American Journal of Pathology, Vol. 137, No. 6, December 1990

Rapid Communication

Study of Neu-protein Expression in Mammary Paget's Disease With and Without Underlying Breast Carcinoma and in Extramammary Paget's Disease

Kurt Meissner,* Alain Rivière,† Gudrun Haupt,* and Thomas Löning†

From the Departments of Dermatology and Gynecopathology† UKE, University of Hamburg School of Medicine, Hamburg, Federal Republic of Germany*

Correlation between neu/c-erbB-2/Her-2 gene amplification and overexpression of the neu gene product has been reported in tumors of glandular origin, especially ductal breast carcinomas. Formalin-fixed and dewaxed sections from 23 cases of mammary (MPD) and 9 cases of extramammary (EPD) Paget's disease were immunohistochemically stained by means of the monoclonal antibody 3B5 directed against an intracellular domain of the neu gene protein. All MPDs exhibited a distinct membrane staining of tumor cells independent of the presence of ductal breast carcinomas found in 18 cases. All these breast carcinomas also were positive for neu staining. In contrast to MPD, all EPDs were negative. Normal epidermis was always negative. Northern blot analysis sustained the immunohistologic findings in that the presence of neu mRNA could be demonstrated in two of three cases with MPD. Negativity in one case was due to dilution effects by nontumor cells. Our results suggest that Paget cells of mammary and extramammary localization, although very similar phenotypically, derive from different genetic accidents. Furthermore neu positivity in all MPDs and all underlying ductal carcinomas suggests common genetic alterations for both tumors. However the finding of five neu protein-positive MPDs without associated ductal breast carcinomas may suggest a somewhat different transformation process. (Am J Pathol 1990, 137:1305–1309)

Proto-oncogenes appear to be involved in the regulation of normal cellular proliferation or differentiation. Germline or somatic mutations (eg, translocation, deletion, or point mutation) or gene amplifications convert these genes into oncogenes that are involved in transforming or tumorigenic processes in target tissues.[1,2] One such gene, known as c-erbB-2, neu, or HER-2, encodes a p185 transmembraneous protein with tyrosinase activity that shows close homology to the epidermal growth factor receptor.[3–5] However a ligand for neu has not yet been identified.

To date, structural changes at the DNA level leading to neu protein alterations have not been found in human tumors.[6] Amplification of the neu gene has been found in breast and gastric adenocarcinoma cell lines,[5,7] in 15% to 40% of primary breast carcinomas[7–10] and in other adenocarcinomas.[11,12] Amplification results in elevated levels of neu mRNA and protein.[9,10,13] About 10% of breast carcinomas with elevated neu transcription levels do not have gene amplification.[10]

Mammary (MPD) and extramammary (EPD) Paget's disease are rare carcinomas *in situ*, the cytogenesis of which has been discussed with controversy (for review see Guarner et al[14]). However recent studies suggest, for both diseases, a malignant transformation of a pluripotent germinative cell of the epidermis.[14–17] Extramammary Paget's disease is most often restricted to the epidermis and adnexal epithelial structures,[14,18] whereas MPD was found to be associated usually with an underlying intraductal or invasive ductal carcinoma of the breast.[19,20] However in some cases of MPD, no underlying carcino-

Supported in part by the Deutsche Forschungsgemeinschaft Lo 285/2-4 and SFB 232D4.

Accepted for publication October 2, 1990.

Address reprint requests to Kurt Meissner, MD, Department of Dermatology UKE, Martinistr. 52, 2000 Hamburg 20, Federal Republic of Germany.

mas were found.[20,21] Because amplification of the neu gene has been observed only in tumors of glandular origin to date,[11,12] we examined a series of MPD and EPD from archival material for neu protein expression by means of a monoclonal antibody to obtain further insight into the cytogenesis and biology of these diseases. Furthermore Northern blot analysis was performed in three cases with MPD.

Material and Methods

Patients

From 9 EPDs, 7 tumors originated in the vulva, 1 in the perianal region, and 1 in the right axilla. Of 23 patients with MPD, 12 patients were diagnosed to have MPD associated with intraductal carcinoma and 6 patients showed MPD associated with primary invasive ductal breast carcinoma. Five patients were observed to have MPD without underlying ductal breast carcinoma. In addition, six biopsies with normal breast tissue were obtained.

Immunohistochemistry

Sections from formalin-fixed and paraffin-embedded biopsies underwent dewaxing, blocking of endogenous peroxidase activity by incubation for 20 minutes in 3% (vol/vol) hydrogen peroxide in methanol, and preincubation for 60 minutes in 5% (vol/vol) normal goat serum in phosphate-buffered saline (PBS). The slides were incubated overnight at 4°C with the primary monoclonal antibody (3B5; diluted 1:70 in PBS, pH 7.4; Dianova, Hamburg, FRG) directed against the amino acid residues 1242-1255 that belong to the inner domain of the neu gene product.[18] Subsequently the slides were exposed to biotinylated goat anti-mouse antibody (diluted 1:200 in PBS, pH 7.4; Vector Laboratories, Burlingame, CA) and to the avidin biotinyl peroxidase complex (diluted 1:100 in PBS, pH 7.4; Vector Laboratories), always for 30 minutes at room temperature. Visualization was done with diaminobenzidine and hydrogen peroxide. Between all steps, slides were washed three times for 10 minutes in PBS. Counterstaining was done with hematoxylin. Appropriate positive and negative controls always were included.

Northern Blot Analysis

Frozen material was available from three cases with MPD that were included in the immunohistologic examination. Before freezing, epidermal areas involved macroscopically in MPD were dissected from surgical specimens to eliminate other tissues as much as possible. After extraction according to the method of Chirgwin et al[22] using cesium chloride, 15 μg of total RNA was separated in denaturing conditions on a 1% agarose gel. After transfer on a nylon membrane, hybridization was done with a ^{32}P-labeled single-stranded RNA probe corresponding to a part of the external and transmembrane domains of the neu protein (Amersham RPN 1323, Arlington Heights, IL). After washing, the membrane was exposed to Kodak X-OMAT films (Kodak, Rochester, NY) at −80°C. For comparison, a biopsy of normal mammary gland tissue and four samples of invasive ductal breast carcinomas also were processed. The MKN7 cell line originating from a gastric adenocarcinoma and showing overexpression of a 4.6-Kb neu mRNA and, in addition, the expression of an aberrant 2.3-Kb mRNA[5] was used as an external control. Its total RNA was extracted according to the method of Perbal[23] for cell cultures and processed as described.

Results

In MPD, all 23 cases exhibited distinct membrane staining of tumor cells (Figure 1A). The intensity of membrane labeling varied. Membrane staining was confined to Paget cells and never observed in normal epidermis. All intraductal and invasive ductal breast carcinomas associated with MPD (18 cases) exhibited unequivocal membrane staining of most tumor cells (Figure 1B). In contrast, all slides with EPD demonstrated negative staining for neu protein (Figure 1C). Normal glandular breast tissue only showed diffuse granular cytoplasmic staining, whereas overlying epidermis was always negative. Northern blot analysis revealed a detectable amount of a 4.6-Kb neu mRNA molecule in two of three cases with MPD (Figure 2A–D). The signal was lower than that for normal mammary gland tissue. Neu mRNA expression was enhanced for three of four invasive ductal breast carcinomas and was absent in the fourth one. In this negative breast carcinoma, heterogeneity of neu-protein staining could be seen in tumor tissue of mixed ductal and solid differentiation with areas of necrosis and fibrosis.

Discussion

In a comprehensive study of neu gene amplification and expression in primary breast carcinomas, Slamon and coauthors[10] demonstrated that immunohistochemistry performed by means of a polyclonal antibody was the method to correlate most consistently with Southern blot results as compared with Northern and Western blots.

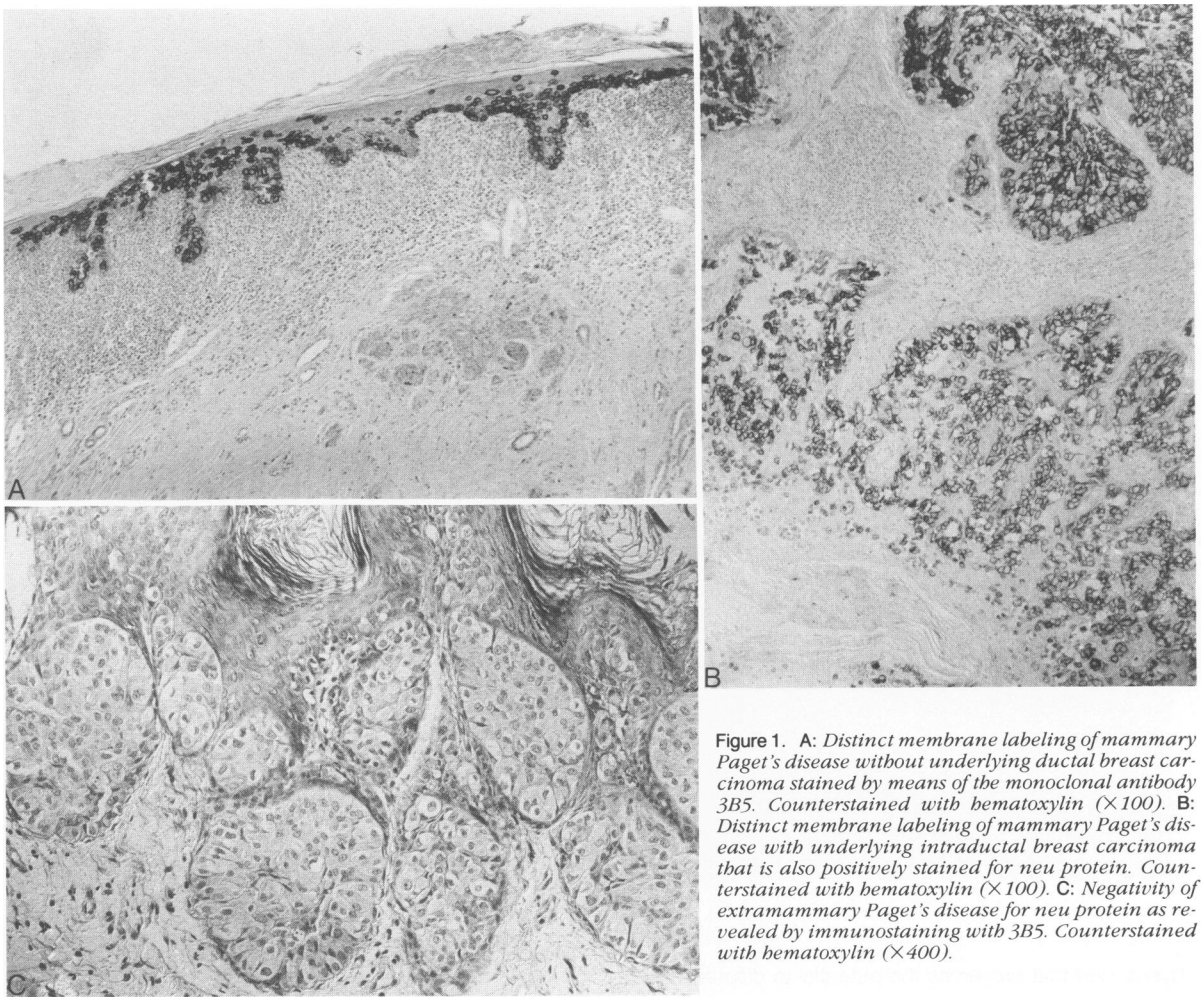

Figure 1. A: *Distinct membrane labeling of mammary Paget's disease without underlying ductal breast carcinoma stained by means of the monoclonal antibody 3B5. Counterstained with hematoxylin (×100).* B: *Distinct membrane labeling of mammary Paget's disease with underlying intraductal breast carcinoma that is also positively stained for neu protein. Counterstained with hematoxylin (×100).* C: *Negativity of extramammary Paget's disease for neu protein as revealed by immunostaining with 3B5. Counterstained with hematoxylin (×400).*

The good correlation between neu amplification and protein overexpression was confirmed by other authors also using polyclonal antibodies and frozen sections.[9,13] These findings were confirmed using formalin-fixed and dewaxed sections and two monoclonal antibodies (called 3B5 and 9G6).[24] Membrane staining was found in all cases with neu gene amplification, whereas no specific staining was observed in all tumors except one with a normal neu gene copy number.[24]

Using the monoclonal antibody 3B5 in our study, we observed distinct membrane staining of most tumor cells in all MPDs and negative labeling in all EPDs. The specificity of the immunohistologic neu membrane staining in our study was sustained by the finding of the expression of a 4.6-Kb neu-specific mRNA in two of three MPD cases examined. The autoradiographic signal was inferior in intensity to that of normal mammary gland tissue and of ductal breast carcinomas. The low signal in two cases and the negativity in the third case with MPD can be explained by the extensive dilution of tumor cells with normal epithelial, connective tissue, and other cells in specimens of MPD. The expression of neu mRNA in normal breast tissue obviously represents a baseline transcription in glandular tissue. Thus our findings underline the necessity of morphologic methods for the understanding of gene expression in Paget's disease to circumvent possible wrong interpretations of blotting results.

All 18 MPD-associated noninvasive and invasive ductal carcinomas showed the same distinct membrane staining as described by others[9,10,13,24,25] and observed for Paget cells in MPD in our study. The absence of associated ductal breast carcinomas in five MPDs[20,21] could suggest that MPD does not result from the spread of ductal tumor cells into the epidermis, at least in some cases. However, with regard to neu expression, no difference exists between MPDs with and MPDs without associated ductal carcinomas. This may suggest that the development of MPD and that of neu-overexpressing ductal breast carcinomas are not independent events. Thus an ancestor cell population with a common genetic alteration may be

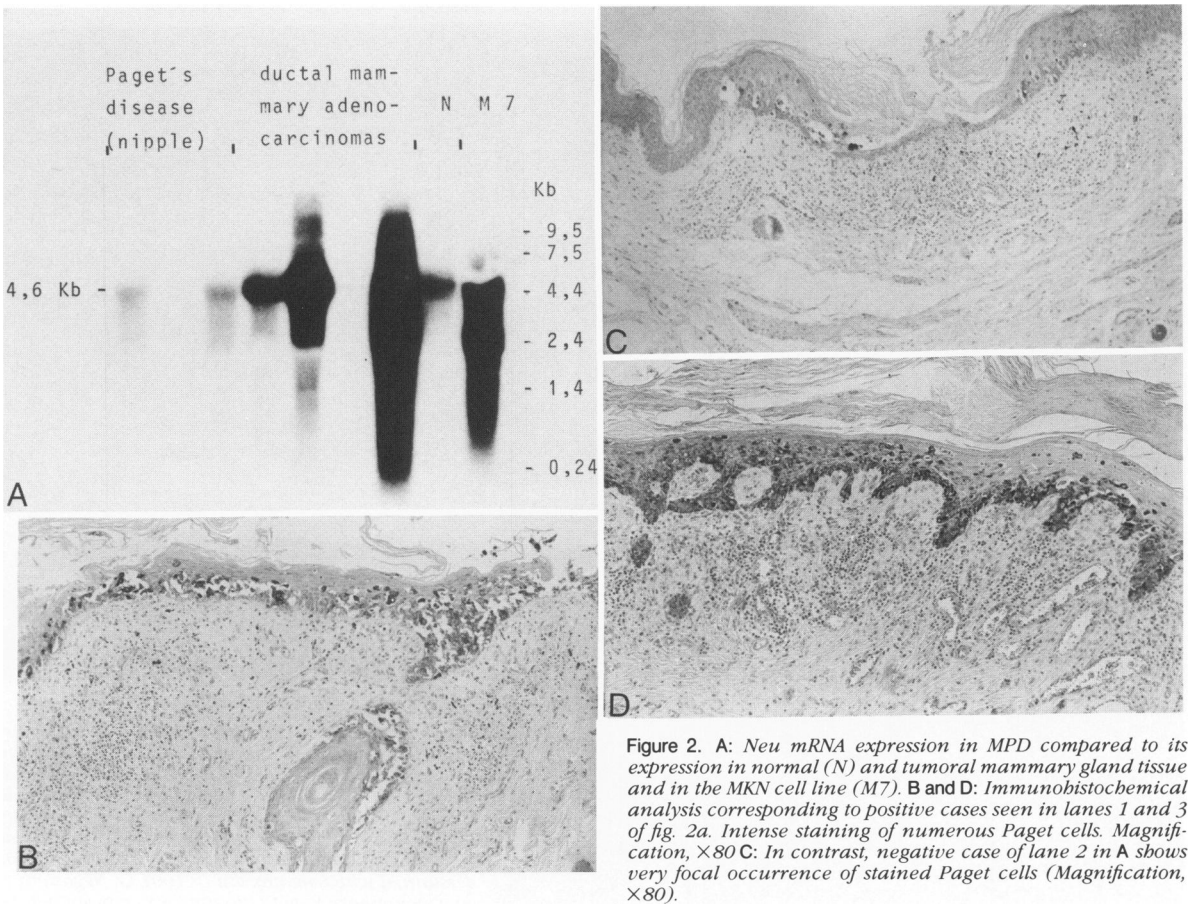

Figure 2. A: *Neu mRNA expression in MPD compared to its expression in normal (N) and tumoral mammary gland tissue and in the MKN cell line (M7).* B and D: *Immunohistochemical analysis corresponding to positive cases seen in lanes 1 and 3 of fig. 2a. Intense staining of numerous Paget cells. Magnification, ×80* C: *In contrast, negative case of lane 2 in* A *shows very focal occurrence of stained Paget cells (Magnification, ×80).*

hypothized that preserves the potential to differentiate to glandular or to nipple epidermis cells. The full tumor phenotype would then depend on an additional genetic event intervening with high probability in the two cell types from which both tumors originate. In any case, our findings suggest a role of the neu gene in the development of MPD and underlying ductal breast carcinomas.

Paget cells in MPD and EPD share many morphologic, histochemical, and immunohistologic characteristics.[14,17] The results of most previous studies have favored a homologous origin of these cells, whereas only very few reports have proposed a nonrelated one (for review, see Guarner et al[14]). Neu gene amplification was found to be limited to tumors of glandular tissues.[11,12] The striking difference in neu expression between MPD and EPD observed in our study, therefor, favors the hypothesis of a different origin. Furthermore it demonstrates that closely related tumor phenotypes may result from different genetic processes.

References

1. Bishop JM: Cellular oncogenes and retroviruses. Ann Rev Biochem 1983, 52:301–354
2. Weinberg RA: Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. Cancer Res 1989, 49: 3713–3721
3. Schechter LL, Hung M-C, Vaidyanathan C, Weinberg RA, Yang-Feng TL, Franke U, Ullrich A, Coussens L: The neu-gene: An erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. Science 1985, 229: 976–978
4. Coussens L, Yang-Feng TL, Chen YC, Gray A, McGrath J, Seeburg PH, Libermann TA, Schlessinger J, Franke U, Levinson A, Ullrich A: Tyrosinase kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science 1985, 230:1132–1139
5. Yamamoto T, Ikawa S, Akiyama T, Semba K, Nomura N, Miyagima N, Saito T, Toyoshima K: Similarity of protein encoded by the human c-erbB-2gene to epidermal growth factor receptor. Nature 1986, 319:230–234
6. Lemoine NR, Staddon S, Dickson C, Barnes DM, Gullick WJ: Absence of activating transmembrane mutations in the c-erbB-2 proto-oncogene in human breast cancer. Oncogene 1990, 5:237–239
7. Van de Vijver M, van de Bersselaar R, Devilee P, Cornelisse C, Peterse J, Nusse R: Amplification of the neu (c-erbB-2) oncogene in human mammary tumors is relatively frequent and is often accompanied by amplification of the linked c-erbA oncogene. Mol Cell Biol 1987, 7:2019–2023

8. Varley JM, Swallow JE, Brammar WJ, Whittacker JL, Walker RA: Alterations to either c-erbB-2(neu) or c-myc protooncogenes in breast carcinomas correlate with poor shortterm prognosis. Oncogene 1987, 1:423–430
9. Berger MS, Locher GW, Saurer S, Gullick WJ, Waterfield MD, Groner B, Hynes NE: Correlation of c-erbB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Res 1988, 47:1238–1243
10. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 1989, 244:707–712
11. Yokota J, Yamamoto T, Toyoshima K, Terada M, Sugimura T, Battifora H, Cline MJ: Amplification of erbB-2 oncogene in human adenocarcinomas in vivo. Lancet 1986, i:765–767
12. Zhou D, Battifora H, Yokota J, Yamamoto T, Cline MJ: Association of multiple copies of the c-erbB-2 oncogene with spread of breast cancer. Cancer Res 1987, 47:6123–6125
13. Venter DJ, Kumar S, Tuzi NL: Overexpression of the human c-erbB-2 oncoprotein in human breast carcinomas: immunohistochemical assessment correlates with gene amplification. Lancet 1987, ii:69–71
14. Guarner J, Cohen C, DeRose P: Histogenesis of extramammary and mammary Paget cells. An immunohistochemical study. Am J Dermatopathol 1989, 11:313–318
15. Mariani-Constantini R, Andreola S, Rilke F: Tumor-associated antigens in mammary and extramammary Paget's disease. Virchow's Arch (A) 1985; 405:333–340
16. Nagle RB, Lucas DO, McDaniel KM, Clark VA, Schmalzel GM: Paget's cells. New evidence linking mammary and extramammary Paget cells to a common cell phenotype. Am J Clin Pathol 1985, 83:431–438
17. Ordonez NG, Awalt H, Mackay B: Mammary and extramammary Paget's disease. An immunocytochemical and ultrastructural study. Cancer 1987, 59:1173–1183
18. Jones RE, Austin C, Ackerman AB: Extramammary Paget's disease. A critical reexamination. Am J Dermatopathol 1979, 1:101–132
19. Azzopardi JG: Major problems in pathology. Problems in Breast Cancer. Vol 11. Philadelphia, WB Saunders, 1979, pp 258–260
20. Haagensen CD: Diseases of the Breast, 2nd Edition. Philadelphia, WB Saunders, 1971, p 554
21. Jones RE: Mammary Paget's disease without underlying carcinoma. Am J Dermatopathol 1985, 7:361–365
22. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 1979, 18:5294–5299
23. Perbal A: A Practical Guide to Molecular Cloning, 2nd Edition. New York, J Wiley, 1988
24. Van de Vijver M, Peterse JL, Mooi WJ, Wisman P, Lomans J, Dalesio O, Nusse R: Neu-protein overexpression in breast cancer. Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med 1988, 319:1239–1245
25. Marx D, Schauer A, Reiche C, May A, Ummenhofer L, Reles A, Rauschecker H, Sauer R, Schumacher M: c-erbB-2 expression in correlation to other biological parameters of breast cancer. J Cancer Res Clin Oncol 1990, 116:15–20

Acknowledgments

The authors thank Prof. Dr. H. E. Stegner and Prof. Dr. M. Jänner for help in tissue sampling and R. Malik and H. Haupt for technical assistance.