Psoriasin Expression in Mammary Epithelial Cells in Vitro and in Vivo

Charlotte Enerbäck,¹ Dale A. Porter, Pankaj Seth, Dennis Sgroi, Justine Gaudet, Stanislawa Weremowicz, Cynthia C. Morton, Stuart L. Schmitt, Robert L. Pitts, Jason Stampl, Kerry Barnhart, and Kornelia Polyak³

Department of Adult Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115 [C. E., D. A. P., P. S., K. P.]; Department of Pathology, Massachusetts General Hospital, Charlestown, Massachusetts 02129 [D. S., J. G.]; Departments of Pathology [S. W., C. C. M.] and Obstetrics [C. C. M.], Gynecology and Reproductive Biology, Brigham and Women’s Hospital, Boston, Massachusetts 02115; Department of Pathology, Beth-Israel Deaconess Medical Center, Boston, Massachusetts 02115 [S. S.]; Harvard Medical School, Boston, Massachusetts 02115 [C. E., D. A. P., P. S., D. S., S. W., C. C. M., S. S., K. P.]; and Imgenex Corporation, San Diego, California 92121 [R. L. P., J. S., K. B.]

Abstract

We determined, by serial analysis of gene expression (SAGE) analysis of normal and DCIS (ductal carcinoma in situ) mammary epithelial cells, that psoriasin and several other genes implicated in psoriasis are aberrantly expressed in high-grade, comedo DCIS. Real-time PCR, mRNA in situ hybridization, and immunohistochemical analysis of breast carcinomas confirmed that psoriasin is frequently overexpressed in estrogen receptor-negative tumors. To gain insight into regulatory pathways that control psoriasin expression, we developed polyclonal and monoclonal antibodies and investigated mechanisms that may account for elevated levels of psoriasin in DCIS. Here, we report that loss of attachment to extracellular matrix, growth factor deprivation, and confluent conditions dramatically up-regulate psoriasin expression in MCF10A mammary epithelial cells. All of these conditions are characteristic of high-grade DCIS and psoriatic skin lesions; therefore, the same mechanisms may be responsible for increased expression of psoriasin in vitro and in vivo.

Introduction

Psoriasin was originally identified as a protein, the expression of which is increased in psoriatic keratinocytes (1). Subsequently, psoriasin was also found to be up-regulated in abnormally differentiating primary keratinocytes, in squamous carcinoma of the bladder, and in a subset of in situ and invasive breast carcinomas (2–5). Psoriasin is a member of the S100 family of calcium-binding proteins (S100A7); it has been shown to bind calcium, and its basal expression is influenced by extracellular calcium levels (6, 7). S100 proteins have been implicated in diverse cellular processes including cell proliferation, apoptosis, differentiation, invasion, and metastasis (7). Among others, the expression of S100A2 and S100A6 are significantly down- and up-regulated, respectively, in breast tumors compared with corresponding normal epithelium, whereas the expression of S100A4 correlates with tumor progression and acquisition of metastatic phenotype (7). The partially secreted nature and restricted expression pattern of psoriasin to abnormal proliferative lesions of squamous epithelia makes it a candidate diagnostic marker (5, 8). Moreover, the high expression of psoriasin in these cell types suggests that it may play a role in the regulation of cell growth, survival, or differentiation.

Materials and Methods

Cell Lines and Culture Conditions. MDA-MB468 and MCF10A cell lines were obtained from American Type Culture Collection and were maintained in 10% fetal bovine serum in McCoy’s medium (Life Technologies, Inc.) and in 5% horse serum in DMEM/F12 medium (Life Technologies, Inc.) supplemented with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin, and 500 ng/ml hydrocortisone, respectively. To determine the effect of serum deprivation on HID-5/psoriasin expression in subconfluent or confluent cultures, MCF10A cells were switched to 0.2% serum containing DMEM/F12 medium and incubated for the indicated time (see Fig. 2B). The effect of confluence was analyzed by maintaining MCF10A cells in confluent conditions for the indicated time (see Fig. 2B) with frequent (every other day) medium changes. For suspension cultures, MCF10A cells were trypsinized, resuspended in fresh medium (1.75 × 10^4 cells/ml medium), plated into poly-2-hydroxy-ethylmethacrylate (Aldrich)-coated (1 mg/cm² in 100% ethanol) Petri dishes, and incubated for the indicated time (see Fig. 2C).

Generation of Polyclonal and Monoclonal Anti-HID-5/Psoriasin Antibodies. Rabbit polyclonal anti-HID-5/psoriasin antibody was generated against a synthetic peptide corresponding to amino acids 83–100 of the human protein (TDYHKQSGHGAAPCSGQS). For the generation of mouse monoclonal antibodies, a PCR-generated BamHI-HindIII cDNA fragment of full-length human HID-5/psoriasin was subcloned into BamHI-HindIII sites of pQE-30 (Qiagen), yielding a construct that encodes HID-5/psoriasin with an NH₂-terminal hexahistidine sequence. The protein was expressed in M15[pREP4] bacteria, purified to homogeneity using denaturing urea buffer and NTA beads (Qiagen). Bound protein was eluted in 50 mM Tris (pH 7.5), 500 mM imidazole, 100 mM EDTA, 1 mM NaCl, 10% glycerol, and 1 mM DTT and used for hyperimmunizing Balb/c mice in collaboration with Imgenex (anti-HID-5/psoriasin monoclonal antibodies are commercially available from Imgenex).

Western Blot Analysis, Immunohistochemistry, and Tissue Microarrays. Western blot analysis of cell lysates and immunohistochemistry were performed using anti-CD45 panleukocyte (Dako), anti-estrogen receptor α, anti-erbB2, and anti-HID-5/psoriasin (clone 1068-1) antibodies as described (10, 11). Tissue microarrays were purchased from Imgenex or were generated as described (12).

FISH, Real-Time PCR, Northern Blots, and mRNA in Situ Hybridization. FISH analysis of metaphase chromosome preparations from peripheral blood lymphocytes obtained from normal human males was performed according to the method described previously (13). Interphase nuclei from disaggregated, formalin-fixed, paraffin-embedded tumor tissue were prepared during recent SAGE analysis of the gene expression profiles of normal and DCIS mammary epithelial cells, we identified HID-5/psoriasin as one of the most abundant transcripts in high-grade DCIS (9). To begin to elucidate the function of psoriasin as it relates to the initiation and progression of breast carcinomas, we developed polyclonal and monoclonal antibodies and examined its expression in vitro.

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² Present address: Department of Clinical Genetics, Sahlgrenska University Hospital SUO, S-416 85, Göteborg, Sweden.

³ To whom requests for reprints should be addressed, at Department of Adult Oncology, Dana-Farber Cancer Institute, D740C, 44 Binney Street, Boston, MA 02115. E-mail: Kornelia_Polyak@dfci.harvard.edu.

4 The abbreviations used are: SAGE, serial analysis of gene expression; DCIS, ductal carcinoma(s) in situ; HID-5, high in DCIS-5; FISH, fluorescence in situ hybridization; ER, estrogen receptor; PR, progesterone receptor.
as described, and FISH was performed according to methods described elsewhere (14). Metaphase chromosomes and interphase nuclei were counterstained with 4,6-diamidino-2-phenylindole-dihydrochloride. Laser capture microdissection, real-time PCR analysis, RNA isolation, and Northern blot analysis were performed as described (10). mRNA in situ hybridizations using 32P-labeled sense or antisense HID-5/psoriasin ribo-probes were performed as described (15).

Results and Discussion

Genes Aberrantly Expressed in DCIS and Psoriatic Lesions.

The generation of SAGE libraries has been described previously (9). Comparison of SAGE libraries generated from normal, intermediate, and high-grade DCIS revealed that HID-5/psoriasin is among the most highly differentially expressed transcripts and is one of the most abundant mRNAs in high grade DCIS (Table 1). Besides psoriasin S100A9, another S100 protein was also highly expressed in high-grade DCIS (Table 1). Both genes are localized to the long (q) arm of chromosome 1, and both have been shown to be up-regulated in psoriatic keratinocytes. This led us to examine the chromosomal localization of other highly differentially expressed genes and the expression level of genes implicated in psoriatic lesions (Table 1 and Fig. 1A). Surprisingly, a significant fraction (13 of 46 reliably mapped transcripts) of genes specifically overexpressed in high-grade DCIS is localized to the long arm of chromosome 1 (Table 1 and Fig. 1A). Structural abnormalities of chromosome 1 are among the most frequent cytogenetic abnormalities in breast carcinomas, and several genes involved in epidermal differentiation map to 1q (16, 17). To determine whether the overexpression of these 13 genes in this high-grade DCIS is attributable to aneuploidy/aneusomy of 1q, we performed FISH using two nonoverlapping bacterial artificial chromosomes containing the psoriasin and the ephrin A4 genes, respectively, on metaphase spreads from a normal individual and interphase nuclei from the DCIS used for SAGE (data not shown). After the confirmation of the chromosomal assignment of the HID-5/psoriasin gene to the long arm of chromosome 1 in band q21, interphase nuclei from the DCIS tumor tissue were hybridized with the bacterial artificial chromosomes containing the gene (data not shown). Two hybridization signals were noted in 31 of 33 (94%) nuclei examined, consistent with a normal number of copies for the genomic region tested. This result indicated that the aberrant expression of HID-5/psoriasin in this high-grade DCIS lesion was not caused by amplification of the HID-5/psoriasin locus. However, the possibility that the psoriasin gene may be amplified in other high-grade DCIS tumors cannot be excluded. In addition to psoriasin, several other genes known to be up-regulated in psoriatic keratinocytes were also aberrantly expressed in high-grade DCIS (Table 1; Refs. 18–20). These genes included S100A9, connexin 43, interleukins 6 and 8, interleukin 6 receptor, amphiregulin, and keratin 6. Squamous cell carcinoma antigen 1 (SCCA1) was also slightly up-regulated in high-grade DCIS, although because of the low abundance of this mRNA, the detected difference did not reach statistical significance. The aberrant expression of these genes in high-grade DCIS and psoriatic keratinocytes may be attributable to hyperproliferation, abnormal differentiation, or lymphocytic infiltration characteristic of both types of lesions (21, 22).

HID-5/Psoriasin Expression in Mammary Epithelial Cells in Vivo and in Vitro.

To evaluate the expression of HID-5/psoriasin in primary breast carcinomas, we performed real-time PCR analysis of 11 laser capture microdissection-purified primary tumors and corresponding normal mammary epithelium (Fig. 1B; Ref. 10). In most tumors, with the exception of two ER- and PR-positive, low- and intermediate-grade lesions (samples 57 and 65), HID-5/psoriasin levels were significantly (≥10 fold) increased relative to corresponding normal mammary epithelium (Fig. 1B). This result is in good correlation with that of a recent study describing a statistically significant association between psoriasin expression and lack of ERα and PR in invasive breast carcinomas (4).

To confirm HID-5/psoriasin expression in high-grade DCIS epithelial cells at the cellular level, we performed mRNA in situ hybridization of two low, two intermediate, and two high-grade DCIS tumors and corresponding normal epithelium (Fig. 1C and data not shown). HID-5/psoriasin is highly and specifically expressed by the tumor cells of the two high-grade comedo DCIS (Fig. 1C). In contrast, no hybridization signal was detected in low- and intermediate-grade DCIS and normal mammary epithelial cells (Fig. 1C and data not shown).

To analyze the expression of HID-5/psoriasin protein, we have generated and characterized polyclonal and monoclonal antibodies (Fig. 2A). Both the recombinant and the endogenous HID-5/psoriasin protein migrate as a Mr~11,000 single band (Fig. 2A). Next, we investigated whether HID-5/psoriasin expression can be detected under various growth conditions in MCF10A cells. MCF10A cells are normal immortalized human mammary epithelial cells that demonstrate no or very low levels of HID-5/psoriasin expression in sparse, exponentially growing cultures. To mimic the in vivo situations likely to occur in high-grade comedo DCIS and psoriatic skin lesions, cells were cultured in high (5%) and low (0.2%) serum containing medium in sparse or confluent conditions. Culture in low serum-containing medium and confluent conditions regardless of the serum concentration led to dramatic up-regulation of HID-5/psoriasin protein levels (Fig. 2B). The highest HID-5/psoriasin protein levels were observed in confluent, serum-deprived cells (Fig. 2B). We also tested the effect of cell detachment from extracellular matrix by culturing MCF10A cells in suspension for 1–3 days. Lack of cell anchorage also dramatically increased HID-5/psoriasin protein levels (Fig. 2C). Northern blot analysis indicated that the up-regulation of HID-5/psoriasin expression by cell suspension and confluency occurred at the mRNA level (Fig. 2D). Cell cycle analysis of MCF10A cells revealed that serum deprivation, confluency, and lack of cell anchorage induces G1 arrest later on, followed by apoptosis (data not shown). The dramatic up-regulation of HID-5/psoriasin expression by these extracellular

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* SCCA1, squamous cell carcinoma antigen-1.
signals indicates that HID-5/psoriasin, similar to other S100 proteins, may play a role in the regulation of these cellular processes. Interestingly, keratinocytes derived from psoriatic lesions have been shown to be resistant to apoptosis compared with those derived from normal skin (23). Similarly, high-grade comedo DCIS tumors demonstrate high apoptotic rates (22), and surviving tumor cells are also likely to be relatively more resistant to apoptosis.

**HID-5/Psoriasin Is a Partially Secreted Cytoplasmic Protein.**
To determine the subcellular localization of the HID-5/psoriasin protein, we performed immunohistochemistry on MDA-MB468 and ex-

**Fig. 1. Chromosome 1 and HID-5/psoriasin expression in DCIS.** A, schematic map of chromosome 1 and localization of genes overexpressed in high-grade, comedo DCIS. The length of the bars indicates fold overexpression. Unigene identification numbers and SAGE tags are listed for each gene. B, real-time PCR analysis of HID-5/psoriasin expression in laser capture microdissection-purified primary breast carcinomas and corresponding normal epithelium. Fold change indicates the ratio of HID-5/psoriasin mRNA levels in normal and cancerous epithelium. Numbers indicate case numbers, and i and inv denote in situ and invasive carcinomas, respectively. The histological grade, ER, PR, and erbB2 status of the tumors are indicated. C, mRNA in situ hybridization using 33P-labeled human HID-5/psoriasin antisense and sense riboprobes in two high-grade comedo DCIS tumors. Hybridization on two low- and two intermediate-grade DCIS gave no signal (data not shown).
Potentially growing and serum-starved MCF10A cells using a monoclonal anti-HID-5/psoriasin antibody (Fig. 3A). Both nuclear and cytoplasmic staining were detected in MDA-MB468 and in serum-starved MCF10A cells, whereas no staining was seen using a negative control antiserum or in exponentially growing MCF10A cells (Fig. 3A). Previous results demonstrated that psoriasin can be detected in the urine of bladder cancer patients and is partially secreted by psoriatic keratinocytes (1, 8). To determine whether HID-5/psoriasin is also secreted by breast cancer cells, we performed immunoprecipitations using anti-HID-5/psoriasin polyclonal antibody and cell lysates and culture medium of MDA-MB468 cells. Immunoprecipitates were then resolved by SDS-PAGE and analyzed by anti-HID-5 Western blot. HID-5/psoriasin protein can be detected both from cell lysates and from the culture medium, whereas no protein was seen using preimmune serum (Fig. 3B). Thus, HID-5/psoriasin protein is also partially secreted or released by breast cancer cells; therefore, it may be detected in the body fluids of breast cancer patients and could potentially be used for breast cancer diagnosis.

**Immunohistochemical Analysis of HID-5/Psoriasin Protein Levels in Primary Breast Carcinomas.** To analyze the *in vivo* expression of the HID-5/psoriasin protein, we performed immunohistochemical analysis of formalin-fixed, paraffin-embedded breast carcinomas using monoclonal anti-HID-5/psoriasin antibodies. To assess the reliability of the staining, we analyzed a high-grade comedo DCIS tumor shown previously to express HID-5/psoriasin based on mRNA in situ hybridization. Intense immunohistochemical staining was detected in the tumor cells using anti-HID5 antibody, whereas no staining was seen using isotype control serum (Fig. 3C). Next, we analyzed two tissue microarrays: one composed of 5 samples of normal breast tissue and 30 primary invasive breast carcinomas (Fig. 3E, array 1); and one composed of 6 samples of normal breast tissue, 3 samples of benign hyperproliferative lesions, and 49 primary invasive ductal carcinomas (Fig. 3E, array 2). On array 1, three punches of each sample were arrayed on the slide, and tumors were grouped according to their histological grade (10 low-, 10 intermediate-, and 10 high-grade tumors). Array 1 samples were also analyzed for the expression of ERα and erbB2 and for the presence of leukocytes using anti-CD45, a panleukocyte antigen, by staining, whereas array 2 was analyzed for expression of the ERs and PRs and p53 (Fig. 3, D and E, and data not shown). A representative tumor is shown in Fig. 3D, whereas results are summarized in Fig. 3E. As expected, low-grade tumors were mostly ERα positive and erbB2 negative, whereas high-grade ones were mostly ERα negative and erbB2 and CD45 positive. No significant HID-5/psoriasin expression was detected in any of the normal breast tissue samples nor in the benign hyperproliferative lesions (Fig. 3E). Correlating with its expression pattern in DCIS lesions and the results of a previous study, HID-5/psoriasin-positive invasive tumors were mostly ERα negative in both arrays (Fig. 3E and data not shown; Ref. 4). Among the 78 tumors examined, 38 were HID-5/psoriasin positive (15 ERα+ and 23 ERα−) and 40 were HID-5/psoriasin negative (26 ERα+ and 14 ERα−). On the basis of these results, HID-5/psoriasin-positive tumors are more likely to be ERα negative (*P* = 0.04, Fisher’s exact test). Similar correlation between HID-5/psoriasin and ERα patterns was observed in breast
cancer cell lines as well (9). The one strong positive sample among the low-grade tumor group on array 1 was later found to be a high-grade DCIS lesion. HID-5/psoriasin is a putative chemoattractant for lymphocytes, and both psoriatic skin and high-grade DCIS lesions demonstrate frequent lymphocytic infiltration (21, 22). Although lymphocytic infiltration, as indicated by CD45 staining, was frequent in high-grade tumors, no clear association was seen between CD45 and HID-5/psoriasin positivity (Fig. 3E). This could be attributable to the relatively small sample size or to the fact that these were invasive and not in situ carcinomas.

In summary, SAGE analysis of gene expression profiles of normal mammary epithelial cells and DCIS tumors revealed that several genes implicated in psoriasis are aberrantly expressed in high-grade comedo DCIS, with HID-5/psoriasin being one of the most abundant transcripts in these tumors. Dramatic up-regulation of HID-5/psoriasin in mammary epithelial cells in vitro is induced by growth factor deprivation, cell confluency, and lack of attachment to extracellular matrix. Because all these conditions are likely to occur in psoriatic skin lesions and high-grade comedo DCIS characterized by high proliferation rates, the high expression of HID-5/psoriasin in these cells could be attributable to the same signals, and HID-5/psoriasin may play a role in the acquisition of apoptosis resistance of these cells.

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References

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