Expression of the 21,000 Molecular Weight ras Protein in a Spectrum of Benign and Malignant Human Mammary Tissues

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ABSTRACT

Monoclonal antibodies RAP-5 and Y13-259, directed against the ras gene product [a protein with a molecular weight of 21,000 (p21)] have been used to evaluate ras p21 expression in malignant and benign mammary tissues as well as in the lesions of intermediate stature such as atypical hyperplasia using immunohistochemical assays. Invasive carcinomas demonstrated enhanced expression of ras p21, with generally decreasing expression in carcinoma in situ, atypical hyperplasia, and nonatypical hyperplasia, respectively. Heterogeneous expression of ras p21 was observed among primary as well as metastatic mammary carcinomas. Carcinomas from postmenopausal patients generally demonstrated higher levels of ras p21 than those from premenopausal patients, but no significant difference in ras p21 expression in carcinomas between estrogen-receptor rich and estrogen-receptor poor patients was found. Normal mammary epithelium in terminal duct lobular units from patients with hyperplasia generally demonstrated higher levels of ras p21 expression than did epithelium in large ducts. This demonstration of enhanced ras p21 expression by the epithelium of peripheral lobular portion of the breast is consistent with the previous hypothesis that these areas preferentially undergo malignant transformation. Analyses of the limited number of specimens available from patients with 15-yr follow-up revealed a generally higher level of ras p21 in hyperplasia from patients who subsequently developed carcinoma, as compared to those from patients without carcinoma development. However, no conclusions regarding the potential for malignant transformation could be drawn for any individual patient on the basis of ras p21 expression. Concomitant analyses of ras p21 expression in mammary carcinomas and benign lesions using liquid competition radioimmunoassay and immunohistochemical assay demonstrated the complementary nature of these alternative approaches. These results suggest that enhanced ras p21 expression may be involved in the early stages of mammary carcinogenesis but is probably not involved in the maintenance of the transformed phenotype.

INTRODUCTION

ras activation and subsequent transformation of a cell to its malignant phenotype has been associated with two mechanisms: (a) a point mutation at position 12 or 61 of the genome (1-3) or (b) enhanced expression of the normal cellular or proto-onc form of ras p21.1 The M, 21,000 product of the ras gene is the ras p21, M, 21,000, 2 the M, 21,000 protein product of the ras gene (4, 5). Mutated ras genes, however, have been identified in only a small percentage of the most common forms of human carcinomas (6). Several investigators have failed to demonstrate point mutated ras gene expression in human mammary carcinomas or cell lines (except the mammary carcinosarcoma cell line, HS578T) using NIH 3T3 transfection assays (6, 7). Recent studies utilizing MAb RAP-5 (RA:p21, P:peptide) generated against a synthetic peptide reflecting amino acid positions 10-17 of the human ras p21, have demonstrated enhanced expression of ras p21 in the majority of human mammary and colon carcinomas using formalin-fixed tissues and immunohistochemical technique, as compared to normal mammary and colon epithelium (8, 9). Furthermore, Spandidos and Agnantis (10) have recently described a significant elevation of Ha-ras transcription in malignant breast tumors as compared to normal breast tissues using dot-blot RNA hybridization techniques.

MAbs reactive with the ras gene product p21 can be utilized with formalin-fixed paraffin-embedded tissues and immunohistochemical techniques. Advantages of this approach include evaluation of a variety of benign, potentially premalignant and malignant tissues, determination of ras p21 expression at the single cell level, and correlation of histological information with ras p21 expression. Atypical hyperplastic lesions of the breast have been associated with an increased risk of subsequent carcinoma development (11-14) many years after a histological diagnosis of atypia has been made on surgically resected biopsy specimens. Hyperplastic lesions without atypia have a similar association, although the risk of subsequent malignant transformation is of lesser magnitude (11, 15). In addition, hyperplastic lesions have been found more frequently in breasts which have undergone malignant transformation than breasts without subsequent carcinoma (12, 16).

Based on morphological studies (16, 17) human mammary carcinomas have been hypothesized to arise in the terminal duct-lobular units. The use of formalin-fixed tissues for identification of particular cell types expressing ras p21 made possible the direct evaluation of surgically resected tissues in an attempt to determine the possible role of the ras gene in the pathogenesis of human mammary carcinoma. This study was designed to define ras p21 expression in (a) benign and malignant mammary tissues as well as the lesions of intermediate stature such as atypical hyperplasia and carcinoma in situ at the single cell level, (b) normal mammary epithelium adjacent to either carcinoma or hyperplasia with reference to pathogenesis of carcinoma, and (c) carcinomas from patients with known estrogen receptor and menopausal status using MAbs RAP-5 and Y13-259 (18) directed against the ras gene protein product, p21. In addition, ras gene expression during mammary tumor progression was also evaluated using primary mammary carcinomas and metastases from the same patients.

MATERIALS AND METHODS

Materials. Tissues used for immunohistochemical analyses of benign and malignant mammary lesions were obtained from 46 patients with fibrocystic change (also known as fibrocystic disease), from 7 patients with carcinoma in situ, and from 47 patients with invasive carcinoma. The specimens containing fibrocystic change could be histologically subdivided into those without hyperplasia (20 cases) and those with hyperplasia (26 cases). The hyperplastic cases were further divided into 2 groups, without atypia (16 cases) and with atypia (10 cases). Hyperplasia is defined as the presence of 3 or more cell layers above the basement membrane without crossing the intraductal space (11, 14).
This designation is largely consistent with the atypical lobule Type A-II and A-III lesions of Wellings et al. (16) and recognizes any increase in cell number relative to the cell membrane (greater than the usual 2 cell layers) except for lesions having some of the features of carcinoma in situ, i.e., atypical hyperplasia (14). Patients with invasive carcinoma had no evidence of regional lymph node or distant metastases, and the size of each tumor was less than 4 cm diameter (Stage I or II: T1,2, N0, M0). The normal mammary duct terminology used was based on the proposals of Wellings et al. (16), and Obuchi et al. (17): from the peripheral portion of ductal system, DTL, ITD, ETD, subsegmental ducts, segmental duct, lactiferous sinus, and collecting duct. The DTL, ITD, and ETD form the TDLU. All portions of the ductal system other than the TDLU were termed collectively as LD (17).

Tissues for immunohistochemical analyses of primary and metastatic mammary carcinomas were obtained from 7 patients with primary and metastatic invasive ductal carcinomas (primary, \( n = 8 \); metastatic, \( n = 26 \)). These patients had metastatic lesions in a variety of different organs from 1 to 8 sites (average of metastatic sites is 3.7).

Tissues for hormonal and immunohistochemical analyses of mammary carcinoma were taken from a different set of 45 patients with invasive ductal carcinoma. Ten of these patients (younger than 40 yr) were premenopausal, while 35 patients (older than 55 yr) were postmenopausal. Twenty-six patients were considered ER rich and 19 were ER poor (see estrogen receptor analysis).

Biopsy tissues for direct binding liquid competition RIA and immunohistochemistry were obtained from 2 patients with invasive ductal carcinoma, one with fibrocystic change with ductal hyperplasia, and one with fibrocystic change. Immediately after collection, tissues were cut into pieces approximately 0.5 g, quick frozen in liquid nitrogen, and stored at \(-70^\circ\)C for direct binding liquid competition RIA analysis. An adjacent piece of tissue was fixed in 10% formalin and embedded in paraffin for histopathological and immunohistochemical studies.

Monoclonal Antibodies. Murine IgG2a Mab RAP-5 was generated using a synthetic peptide reflecting amino acid positions 10–17 of the human ras gene product p21, from the T24 human bladder carcinoma cell line (8). This antibody reacts with both the point-mutated and proto-onc forms of ras p21. Mab Y13-259, generated by Furth et al. (18), is a rat-derived Mab which immunoprecipitates both the point-mutated and proto-onc forms of ras p21 of the human, rat, and mouse. MAbs UPC-10 (a purified murine myeloma IgG2a protein) and MOPC-21 (a purified murine IgG1 myeloma protein) (19) were obtained from Litton Bionetics (Charleston, SC).

Immunohistochemical Assays. Immunohistochemical assays were performed using a modification of the methods of Hsu et al. (20) as described previously (21). In brief, 5-μm sections of formalin-fixed paraffin-embedded tissues mounted on gelatin-coated slides were deparaffinized and immersed in methanol containing 0.3% H2O2 to eliminate endogenous peroxidase activity. The sections were rinsed in 10 mM PBS without CaCl2, MgCl2, pH 7.4. In assays using Mabs RAP-5 and UPC-10, sections were incubated with 10% normal horse serum for 15 min. This and all subsequent reagents were diluted in PBS containing 0.1% BSA and added at 200 μl/slide. After removal of the horse serum, purified Mab RAP-5 was added to each section at 5 μg/ml, and the slides were incubated for 30 min at room temperature. Purified Mab UPC-10 (5 μg/ml) was used as an isotype identical (IgG2a) control for primary antibody RAP-5 on the serial sections. The sections were washed in PBS and incubated with biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA) for 30 min. After a PBS wash, the slides were treated with avidin dehydrogenase and biotinylated horseradish peroxidase H complex for 30 min. Following another rinse with PBS, the slides were treated with 0.06% diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and 0.01% H2O2 for 5 min and rinsed with PBS. The sections were counterstained with hematoxylin.

Immunohistochemical assays using Mab Y13-259, a rat-derived IgG (subtype unknown) Mab, were performed using the same techniques as described for Mab RAP-5, except for pretreatment (with 10% normal rabbit serum for 15 min), primary antibody incubation (with rat ascites fluid of Mab Y13-259 at 1:500 dilution or purified Mab at 60 μg/ml for 30 min), and second antibody incubation (with biotinylated rabbit anti-rat IgG; Vector Laboratories, Inc.). Mab MOPC-21, a murine myeloma protein of IgG1 isotype, was used at 60 μg/ml as a control for nonspecific binding of immunoperoxidase reagents to the tissues. In the assays with Mab MOPC-21, 10% normal horse serum was used as a pretreatment of tissue section, and biotinylated horse anti-mouse IgG was used as a second antibody.

The specimens which D. L. P. and S. A. H. selected for preservation of paraffin blocks as well as clearly documented clinical data were studied by N. O., A. T., P. H. H., and J. S. using immunohistochemistry without any knowledge of their clinical history. All tissues from the 2 institutions (Vanderbilt University, Nashville, TN and George Washington University, Washington, DC) were utilized; hence, bias was excluded. It is important to point out that formalin-fixed tissues obtained from certain institutions were unsuitable for ras p21 detection (high background) using the reagents and methodology described here. These tissues have since been shown to be either overheated or incompletely baked. The tissues from these institutions were excluded from this study.

Counting Methods for Immunohistochemical Evaluation. The percentage of Mab reactivity given was an estimation of the number of epithelial cells reactive with each Mab divided by the total number of epithelial cells of the same histological lesions × 100. The tissues were then subdivided into epithelial component categories (i.e., hyperplasia, hyperplasia with atypia, carcinoma in situ, invasive carcinoma, etc.). The epithelial cells within each category were scored for Mab reactivity. For Figs. 1 and 4, and Tables 1, 3, and 5, scoring of the most severe histological category was utilized. All of the epithelial cells included in tissue sections was examined. At least 5 histological lesions or more than 200 epithelial cells of benign or malignant lesions for each specimen were evaluated for Mab reactivity. As with all immunological methods, tissues scoring “negative” for Mab reactivity must be interpreted with full knowledge of the limitations of the sensitivity of the assay conditions used. No certainty as to the absolute lack of expression of ras p21 was intended by a negative designation. Negativity denotes relative lack of reactivity in comparison to the “positive” reactivity obtained with other tissues at the same Mab concentration and conditions. The evaluation of reactivity of Mabs with these specimens was done without knowledge of subsequent disease outcome or hormonal status of the patients.

Statistics. The average percentage of reactivity of Mabs with mammary tissues are presented as mean ± SD. Results are compared by Wilcoxon rank sum test for nonparametric methods (22).

Estrogen Receptor Analysis. Estrogen receptor content was determined on the same specimens which were evaluated for immunoreactivity with Mab RAP-5, using the dextran-coated charcoal method with tritium-labeled 17β estradiol (23). Results were obtained by Scatchard analysis of the binding data. Tumors with ER levels greater than 20 fmol/mg protein were considered ER rich and tumors with ER levels less than 5 fmol/mg protein were considered ER poor.

Direct Binding Liquid Competition Radioimmunoassays. Fifty μl of a protein extract of the Ha-transformed NIH 3T3 cell line (10 μg) were added to each well of 96-well microtiter plates and allowed to dry overnight by incubation at 37°C (detection plate). To minimize non-specific protein adsorption, microtiter wells of detection plate and the reaction plate (a plate with no extract adsorbed to wells) were treated with 100 μl of 5% BSA in PBS and incubated for 1 h at 37°C. The BSA was removed and the wells were washed with 1% BSA in PBS. For each well of the reaction plate, 40 μl of 125I-labeled Mab Y13-259 (75,000 cpm) diluted in PBS containing 1% BSA, 0.05% Tween 20 (assay buffer), and either 40 μl of competitor antigen (diluted in assay buffer containing 0.02% Empigen) or assay buffer containing 0.01% Empigen were added. The final detergent concentration in each well was 0.05% Tween and 0.01% Empigen. The reaction plates were incubated for 1 h at room temperature. Thirty-μl aliquots were transferred from each well of the reaction plate to the detection plate in duplicate. The detection plates were then incubated 16–18 h at 4°C. The wells were washed three times with assay buffer and cut from the plate. 125I-labeled Mab Y13-259 IgG bound to the wells was measured in a gamma counter. The percentage bound was determined by dividing the average of the cpm bound to wells of the detection plate in the
RESULTS

Immunohistochemical Reactivity of MAb RAP-5 with Benign and Malignant Mammary Tissues. Formalin-fixed tissue sections of biopsy material from a spectrum of mammary lesions were analyzed for ras p21 expression using MAb RAP-5 and the avidin-biotin-peroxidase complex immunohistochemical method. As shown in Figs. 1A and 2A, 10 of 20 specimens containing fibrocystic change without hyperplasia were completely negative for reactivity to MAB RAP-5. Another 10 specimens reacted very slightly (<5% of positive epithelial cells), demonstrating low levels of ras p21 expression in the epithelium which was anatomically located in the TDLU. Specimens with fibrocystic change represent a spectrum of epithelial and stromal variations considered “normal” maturational events in the postpubertal breast. In specimens obtained from women in this category hyperplasia was not identified. Hyperplastic lesions without atypia demonstrated slightly higher levels of ras p21 expression than did non-hyperplasia (P < 0.01) with an average reactivity of 18% epithelial cells positive (Fig. 1B). In 8 of 16 specimens, hyperplastic epithelium demonstrated ≥20% of cells positive, whereas 3 of 16 were completely negative. Hyperplastic lesions with atypia, i.e., atypical ductal hyperplasia (4 cases) and atypical lobular hyperplasia (6 cases) generally demonstrated slightly higher levels of ras p21 expression than did nonatypical hyperplastic epithelium (P < 0.05) and nonhyperplastic epithelium (P < 0.01) with an average reactivity of 34% of epithelial cells present (Fig. 1C). Atypical ductal hyperplasias (which had some but not all of the requisite features of ductal carcinoma in situ) reacted strongly with MAb RAP-5, particularly in the most histologically atypical cells (Fig. 2B). These findings were also observed in atypical lobular hyperplasias (Fig. 2C), which are similar to lobular carcinoma in situ but lack the complete histological criteria for that diagnosis (14). Three of 4 specimens containing atypical ductal hyperplasia and 5 of 6 specimens with atypical lobular hyperplasia demonstrated ≥20% of positive cells using MAB RAP-5. Early cancerous lesions without invasion (carcinoma in situ) generally demonstrated slightly higher ras p21 expression (Figs. 1D and 2D) than did atypical hyperplasias, but the difference is not statistically significant (P < 0.1). Of the 7 ductal carcinoma in situ, 5 contained ≥60% of is in situ carcinoma cells expressing detectable levels of ras p21. Invasive carcinomas, i.e., invasive ductal carcinoma and invasive lobular carcinoma, demonstrated enhanced levels of ras p21 expression, averaging 62% of carcinoma cells positive with MAB RAP-5 (Fig. 1E). The average reactivity of invasive carcinomas is not significantly different from that of carcinoma in situ (P > 0.1) but is statistically different from that of atypical hyperplasias or nonatypical hyperplasias (P < 0.01). The percentage of carcinoma cells reactive varied from 1 to 95%, with the majority of cases (36 of 47) demonstrating at least 50% of the carcinoma cells expressing ras p21. As shown in Fig. 1, it is important to note that antigenic heterogeneity of ras p21 expression was observed in the vast majority of mammary carcinomas as well as hyperplastic lesions.

The cellular reactivity of MAB RAP-5 with specific histological lesions concurrent with invasive ductal carcinoma was also examined (Fig. 3). Thirteen of 47 patient specimens with invasive carcinoma contained a myriad of histological lesions as well as normal mammary epithelium. These included ductal carcinoma in situ and hyperplasias of various type. The invasive ductal carcinomas demonstrated enhanced cellular ras p21 expression with an average of 63% of carcinoma cells reactive (Fig. 3D). The ductal carcinoma in situ component reacted slightly less than its invasive counterpart, with 58% of the in situ carcinoma cells expressing detectable levels of ras p21 (Fig. 3C). MAB RAP-5 reactivity was also observed in hyperplastic epithelial cells (Fig. 3B) as well as normal epithelial cells (Fig. 3A) adjacent to carcinomas. The cytoplasmic staining reaction noted in hyperplastic or normal epithelium, however, was generally weaker in intensity than that observed in carcinomas. Statistically, the differences in the average percentage of reactivity were significant among Fig. 3, B and C (P < 0.05), Fig. 3, A and B (P < 0.05), Fig. 3, B and D (P < 0.01) and D, B and D (P < 0.02) but not significant among Fig. 3, A and B (P < 0.1) and Fig. 3, C and D (P > 0.1).

ras p21 Expression in Hyperplastic Lesions with Subsequent Carcinoma Development. Of the hyperplastic disease patients evaluated above, 8 with nonatypical hyperplasia and 10 with atypical hyperplasia were clinically followed for 15 yr. These patients (n = 18) with hyperplasia were subdivided into 2 groups based on their clinical outcome (Table 1). Four of 10 patients with atypical hyperplasia (2 with atypical ductal hyperplasia and 2 with atypical lobular hyperplasia) subsequently developed carcinoma of the ipsilateral breast, and one of 8 patients with nonatypical hyperplasia subsequently developed ductal carcinoma in both breasts. Hyperplastic lesions from the patients without subsequent carcinoma development reacted with MAB RAP-5 in the range of 5–40% of hyperplastic epithelial cells positive, with an average reactivity of 21% (Fig. 4A). Nine of 13 specimens demonstrated ≤20% reactivity. In contrast, higher levels of ras p21 expression were generally observed in hyperplastic lesions from 5 patients who subsequently developed carcinoma, ranging from 25–55%, with an average of 40% of epithelial cells reactive (Fig. 4B). There was a significant difference of MAB RAP-5 reactivity between the 2 patient groups at P < 0.01 (Table 1). It is important to emphasize, however, that due to the nature of this study (i.e., the long term follow-up as well as preserved tissues) a relatively few samples have been evaluated, and no conclusions regarding the potential for carcinoma development can be drawn for any individual patient on the basis of ras p21 expression.
**ras p21 Expression in Normal Mammary Epithelium.** ras p21 expression was evaluated in normal mammary epithelium from the 18 patients with hyperplasia who were followed up for 15 yr. Normal mammary epithelial and myoepithelial cells adjacent to hyperplastic lesions demonstrated a moderately high reactivity with MAb RAP-5 in the DTL, and the reactivity decreased gradually as one moved to the LD, i.e., from the DTL to ITD, ETD, and LD (Table 2). The epithelium of TDLU which includes the DTL, ITD and ETD (16, 17) generally demonstrated higher levels of ras p21 expression than the LD (Fig. 5, A and B). This finding was most striking in the normal epithelium adjacent to hyperplasia from the patients who subsequently developed carcinoma. Of particular interest is the fact that normal epithelium of TDLU from the patients with subsequent carcinoma development generally demonstrated higher levels of ras p21 than did anatomically similar epithelium from the patients without carcinoma development ($P < 0.02$).

**Immunohistochemical Comparison of ras p21 Expression Using MAbs RAP-5 and Y13-259.** MAb Y13-259 has been used previously to detect ras p21 in a variety of tissues using Western blotting and immunoprecipitation techniques (18). We have now adapted this MAb for use with formalin-fixed tissue sections. To compare the reactivity of MAbs RAP-5 and Y13-259 with human mammary tissues, 7 cases (2 invasive ductal carcinomas, one ductal carcinoma in situ, 2 atypical hyperplasias, one hyperplasia without atypia, and one fibrocystic change without hyperplasia) were selected. The 2 atypical hyperplasia specimens were from patients who subsequently developed carcinoma. Table 3 shows the reactivity (both intensity and percentage of positive epithelial cells) of MAb RAP-5 and Y13-259 as well as the control MABs UPC-10 and MOPC-21 using serial sections of the various breast tissues. Although the percentage of reactivity of MAb Y13-259 with breast tissues was not exactly the same as that of MAb RAP-5, the staining pattern and anatomic location of immunoreactivity were almost iden-
ras p21 Expression in Mammary Carcinomas and Its Relationship to Estrogen Receptor and Menopausal Status. Forty-five mammary carcinomas from the patients whose menopausal and ER status were determined were evaluated for immunohistochemical reactivity with MAb RAP-5 (Table 4). Although a relatively small number of patients was analyzed in each group, carcinomas from postmenopausal patients generally demonstrated higher ras p21 expression than those from premenopausal patients (Fig. 7; P < 0.01). However, no significant difference of ras p21 expression in carcinomas between ER-rich and ER-poor patients was found.

ras p21 Expression in Primary and Metastatic Mammary Carcinomas. Tissues from 8 primary mammary carcinomas and 26 metastases from the same patients (n = 7) were examined for their immunohistochemical reactivity with MAbs RAP-5 and Y13-259. As shown in Fig. 8 there was a wide variety of reactivity of MAbs with metastatic carcinomas as compared to their respective primary carcinoma, although the reactivities of both MAbs were comparable in each lesion. For example, in case 1, the metastases to lymph node, skin, bone, colon, lung, and pancreas showed lower expression of ras p21 than the primary carcinoma, but the metastasis to appendix demonstrated higher ras p21 than the primary, and the metastasis to ovary demonstrated the same reactivity as the primary. Although in every case at least one metastatic mammary carcinoma showed a lower level of ras p21 expression than the respective primary carcinoma, in 6 of 7 patients the metastases to other organs demonstrated no change or even higher levels of ras p21 than their primary carcinomas. There is therefore a marked heterogeneous expression of ras p21 among metastatic as well as primary mammary carcinomas.

Comparison of ras p21 by Liquid Competition Radioimmunoassay and Immunohistochemistry. To determine if a correlation exists between the evaluation of ras p21 expression by immunohistochemical analyses and quantitative liquid competition RIA, both methods were utilized to evaluate ras p21 expression in the same specimens. Biopsies of benign breast from patients exhibiting fibrocystic change with and without hyperplasia demonstrated 11.9 and 3.5 pg ras p21/µg protein respectively by liquid competition RIA (Table 5). In contrast, approximately 3- to 12-fold more ras p21 was observed in 2 biopsies from patients with invasive ductal carcinoma. As a comparison immunohistochemical studies were performed and demonstrated elevated expression of ras p21 in the 2 carcinomas as well as lower expression in the 2 benign breast lesions. The immunohistochemical reactivity of MAb RAP-5 with each specimen was approximately equivalent to that of MAb Y13-259.

DISCUSSION

The utilization of MAbs directed against the ras gene protein product p21 has allowed the definition of relative levels of ras p21 expression at a cellular level using immunohistochemical techniques. We have previously reported enhanced expression of ras p21 in human colon and mammary carcinomas, as compared with their respective normal epithelium using the RAP MAbs which have been shown to react with both point-mutated and proto-onc forms of ras p21 (8, 9). Enhanced cellular protooncogene has been implicated in the development of certain human cancers (6, 24), suggesting that increased amounts of cellular oncogene proteins may alter the regulatory controls of cell transformation. Previous studies (9, 25) have suggested a lack of correlation between cell proliferation and
enhanced \textit{ras} p21 expression. Fasano \textit{et al.} (26) have recently demonstrated amplification of the nonmutated \textit{N-ras} gene expression in a human mammary carcinoma cell line (MCF-7) using a tumorigenicity assay. Moreover, Pulciani \textit{et al.} (27) have observed that transformation of NIH3T3 cells results from the combined effect of multiple copies of Ha-\textit{ras}-1 protooncogene rather than from spontaneous mutation of one of the transformed Ha-\textit{ras}-1 clones. Mammary carcinomas have been consistently negative in the NIH3T3 DNA transfection assay system, which is consistent with the evidence that enhanced \textit{ras} p21 expression reflects an increased expression of the cellular, or proto-onc form of \textit{ras} p21 (6, 7, 10).

MAbs RAP-5 and Y13-259 have been utilized in immunohistochemical assays using formalin-fixed paraffin-embedded human mammary tissues in order to define qualitative levels of \textit{ras} p21 expression in benign, potentially premalignant, and malignant mammary tissues at the single cell level. Our results show that there is a certain trend of \textit{ras} p21 expression, that invasive carcinoma demonstrates enhanced \textit{ras} p21 expression with generally decreasing expression in carcinoma in situ, atypical hyperplasia, and nonatypical hyperplasia. Of particular interest is the finding that atypical hyperplasia showed higher levels of \textit{ras} p21 (determined by the percentage of cell reactivity and intensity of staining) when compared to nonhyperplastic specimens (i.e., fibrocystic change). Hyperplasia generally demonstrated higher levels of \textit{ras} p21 than tissues without hyperplasia. Lesions without hyperplasia containing fibrocystic change or no histological abnormality were almost negative. The reactivity of MAb RAP-5 with carcinomas and adjacent benign tissues was also examined in attempt to define the extent and anatomic distribution of \textit{ras} p21 expression in epithelial cells of various histological types (Fig. 2). The data show that a trend that \textit{ras} p21 expression is higher in invasive ductal carcinoma with generally decreasing expression in carcinoma in situ, hyperplastic, and normal epithelial components of the same breast. These results are very preliminary, but suggest (a) an enhanced \textit{ras} p21 expression in carcinoma in situ and atypical hyperplasia as well as invasive carcinoma of the breast.

### Table 2

<table>
<thead>
<tr>
<th>Normal epithelium</th>
<th>Tissues from patients without subsequent carcinoma development (13 cases)</th>
<th>Tissues from patients with subsequent carcinoma development (5 cases)</th>
<th>Total (18 cases)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>DTL</td>
<td>ITD</td>
<td>ETD</td>
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<tr>
<td>TDLU includes DTL, ITD, and ETD.</td>
<td></td>
<td></td>
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<tr>
<td>LD includes collecting duct, segmental duct, and subsegmental ducts.</td>
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<tr>
<td>Total percentage was obtained by (DTL + ITD + ETD + LD)</td>
<td></td>
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<tr>
<td>Mean ± SD. Each percentage represents (reactive epithelial cells with MAb RAP-5) (total epithelial cells in different level of mammary structure) × 100.</td>
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<tr>
<td><strong>P &lt; 0.05.</strong></td>
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<td>*P &lt; 0.02.</td>
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<tr>
<td><strong>P &lt; 0.01.</strong></td>
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<tr>
<td><strong>P &lt; 0.001.</strong></td>
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### Table 3

<table>
<thead>
<tr>
<th>MAb and type</th>
<th>RAP-5 IgG2a</th>
<th>UPC-10 IgG2a</th>
<th>Y13-259 IgG</th>
<th>MOPC-21 IgG1</th>
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<tbody>
<tr>
<td>Invasive ductal carcinoma (1)*</td>
<td>++ (75)</td>
<td>−</td>
<td>++ (70)</td>
<td>±</td>
</tr>
<tr>
<td>Invasive ductal carcinoma (2)</td>
<td>± (1)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ductal carcinoma \textit{in situ}</td>
<td>++ (60)</td>
<td>−</td>
<td>++ (50)</td>
<td>−</td>
</tr>
<tr>
<td>Hyperplasia with atypia* (1)</td>
<td>+ (45)</td>
<td>−</td>
<td>+ (30)</td>
<td>−</td>
</tr>
<tr>
<td>Hyperplasia with atypia* (2)</td>
<td>+ (40)</td>
<td>−</td>
<td>+ (30)</td>
<td>−</td>
</tr>
<tr>
<td>Hyperplasia without atypia</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Fibrocystic change</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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* Numbers in parentheses, patient number. |
* Intensity of reactivity: ++ (strong); + (moderate); ± (weak); − (no reactivity). |
* Numbers in parentheses, % positive cells |
* From patients who subsequently developed carcinoma.
ras p21 EXPRESSION IN HUMAN MAMMARY TISSUES

**Fig. 6.** Comparable immunohistochemical staining of serial sections of invasive ductal carcinoma using MABs RAP-5 and Y13-259. A, staining pattern of MAB RAP-5 with the carcinoma. ×330. B, serial section treated with MAB Y13-259. ×330. Almost identical reactivity of MABs RAP-5 (A) and Y13-259 (B) was demonstrated in the cytoplasm of carcinoma cells.

<table>
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<tr>
<th>Table 4: Average percentage of reactivity of MAb RAP-5 with invasive ductal carcinomas of the breast according to ER and menopausal status</th>
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<tbody>
<tr>
<td>ER rich</td>
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<td>---------</td>
</tr>
<tr>
<td>Premenopausal</td>
</tr>
<tr>
<td>Postmenopausal</td>
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<tr>
<td>Premenopausal and postmenopausal</td>
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* Mean ± SD.
† Numbers in parentheses, number of patients examined.

and (b) a trend of correlation between ras p21 and degree of histological abnormality of the breast. Hence, ras gene activation and p21 expression may be involved in the early stages of mammary carcinogenesis.

In a previous report (8), elevated ras p21 was detected in young women with multiple fibroadenomas, a syndrome in which hormonal factors have been implicated. In light of previous studies which failed to demonstrate point mutations of the ras gene in human mammary carcinomas, the mechanism of ras activation in malignant and potentially premalignant lesions probably involves the enhanced expression of the normal cellular ras gene. This may be mediated by hormonally controlled promoter sequences because hormonal factors have been implicated in the development of these lesions (28), although the exact mechanism of enhanced gene expression in human mammary epithelium has not yet been clearly defined. Epidemiological and histological studies have shown that mammary carcinoma is often multifocal and is associated with other histological lesions in the same and/or contralateral breasts (29, 30). These observations are in concordance with our data.
showing multifocal ras p21 expression in mammary carcinoma and adjacent benign epithelium.

Higher levels of ras p21 expression were generally observed in hyperplasias (particularly in atypical hyperplasias) from patients who subsequently developed carcinoma, as compared to those from patients without subsequent carcinoma development. Clinical epidemiological experience has shown that atypical hyperplasias are associated with a higher risk of subsequent carcinoma development. Dupont and Page (11) and Page et al. (14) reported in their long term follow-up studies that the risk of invasive ductal carcinoma after a diagnosis of atypia was 4 to 5 times that of the general population. Although only the limited number of cases were analyzed (because most of the primary biopsy tissues have not been kept for more than 15 yr), our findings suggest that ras gene activation may be an early event in the process of cellular transformation from benign to malignant. These preliminary studies, however, must eventually be expanded before any conclusions can be drawn.

Normal mammary epithelium anatomically located in the TDLU generally demonstrated higher levels of ras p21 expression than that of the LD, particularly in specimens from patients who subsequently developed carcinoma. Previous morphological studies have suggested that the TDLU of human mammary gland (16, 17), and the terminal end buds of rat mammary gland (31) contain the epithelial cells which give rise to carcinoma. A spectrum of change from nonatypical hyperplasia through atypia to carcinoma within papillary lesions has been demonstrated in the TDLU by a 3-dimensional reconstruction study (17). In light of the findings reported here one might speculate that epithelial cells located in the TDLU expressing elevated ras p21 may be different from epithelium in the LD. This expression may indicate a transitional cellular state before the malignant phenotype, i.e., somewhere between the initiation, promotion, and transformation phases of carcinogenesis. This interpretation, however, must be viewed as preliminary because the data presented included only a limited number of patients with long term follow-up. In fact, until a much larger pool of specimens is obtained for both retrospective and prospective studies, no prediction in terms of carcinoma development for any individual patient on the basis of the level of ras p21 expression can be drawn.

The specificity of MAb RAP-5 for ras p21 expression was confirmed by parallel assays with MAb Y13-259 using same tissues. We detected ras p21 expression in formalin-fixed paraffin-embedded carcinoma and atypical hyperplastic tissues using MAb Y13-259 and ABC immunohistochemical assay, and obtained qualitatively similar cellular reactivity with MAB RAP-5. In addition, absolute values of ras p21 in breast biopsy specimens were determined using a liquid competition RIA, and the results were compared with the immunohistochemical reactivity of the same specimens using MABS RAP-5 and Y13-259. Quantitative evaluation of ras p21 by competition RIA correlated with reactivity observed using immunohistochemical analyses. These results validate the utility of immunohistochemical methods for the evaluation of relative levels of ras p21 in tissue specimens and demonstrate the potential value of concurrent analyses of tissues using both methods.

Kasid et al. (32) transfected viral Ha-ras DNA into MCF-7 human breast carcinoma cells and found that the cells acquiring an activated oncogene are estrogen independent in the nude mouse tumorigenicity assay. However, they were unable to find any effect of estrogen stimulation on cellular Ha-ras, myc, mht, erb, sis, or myb gene expression in MCF-7 cells. In this study, the relationship of ER, menopausal status, and ras p21 expression in human mammary carcinomas was evaluated using MAB RAP-5 and immunohistochemical assay. Generally higher levels of ras p21 were found in carcinomas from postmenopausal patients when compared to tumors from premenopausal patients. However, we could not find any significant differences in ras p21 expression between carcinomas in ER-rich and ER-poor patients. Our results suggest that estrogen stimulation may not effect levels of ras gene expression in human mammary carcinogenesis.

Activation of cellular oncogenes may be important events in tumor initiation, promotion, and progression. Whether or not subtle alterations in the regulation of cellular ras gene expression provide an impetus for further neoplastic events requires comprehensive analyses of different stages of tumor progression. In this study, tumors from primary mammary carcinomas as well as metastatic carcinomas from the same patients have been examined for the expression of ras p21 using MABs RAP-5 and Y13-259. In every case examined at least one metastatic mammary carcinoma showed a lower level of ras p21 than respective primary carcinoma, while in 6 of 7 cases metastases to other sites demonstrated no change or even higher levels of ras p21 expression than did their respective primary tumors. Gallick et al. (33) reported that in distant metastases from primary colon tumors, the level of ras p21 in tumor tissue was considerably reduced with respect to that observed by immunoblotting technique in primary colonic tumor tissue. However, they also found that metastases to the lung from 2 patients with mammary tumors and one patient with a sarcoma showed no changes in the level of ras p21 expression versus that observed in the primary tumor. The data reported here suggest that if ras gene activation is involved in cellular transformation of human mammary epithelium, a continuous expression of ras p21 in all tumor cells does not appear to be necessary for the maintenance of the transformed phenotype.

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REFERENCES


Expression of the 21,000 Molecular Weight ras Protein in a Spectrum of Benign and Malignant Human Mammary Tissues


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