulting protein migration patterns are shown: A and B, human ductal tumor; C and D, human lobular tumor; E, nonmalignant human breast tissue; and F, mouse tumor. The nonmalignant breast tissue (E) is derived from the same source as the human lobular tumor in D. Arrow, form of PCNA that is unique to malignant breast cells.

single form that exhibited a basic pi (Fig. 1, B and D, respectively). The PCNA form with the acidic pi was not detectable in the nonmalignant cells.

In a separate experiment, 2D PAGE analysis was performed using a sample containing isolated DNA synthesome from both MCF7 and MCF10A cells. The resulting protein migration pattern showed only one basic form and one acidic form of PCNA (data not shown). This result indicated that the basic form of PCNA was identical in both the malignant and nonmalignant cells, whereas the acidic form of PCNA was unique to the malignant cells.

**Breast Tumors Contain the Unique Form of PCNA.** Studies were initiated to determine whether the DNA synthesome derived from nonmalignant and malignant breast tissue exhibited the same 2D PAGE profile for PCNA as that observed in the non-malignant and malignant breast cell cultures. The DNA synthesome was isolated from a virally induced mouse breast tumor (20). The DNA synthesome isolated from six human lobular breast cancer tissues and from four ductal breast cancer tissues was also analyzed. For comparison, the DNA synthesome-associated PCNA isolated from nonmalignant breast tissue from two sources (breast reduction tissue and genetically matched nonmalignant tissue taken from the patients with malignant breast tumors) was examined. The purified DNA synthesome derived from these tissues was resolved by 2D PAGE. The resolved polypeptides were transferred to nitrocellulose membranes and probed with an antibody directed against PCNA. PCNA derived from malignant mouse and human tumor tissue had a 2D PAGE profile consistent with that of the malignant breast cell lines (Fig. 2, A–D and F). There were two forms of PCNA present, an acidic form and a basic form. The PCNA from the nonmalignant breast tissue was in the basic form (Fig. 2E). These findings were consistent with the 2D PAGE profile of PCNA from the MCF10A cells.

The **Unique Form of PCNA Is Not Proliferation Dependent.** To demonstrate that the abundant levels of the form of PCNA with a acidic pi was a property unique to malignant breast cells as opposed to a proliferation response, we analyzed the 2D PAGE profile of PCNA isolated from benign proliferative breast tumors and estrogen-stimulated MCF7 cells. Estrogen has a stimulatory effect on cellular proliferation (25). We found that the estrogen-stimulated cells had an increased rate of proliferation compared to control cells, as demonstrated by several parameters (Table 1). Similar findings have been reported by other investigators (25–27). The DNA synthesome was isolated from these cells, and the components were resolved by 2D PAGE followed by Western analyses for PCNA. There was an overall increase in the level of PCNA in estrogen-stimulated cells; however, there was no effect on the 2D PAGE profile for PCNA (Fig. 3, A and B).
Table 1  Stimulation of cell proliferation following treatment with 17-ß-estradiol

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control cells*</th>
<th>17-ß-Estradiol (E2)-treated cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H] Thymidine uptake</td>
<td>1,548 cpm/10^5 cells</td>
<td>10,564 cpm/10^5 cells</td>
</tr>
<tr>
<td>DNA polymerase α activity</td>
<td>496 ± 80 cpm/mg</td>
<td>1,359 ± 118 cpm/mg</td>
</tr>
<tr>
<td>Cells in S phase</td>
<td>10.7%</td>
<td>60.1%</td>
</tr>
</tbody>
</table>

* Control cells are MCF7 cells that were grown in phenol red-free DMEM, which was supplemented with 10% dextran-coated, charcoal-treated fetal bovine serum, 1% penicillin/streptomycin, and nonessential amino acids.

DNA polymerase α activity was measured as described by Malkas et al. (12).

Cell cycle distribution analyses of the cultured cells grown in the presence or absence of 17-ß-estradiol were performed as described by Lin et al. (35).

The DNA synthesome was also isolated from several benign breast tumors. In general, the 2D PAGE profile for PCNA from the benign tumors was identical to that of nonmalignant cells in culture and in nonmalignant breast tissue (Fig. 3C). These data provide compelling evidence that the acidic form of PCNA is characteristic of only malignant breast cells.

Genetic Mutation Is Not Responsible for the Acidic Form of PCNA in Malignant Breast Cells. Total cellular RNA isolated from MCF7 and MCF10A cells was used to clone the cDNA encoding the entire PCNA translation unit from each cell line. Four independent clones encoding the PCNA gene derived from MCF7 cells and four independent clones from MCF10A cells were sequenced. Sequence analysis indicated that these eight independent clones have an identical nucleotide sequence (Fig. 4). Furthermore, this nucleotide sequence does not differ from that of the sequence for the PCNA gene cloned from the human lymphoma cell line MOLT-4 (Ref. 28; Fi. 4).

Discussion

Here, it was demonstrated that a unique form of PCNA with an acidic pI is present in malignant breast cells. This unique form is found in malignant breast cell cultures, as well as malignant breast tumors, but not in nonmalignant breast cell cultures, nonmalignant tissue, or a sampling of benign breast tumors. It was shown that the cancer-specific form of PCNA was not due to a proliferation response or genetic mutation. This novel form of PCNA is most likely a result of an altered posttranslational modification in the malignant breast cells.

One of the hallmarks of breast cancer is the accumulation of genetic mutations that lead to genomic instability (29). These mutations may contribute to uncontrolled cellular proliferation, resistance to antiproliferative processes, and metastasis (4, 7, 30). The accumulation of mutations in breast cancers is high, considering that normally mutations occur infrequently. This has lead to the hypothesis of a "mutator phenotype" (31). It was proposed that a cell's progression to malignancy is accompanied by the accumulation of multiple genetic mutations created by error-prone DNA replication and a reduction in the efficiency of the DNA repair processes in the cell. Sekowski et al. (10) have recently found that the DNA replication apparatus from malignant breast cells is mutagenic, resulting in a decreased replication fidelity. Due to the essential role PCNA plays in both DNA replication and DNA repair, the unique form of PCNA in malignant cells is a likely contributor to the accumulation of genetic mutations and genomic instability.

An implication of the described findings is that posttranslational modification of PCNA is most likely responsible for the development of the acidic form of the protein found in the cancer cells. Bravo and Celis (32) demonstrated that PCNA from HeLa cell extracts was not posttranslationally modified by phosphorylation and that acetylation, glycosylation and sialylation are not likely contributors to the migration pattern of PCNA. Simbulan et al. (33) have recently shown that PCNA is modified by poly(ADP) ribosylation. Further studies to determine how the posttranslational modification of PCNA affects its ability to interact with proteins involved in DNA replication and DNA repair are underway and should provide crucial insights into the role...
The nucleotide sequence of the PCNA cDNA cloned from the two breast cell lines pCR2.1 vector. Ampicillin-resistant colonies containing the cDNA were chosen using the exponentially growing MCF7 and MCF10A cells using reverse transcriptase PCR and the nucleotide sequences shown are those of MOLT-4 (A), MCF7 (B), and MCF10A (C). is aligned with the sequence reported for an acute lymphoblastic leukemia cell. The PCNA

CACTTAAAATACTACTTGGCTCCCAAGATCGAGGATGAAGAAGGATCTTAGGCATTCTTAAAATT
AATGAACCAGTTCAACTAACTTTTGCACTGAGGTACCTGAACTTCTTTACAAAAGCCACTCCACTCTCT
CACTTAAAATACTACTTGGCTCCCAAGATCGAGGATGAAGAAGGATCTTAGGGATTCTTAAAATTCAAG
AATGGAAACATTAAATTGTCACAGACAAGTAATGTCGATAAAGAGGAGGAAGCTGTTACCATAGAGATG
AATGGAAACATTAAATTGTCACAGACAAGTAATGTCGATAAAGAGGAGGAAGCTGTTACCATAGAGATG
TCAACGGTGACACTCAGTATGTCTGCAGATGTACCCCTTGTTGTAGAGTATAAAATTGCGGATATGGGA
ATTGGAGATGCTGTTGTAATTTCCTGTGCAAAAGACGGAGTGAAATTTTCTGCAAGTGGAGAACTTGGA
CAGGAGTACAGCTGTGTAGTAAAGATGCCTTCTGGTGAATTTGCACGTATATGCCGAGATCTCAGCCAT
CAGGAGAAAGTTTCAGACTATGAAATGAAGTTGATGGATTTAGATGTTGAACAACTTGGAATTCCAGAA
CAGGAGAAAGTTTCAGACTATGAAATGAAGTTGATGGATTTAGATGTTGAACAACTTGGAATTCCAGAA
GAAGATATCATTACACTAAGGGCCGAAGATAACGCGGATACCTTGGCGCTAGTATTTGAAGCACCAAAC
GAAGATATCATTACACTAAGGGCCGAAGATAACGCGGATACCTTGGCGCTAGTATTTGAAGCACCAAAC
AGCATGGACTCGTCCCACGTCTCTTTGGTGCAGCTCACCCTGCGGTCTGAGGGCTTCGACACCTACCGC
TGCGACCGCAACCTGGCCATGGGCGTGAACCTCACCAGTATGTCCAAAATACTAAAATGCGCCGGCAAT
AGCATGGACTCGTCCCACGTCTCTTTGGTGCAGCTCACCCTGCGGTCTGAGGGCTTCGACACCTACCGC
TGCGACCGCAACCTGGCCATGGGCGTGAACCTCACCAGTATGTCCAAAATACTAAAATGCGCCGGCAAT
TTGCAGGCACTCAAGGACCTCATCAACGAGGCCTGCTGGGATATTAGCTCCAGCGGTGTAAACCTGCAa
C) ATCTTCGAGGCGCGCCTGGTCCAGGGCTCCATCCTCAAGAAGGTG

Fig. 4. Nucleotide sequence of the PCNA cDNA cloned from MCF7 and MCF10A

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Another implication of this work is that the malignant cell-specific form of PCNA may be a more fundamental characteristic of cancer cells than any single type of genetic mutation. This supposition is based on the observation that the cancer-specific form of PCNA was found in 100% of malignant breast cells examined to date. This correlation is higher than that observed for other molecular markers for malignancy currently examined for in breast tumors (i.e., p53, BRCA, her2/neu, and progesterone and estrogen receptor status), which are only found in a fraction of all breast tumors.

The findings described here are novel in that this is the first time it has become possible to link the decreased fidelity of cellular DNA replication with a specific alteration to a component of the DNA synthetic apparatus of breast cancer cells. PCNA is currently used as a marker for cellular proliferation. The novel form of PCNA that we have identified may prove to be a new signature for malignant breast cells. Our findings provide the first evidence that the unique form of PCNA in breast cancer cells could potentially serve as a powerful marker for the detection of malignancy.

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