p16 expression in Paget's disease of the breast

F. Buxant, *M.D.*; **J.C. Noël,** *Ph.D.*

Breast Unit, Erasme Hospital, Free University of Brussels (ULB) (Belgium)

Summary

Background: Paget's disease of the nipple is generally associated with an underlying invasive cancer or an underlying ductal carcinoma in situ. Epidermotropic theory maintains that Paget's cells are derived from an underlying mammary in situ adenocarcinoma. Because p16 protein plays a major role in cell-cycle control and in tumoral cell mobility, we analyzed p16 expression in Paget's disease of the nipple and in associated underlying ductal carcinoma in situ. *Methods:* The expression of p16 protein was analyzed by immunohistochemistry in eight cases of Paget's disease of the nipple with associated underlying ductal carcinoma in situ. The Student's t-test (2-tailed) was used to establish the equality of means. *Results:* The expression of p16 protein was observed in 87.5% (7/8 cases) both in the nipple disease and in the associated underlying ductal carcinoma in situ. The difference between the two populations was not statistically significant. In normal breast tissue, no expression of the protein was observed. *Conclusion:* The positive p16 expression in Paget's disease of the nipple and the underlined ductal carcinoma in situ and its role in cell motility lead us to propose a role of p16 in the spread of this disease.

Key words: p16; Paget's disease; Breast; Carcinoma; Intraductal carcinoma.

Introduction

Paget first described Paget's disease of the nipple in 1874 after he noticed that changes in the skin adjacent to the nipple preceded the development of an underlying breast cancer [1]. In 1881, Thin observed that the nipple lesion contained malignant cells that were related to an underlying cancer [2] and suggested the process of intraductal extension of cancer through the major lactiferous sinuses that we know today as "pagetoid spread". Paget's disease may occur in the nipple in conjunction with an invasive cancer mass, with underlying ductal carcinoma in situ (DCIS), or alone without any underlying invasive breast carcinoma or DCIS. The associated underlying cancer may be located centrally in the breast adjacent to the nipple or peripherally in the breast. The most widely accepted hypothesis regarding the origin of Paget's cells is the epidermotropic theory, which maintains that Paget's cells are derived from an underlying mammary in situ adenocarcinoma [3, 4].

The p16 protein plays a major role in the cell-cycle control by way of cyclin D-dependant kinase4 (cdk4). Despite conflicting results, it has been suggested that p16 protein expression is associated with accelerated tumor growth and poor clinical outcome in breast carcinoma [5]. In addition, in uterine cervical cancer, colorectal cancer, and basal cell carcinoma, it has been demonstrated that elevated p16 expression accompanies the tumor invasion front and could play a role in tumor cell hypermobility [6-9]. As Paget's cells are considered to be intraepidermally hypermobile and migratory tumoral cells originating from underlying in situ intraductal carcinoma, the aim of the present study was to investigate p16 expression in Paget's disease of the nipple and in the associated underlying DCIS to determine if p16 could play a role in this disease by modulation of cell invasion. These data have not yet been published.

Materials and Methods

Patients

Tumor sample were obtained from eight patients with primary Paget's disease of the breast who had undergone mastectomy or central lumpectomy with sentinel lymph node dissection at the Erasme Hospital (Brussels, Belgium). All patients gave their consent for this study and the experimental research was performed with the approval of the local ethics committee.

Histologic evaluation

To establish immunohistochemistry, 4-µm sections were cut sequentially from the archival specimens and mounted onto superfrost-treated slides (Menzel-Glaser, Braunschweig, Germany). The slides were dried overnight at 37°C before deparaffinization in xylene and and rehydrated with graded ethanols. For the p16 antibody, the antigen retrieval method was used with an incubation period of 60 min in a hot water bath at 95-99°C with citrate buffer (pH 6.0). The slides were then cooled in the buffer for 20 min at room temperature; 0.3% H_2O_2 was added to the slides, which were incubated at room temperature for 30 min. After rinsing with tris-buffered saline (TBS), pH 7.6, normal horse serum was added to each slide for 20 min. The mouse monoclonal antibodies diluted in TBS (monoclonal p16 antibody, clone 16P04, dilution 1/50; Neomarkers, Fremont, CA, USA) were added to each slide and incubated for 60 min at room temperature. After rinsing, twice for 5 min in TBS, the slides were incubated for 30 min with diluted biotinylated secondary antibody. The sections were again washed for > 5 min in TBS buffer and incubated with an avidin-biotin-peroxydase complex for 30 min (Vectastain Elite ABC kit; Vector,

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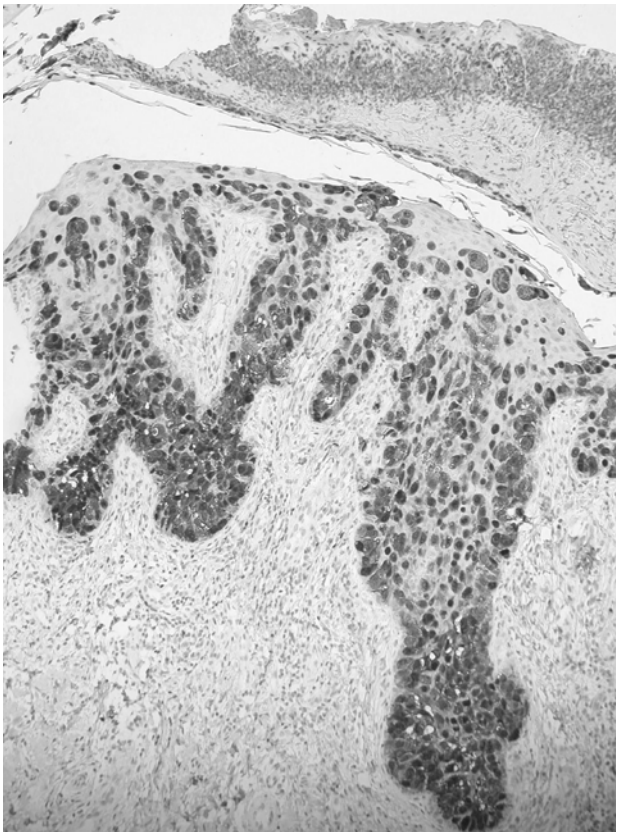

Figure 1. — Nipple Paget's disease. Strong overexpression of p16 protein. Note the negativity of adjacent keratinocytes.

Burlingame, CA, USA). After they were rinsed again, a peroxydase substrate solution (diamino-benzidine-tetrahydrochloride) was used to stain the slides. The sections were rinsed in tap water and counterstained in Gill's modified hematoxylin for 2-5 min, cleared, and mounted. As a negative control, normal mouse immunoglobulin G at a comparable concentration instead of the p16 antibody was included.

Evaluation of p16 expression was performed independently by two pathologists by using the H-score system [10]. The H score is based on a summation of the proportion of tumor cells showing different degrees of reactivity: 0 x % tumor cells negative + 1 x % tumor cells weakly positive + 2 x % tumor cells moderately positive + 3 x % tumor cells strongly positive. This gives a maximum total score of 300 if 100% of the tumor cells show a strong reactivity. In all cases, 500 cells were randomly counted.

Statistical analysis

The data were compared with the Student's t-test (2-tailed) to test for equality of means.

Results

The eight patients ranged in age from 54 to 90 years (median 54 years). All Paget's diseases of the nipple were associated with DCIS, and three were associated with an invasive carcinoma. Immunoreactivity of the p16 antibody was also positive in seven of eight cases of Paget's disease of the nipple and associated DCIS (Figure 1). One of the three underlying invasive carcinomas was positive (Table 1).

Table 1.

	Paget's nipple	Associated DCIS	Associated invasive carcinoma
p16 positivity	7/8	7/8	1/3
H score	165 ± 126	110 ± 97	33 ± 57

p value for H score Paget nipple/DCIS: p = 0.34.
p value for H score Paget nipple/invasive carcinoma: p = 0.04.

No difference was found to be significant (t-test for equality of means, $p > 0.02$) between Paget's disease of the nipple and the associated DCIS or invasive carcinoma.

Discussion

Both decreased and increased p16 expression have been described in primary human breast cancer, but these differences in expression have not been well correlated with clinical outcome [5]. In other human cancers, the loss of p16 expression, regardless of the mechanism, appears to confer a grave prognosis presumably because of more rapid cell growth and an increased mutation rate in p16 null cells [11].

Our study is the first to describe p16 overexpression in Paget's breast disease and in the underlying DCIS. High levels of p16 expression have been associated with loss of retinoblastomo-protein (pRB) expression in both primary cancers and cell lines of various kinds, presumably due to loss of a feedback loop and to pRB expression being transcriptionally repressed by ectopic p16 expression [12]. This loss of feedback would be associated with a higher cell proliferation rates and could explain why p16 without gene mutation is associated with larger primary tumors with a poor prognosis in breast cancers [5].

Because p16 is up-regulated at the invasive front of the majority of basal cell carcinomas with an infiltrative pattern and accompanied by cessation of proliferation [8], a similar role could be associated with the spread of the underlying DCIS to the nipple.

We may also consider the possibility that mutations in p16 might result in an effective protein that was overexpressed in a compensatory manner. In vitro, adenovirus-mediated p16/CDKN2 gene transfer suppresses glioma invasion and growth [13, 14]. Therefore, the loss of p16-controlled cell proliferation and invasion in the underying DCIS could suggest the process of intraductal extension of cancer through the major lactiferous sinuses that is known today as "pagetoid spread" [15].

Research into the proliferation index regarding p16 expression in DCIS and in Paget's lesions as well as into gene mutations in p16 may contribute to an understanding of this protein in Pagets's breast disease.

In summary, the similar p16 overexpression in the two cell populations confirms that they share the same

immunohistochemical profile [16]. The leading role of p16 in cell invasion and motility could explain the pagetoid spread of this disease but this will have to be verified in future studies.

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Address reprint requests to:
F. BUXANT, M.D.
Department of Obstetrics and Gynecology
CUB Hospital Erasme
1070 Brussels (Belgium)
e-mail: fbuxant@ulb.ac.be