Immunohistochemical detection of *ras* oncogene p21 product in benign and malignant mammary tissue in man

ANNA K GHOSH,* M MOORE,* M HARRIS†

From the Department of *Immunology, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester and the †Department of Pathology, Christie Hospital and Holt Radium Institute, Manchester

SUMMARY The monoclonal antibody RAP-5 generated against a synthetic peptide corresponding to amino acid positions 10-17 of the *ras* p21 protein was used in an immunohistochemical study of the expression of *ras* in normal, benign, and malignant breast epithelium in man. The staining intensity and intracellular distribution of RAP-5 was similar in the three epithelial populations and extended to other tissue elements including myoepithelial cells, smooth muscle, myelin, capillary endothelium, and stromal fibroblasts, as well as sebaceous glands and sweat glands overlying the breast. These results suggest that RAP-5 recognises a normal cellular component, the expression of which is not more enhanced in hyperplastic or neoplastic conditions. The detection of mutant forms of p21 exclusively expressed in malignant tumours requires that alternative reagents be developed.

At least 25 viral oncogenes with known cellular homologues have been described, and several of them are thought to have important roles in the initiation, promotion, or progression of tumours.^{1 2} The cellular oncogenes most commonly implicated in many different malignancies in man belong to the *c-ras* gene family. Activation of *ras* genes is usually a consequence of structural rather than regulatory mutations.³ Activated cellular Kirsten sarcoma virus oncogene (*c-Ki-ras*) has been detected in lung, colon, and ovarian tumours; cellular Harvey sarcoma virus oncogene (*c-Ha-ras*) in bladder and urinary tract tumours; and *N-ras* in haemopoietic malignancies and neuroblastomas.³⁻⁶

Ras genes code for a family of 21 000 dalton proteins (designated p21s) that seem to be situated beneath the cell membrane and have GTP-binding and GTP-ase enzyme activity.⁷⁻¹⁰ They are thought to participate in normal cellular proliferation, probably in the transmission of signals from a receptor at the cell surface. One mechanism by which the *ras* genes may become activated includes a point mutation resulting in a single amino acid change in the p21 product. Mutated forms of *ras* genes, as detected by transfection assays, have been estimated to occur in only 10%-15% of tumours in man. Raised values of *ras* mRNA have been detected by spot hybridisation analysis in various tumours in

man, including those of the breast and colon.^{11 12}

At the tissue level DNA and RNA hybridisation is presently difficult to achieve with fixed embedded pathological specimens, not least on account of the low copy number of the genes and message. Furthermore, hybridisation techniques provide no information on the ultimate concentration of a given gene product or its cellular distribution.

Accordingly, considerable effort has recently been devoted to the development of monoclonal antibodies against oncogene products and to their immunohistochemical application on pathological material. One such series of reagents has been generated against synthetic peptides corresponding to amino acid positions 10-17 of the *ras* p21 protein.¹³ The reactivity of one of these antibodies (RAP-5) with normal, benign, and malignant epithelium of the human breast is the subject of this study. We show that, in common with reagents reactive with other *ras* p21 epitopes, the product detected by RAP-5 is a normal component of several cell types and is neither limited to normal epithelial cells undergoing proliferation nor to their neoplastic counterparts.

Material and methods

Human breast tissue samples were obtained from the surgical pathology files of the histopathology department at this hospital. They had been fixed in

Table 1 Immunohistochemical staining reaction of RAP-5 monoclonal antibody with normal, benign, and malignant breast tissue

Histological diagnosis	No studied	Staining intensity			
		+/-	+	++	+++
"Normal"	2			1	1
Fibroadenoma	7			2	5
Fibrocystic disease	12	2	1	6	3
Periductal inflammation	1			1	
Duct papilloma	3			2	1
Medullary carcinoma	1		1		
Mucoid carcinoma	2		1	1	
Infiltrating lobular carcinoma	9	1		4	4
Intraductal carcinoma	3	1	1 ^a		1
Infiltrating ductal carcinoma	7	1			6
Total	47				

^a = heterogeneous.

formol saline and embedded in paraffin wax. Cases were chosen to represent a wide range of mammary pathology and included two cases designated "normal" breast tissue from patients with fibroadenoma, 23 benign, and 22 malignant. Table 1 shows the histological diagnoses.

ANTIBODIES

The monoclonal antibody RAP-5 was obtained from Dr J Schlom and was used at a dilution of 1/1000. This antibody has been raised against a synthetic peptide reflecting amino acid positions 10-17 of the *Hu-ras*^{T24} gene product Gly-Ala-Val-Gly-Val-Gly-Lys-Ser and has been shown to react with the *ras* gene product p21 by radioimmunoassay.¹³ Rabbit anti-mouse immunoglobulin was obtained from Dako-patts A/S and mouse monoclonal antialkaline phosphatase was kindly donated by Dr DY Mason.

IMMUNOENZYMATIC STAINING

Sections (5 µm) were picked up on gelatinised slides and stained by an immunoalkaline phosphatase technique.¹⁴ Briefly, this entailed successive incubation with primary mouse monoclonal antibody, rabbit anti-mouse immunoglobulin, and alkaline phosphatase-mouse monoclonal antialkaline phos-

phatase (APAAP) immune complexes. These last two steps were then repeated with shorter incubation times. Slides were washed in Tris buffered saline, pH 7.6, between each incubation step. The alkaline phosphatase reaction product was visualised using naphthol As Mx phosphate and fast red as substrates. Endogenous alkaline phosphatase was inhibited by adding 1 mM levamisole to the alkaline phosphatase substrate. A control section in which Tris buffered saline replaced the RAP-5 antibody was included in each run. No control sections showed staining.

Results

The results of staining of the "normal", benign, and malignant breast tissue samples are shown in Table 1. The staining intensity was graded from weak (+/-) to intense (+++).

NORMAL BREAST

The two cases of "normal" breast examined showed intense staining of the glandular epithelium. Both lobules and ducts were positive and the stain was generally uniformly distributed throughout the cytoplasm (Fig. 1). Areas of normal breast tissue adjacent to carcinomas showed a similar staining reaction.

Table 2 Titration of RAP-5 with malignant breast lesions showing approximate percentage of epithelial cells with positive reaction

Dilution of RAP-5	Intraductal carcinoma		Infiltrating ductal carcinomas		
1/1000	(95)+++	(90)++	(100)+++	(95)+	(100)++
1/2000	(80)+++	(20)+	(100)+++	(90)+	(100)++
1/4000	(80)+++	(5)+/-	(100)++	(60)+/-	(90)++
1/8000	(90)+++	(<1)+/-	(100)+++	(60)+/-	(80)+
1/16000	(40)+	0 Negative	(90)+/-	0 Negative	(50)+/-
1/32000	(20)+/-	0 Negative	(50)+/-	0 Negative	(20)+/-

The intensity of immunocytochemical staining was scored on a +/- to +++ scale; where considerable variation exists within a case the two extremes have been shown.

Each column represents one case.

FIBROADENOSIS AND CYSTIC DISEASE

The 12 cases in this group showed a consistent staining pattern similar to that observed in normal breast epithelium. Both ducts and lobules were generally uniformly stained, although ducts gave a more intense stain in some cases. Where epitheliosis and papillomatosis occurred a similar pattern of staining was observed. One case of cystic disease included a radial scar that also showed positive staining. Apocrine epithelium showed an intense cytoplasmic staining reaction usually stronger than that observed in normal epithelium (Fig. 2).

FIBROADENOMA

The epithelium in the fibroadenoma cases gave a consistent staining pattern similar to that observed in other benign breast conditions. The ductules gave a uniform cytoplasmic staining reaction (Fig. 3).

DUCT PAPILLOMA

The three cases of duct papilloma examined showed a similar staining reaction to that of other benign conditions, with intense cytoplasmic staining of the epithelium.

PERIDUCTAL CHRONIC MASTITIS

This case showed a similar staining reaction to that of other non-malignant conditions, with intense staining of the ducts and lobules.

CARCINOMAS

Carcinomas showed a more variable staining pattern with weak staining observed in three cases—one lobular carcinoma, one intraductal carcinoma, and an infiltrating ductal carcinoma); weak to moderate staining in the medullary carcinoma and one of the two mucoid carcinomas; and moderate to intense staining in the remaining 17 cases. Where the staining was intense most (over 90%) of carcinoma cells were positive. Where clumps of carcinoma cells were observed occasional heterogeneity of staining occurred, with the cells in the centre of the clumps showing weaker staining (Figs. 4 and 5).

As the intensity of stain with RAP-5 monoclonal antibody on benign and malignant breast tissue was similar at the original dilution used, dilution experi-

ments were carried out on a representative selection of cases. These results are shown in Tables 2 and 3. There was a gradual decrease in the intensity of stain with dilution on both benign and malignant breast lesions.

In addition to positive staining of epithelial cells with the RAP-5 monoclonal antibody, other tissue elements also stained. Myoepithelial cells gave variable staining, smooth muscle and myelin were positive in most cases (Fig. 6), and capillary endothelium and stromal fibroblasts were positive in some cases. Sebaceous glands and sweat glands in the skin overlying the breast also showed positive staining with the RAP-5 antibody.

Discussion

The activity of cellular *ras* proto-oncogenes has hitherto been associated with cell growth and proliferation and in an activated form with oncogenesis. The p21 proteins are thought to function as transducers of signals from the extracellular environment to the nucleus in a system with an intimate role in the control of cellular proliferation.¹⁵⁻¹⁷ Activation of *ras* seems to result in the delivery of a continuous, as opposed to an intermittent, regulated signal.¹⁸ Theoretically, this could occur via amplification of the normal p21 product, or by the synthesis of mutant forms. A monoclonal antibody that fails to distinguish between normal and mutated variants is likely to be useful only for the quantification of differences between normal, benign, and malignant tissues. The salient features of this study were that reactivity of RAP-5 with all three tissue categories was indistinguishable by the criteria of staining intensity, dilutional analysis, and intracellular distribution. The implications of these findings are broadly two fold: RAP-5 clearly recognises a normal cellular component, the expression of which is not more amplified in hyperplastic or neoplastic conditions; and mutant forms of p21 exclusively expressed in malignant tumours would not be detected by this reagent.

Several properties of RAP-5 relevant to this investigation have been described previously.¹³ Although it seemed to react differently with Hu-

Table 3 Titration of RAP-5 with benign breast lesions showing approximate percentage of epithelial cells with positive reaction

Dilution of RAP-5	Normal	Fibroadenoma	Cystic disease	Duct papilloma
1/1000	(95) + + +	(95) + + +	(100) + + +	(95) + + +
1/2000	(95) + +	(95) + +	(100) + + +	(90) +
1/4000	(80) + + +	(90) +	(90) + + +	(90) +
1/8000	(20) + -	(70) +	(90) + +	(70) + -
1/16000	(10) + -	(60) + -	(70) + -	(50) + -
1/32000	(5) + -	(20) + -	0 Negative	(4) + -

Fig. 1 Normal breast reacted with RAP-5 monoclonal antibody showing strong staining of lobules. (Haematoxylin counterstain.) $\times 400$.

Fig. 2 Epitheliosis showing strong staining of apocrine epithelium. $\times 250$.

Fig. 3 Fibroadenoma showing ductal epithelium positive with RAP-5. $\times 250$.

Fig. 4 *Infiltrating lobular carcinoma with foci of residual lobular carcinoma in situ showing positive cytoplasmic staining with RAP-5. $\times 250$.*

Fig. 5 *Infiltrating ductal carcinoma showing strong cytoplasmic staining with RAP-5. $\times 400$.*

Fig. 6 *Small nerve: myelin sheaths stain positively with RAP-5. $\times 400$.*

ras^{T24} in comparison with the normal Hu-*ras*^{Hu} peptide by solid phase radioimmunoassay, RAP-5 could not discriminate between Hu-*ras* gene products (Ha-*ras*, Ki-*ras*, and N-*ras*). The pattern of reactivity should, therefore, be regarded as "group specific" rather than "type specific", and the possibility of detecting mutant p21 by immunohistochemistry should be correspondingly diminished.

Less readily explained, however, is the finding of heterogeneous *ras* p21 expression in most of the primary mammary carcinomas, contrasting with its virtual absence from fibroadenomas and fibrocystic disease.¹³ These data suggest that *ras* p21 participates in the neoplastic transformation of mammary epithelium. In our hands dilutional analysis and comparison of staining intensities have consistently detected the product in normal and benign tissues, not only of breast as in the present study, but also in colorectal epithelium (unpublished data).

The numbers of specimens in the two studies (aggregate 98) are such that selection of material is unlikely to account for the differences. We therefore attribute the disparity to some hitherto unrecognised nuance of technique either at the level of tissue processing or storage, or both, or sensitivity of staining. Whatever the explanation, it seems that RAP-5, while undoubtedly of intrinsic interest for the study of *ras* gene translation systems, is of limited usefulness in routine immunopathology.

Other features of this study are that RAP-5 does not differentiate subpopulations of proliferating cells, nor cells with an increased propensity for metastasis. P21 is also reported to be membrane associated, so that the importance of the predominantly cytoplasmic staining we found is unclear.

One possibility is diffusion of the membrane associated protein into the cytoplasm as an artefact of the fixation process. There is even the possibility, however, not yet formally excluded, that the peptide sequence recognised by RAP-5 is unmasked at the tissue level in widely distributed proteins other than *ras* p21.

Our study compares with that of recent immunohistochemical experience in human colorectal neoplasia with another monoclonal antibody to *ras* p21 protein.^{19,20} The monoclonal antibody Y13 259 is one of a series produced in rats bearing sarcomas induced by Harvey virus²¹ and reactive with p21 protein species encoded by both the Harvey and Kirsten strains of the virus. The close homology of the p21 products of viral and cellular *ras* genes is such that the proteins are identical in all but three of the 189 amino acid residues.²² Y13 259 binds to a single protein band with an apparent molecular weight of 21Kd on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) which is present in substantial

quantities in *ras* transformed cells but at much lower levels in non-transformed cells. The pattern and intensity of *ras* p21 staining with this reagent, however, was comparable with that of colonic adenocarcinomas and normal mucosa,¹⁹ while adenomas reacted more strongly.²⁰ These results again differed from those obtained with the RAP series of monoclonal antibodies^{13,23} on colorectal tissue, which found that *ras* p21 expression correlated with the depth of invasion of colonic carcinoma within the bowel wall.

In conclusion, the inability of RAP-5 to distinguish between p21 proteins neither confirms nor denies the role of *ras* genes in mammary carcinogenesis in man. By the same evidence considerable overproduction of the normal p21 product does not seem to occur in this disease. Slamon *et al*⁶ and Tatosyan *et al*²⁴ showed that more than one oncogene may be activated in tumours arising from the same site. Different patterns of oncogene activation in tumours, consistent with current views on multiple carcinogenesis,²⁵ may conceivably be of different clinical importance, and such correlates may eventually be clarified by comparing oncogene activation profiles with clinical outcome. Antibodies to oncogene products might have a role in such studies, provided reagents of appropriate specificity at the tissue level could be generated. In situ hybridisation, or analysis of restriction fragment polymorphism of tumour DNA, however, might ultimately be more promising approaches.

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Request for reprints to: Dr AK Ghosh, Department of Immunology, Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, England.