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Immunolocalization of Cytoskeletal Elements in Human Mammary Epithelial Cells

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Abstract—*The expression of cytokeratins, vimentin and actin was analysed by immunohistochemical methods in primary cultures derived from breast carcinomas and non-malignant breast tissues, in tissue sections and in permanent breast cancer cell lines. A polyclonal antibody specific for cytokeratins bound to the majority of cells in all primary cultures. Cells positive for simple-epithelial cytokeratins were less frequent in primary cultures derived from malignant tissues than in cultures derived from non-malignant tissues. Established cell lines MCF-7 and BT-20 expressed high amounts of simple-epithelial cytokeratins.*

All outgrowing cells in primary cultures expressed high amounts of vimentin whereas vimentin expression in established carcinoma lines was variable but generally low. When paraffin sections of the original mammary tissues were analysed, epithelial cells were devoid of vimentin as expected.

Anti-actin antibody decorated fine fibres in all cells of primary cultures in contrast to the diffuse staining found in established mammary carcinoma cell lines.

INTRODUCTION

TECHNIQUES have been described recently which allow the growth of human breast epithelial cells *in vitro* in a reproducible fashion [1–3]. The mammary epithelial origin of these cells has been verified by ultrastructural analysis and by immunological staining with antibodies against cytokeratin filaments [1, 4]. The heterogeneity of cells in primary human mammary cultures derived from benign and malignant breast tissues has not been determined. In a recent report Bartek *et al.* [5] described a homogeneous staining pattern with monoclonal antibodies to cytokeratin 19 in eight human breast carcinoma cell lines, while staining of cells in human milk derived cultures was variable.

The structural components of the cellular cytoskeleton have proved to be useful markers for discriminating amongst different cell types. This is especially true for the intermediate filaments (IF), which have been shown to be expressed in a tissue type specific fashion: (1) cytokeratins, characteristic of true epithelia, (2) glial fibrillary acidic protein, found in astrocytes, (3) desmin, typical for muscle cells, (4) neurofilaments, detected in neurons, and (5) vimentin, a marker for mesenchymal origin (for reviews see [6–10]).

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Cytokeratins represent a family of 19 proteins which are expressed in various combinations depending upon type of epithelial cell and the state of differentiation [7, 8]. Simple epithelia express cytokeratins 7, 8, 18 and 19, stratified epithelia cytokeratins 1–6 and 9–17. Complex epithelia as found in the human mammary gland display in addition to the simple epithelial cytokeratins varying types of the stratified epithelial cytokeratins 4–6 and 14–17 [7, 8]. It has been recently shown that in the mammary gland luminal cells can be distinguished from basal cells by their expression of keratins 18 and 19 [11, 12].

Two different types of IF expressed in the same cell are found rarely *in vivo* [10, 13–16]. In the case of mammary epithelial cells vimentin has been found in addition to cytokeratin in cells of short term cultures derived from normal breast tissues [4], in established breast carcinoma cell lines [17] and in pleural effusions of metastatic breast tumour cells [13]. The physiological significance of the expression of different IF combinations is as yet unclear. These data demonstrate that intermediate filaments offer a set of markers which allow one to differentiate among subpopulations of epithelial cells and to analyse the physiological state of cells under certain *in vitro* growth conditions.

In the present study we have used several antibodies directed to cytoskeletal proteins to identify

and characterize the cell types in cultures derived from benign and malignant breast tissues. The findings in primary cultures were compared with the patterns of cytoskeletal elements expressed in established breast cell lines and in the tissue sections of the surgical specimens from which our primary cultures were initiated.

MATERIALS AND METHODS

Tissue sources

The following human surgical specimens were kindly supplied by the Institute of Pathology, University of Basel, Basel, Switzerland: four non-malignant tissue specimens (MP-6, -8, -9 and -11) taken from areas distal to the primary carcinoma, one non-malignant tissue specimen (MP-10) from an exploratory lobectomy without a malignant tumour and three malignant tissue specimens, all invasive ductal carcinomas (MaCa 83–85). MP-8/MaCa-83 and MP-11/MaCa-85 are corresponding pairs of two different patients' mastectomy specimens.

Preparation of epithelial cultures

Tissue was first freed from grossly fatty areas, minced into small pieces and then digested with collagenase (Flow Laboratories, Baar, Switzerland) and hyaluronidase (Sigma, St. Louis, MO) at 37°C with gentle rotation as described by Stampfer *et al.* [1]. The filtering procedure was omitted. The cell suspension was washed twice with culture medium, as described below, and subsequently cultured in Falcon tissue culture flasks (Becton Dickinson Lincoln Park, NJ) and/or cryopreserved in culture medium containing 10% DMSO (Merck, Damstadt, F.R.G.).

Cells and organoids were cultured using a slight modification of the medium designed for human mammary epithelial cells by Stampfer *et al.* [1]. Briefly, culture medium contained the following components: 32.5% Dulbecco's modified Eagle's medium (DMEM, Gibco, U.K.); 32.5% Ham's F-12 medium (Gibco, U.K.); 34% conditioned medium from human foetal intestinal epithelial cell line Hs 74 Int [18], human myoepithelial cell line Hs 578 Bst [19] and from mouse fibroblast cell line NIH/3T3; 0.5% foetal calf serum (FCS, Amimed, Basel, Switzerland); 10 μg/ml insulin (Sigma, St. Louis, MO); 5 ng/ml epidermal growth factor (Collaborative Research, Lexington, MA); 10^{-8} M triiodothyronine (Sigma, St. Louis, MO); 10^{-9} M estradiol (Sigma, St. Louis, MO); 0.1 μg/ml hydrocortisone (Sigma, St. Louis, MO); 1 ng/ml cholera toxin (Sigma, St. Louis, MO).

Human mammary cell line

The human carcinoma lines MCF-7 [20], ZR-75-1 [21] and T-47-D [22] were cultured in IMEM-ZO [23] (Amimed, Basel, Switzerland), 5% FCS, 5 μg/ml insulin, 2 mM L-glutamine (Gibco, U.K.), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco, U.K.).

The mammary carcinoma line BT-20 [24] was cultured in Eagle's minimal essential medium (MEM, Gibco, U.K.), 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin. Hs 578 Bst [19], a myoepithelial line, was cultured in a medium consisting of 50% DMEM, 50% Ham's F-12, 5% FCS, 5 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin.

Cells were incubated at 37°C, 5% CO_2 in air. Cell lines ZR-75-1 and HS 578 Bst were from ATCC (Rockville, MD), MCF-7 an BT-20 from Dr. L.J. Old; Sloan Kettering Institute, NY. T-47 D cells were from Dr. U. Eppenberger, Kantonsspital Basel, Switzerland.

Immunohistochemical staining

Cells were cultured on plastic slides (Lab-Tek, Miles, Lausanne, Switzerland), containing four wells, for 3–7 days and fixed with methanol for 5 min and with acetone for 2 min both at −20°C.

Antibodies used are listed in Table 1. The reactivity of these antibodies was visualized with the peroxidase–antiperoxidase method (PAP) [25], using PAP kits directed against mouse or rabbit antibodies (Dakopatts, Denmark). For each antibody the optimal dilution was determined by titration. Briefly, the slides were washed by TBS buffer (0.05 M Tris–HCl, pH 7.6 and 0.9% NaCl 1 : 9 v/v), incubated for 20 min in 3% H_2O_2, rinsed with water, incubated for 20 min in normal rabbit or swine serum, incubated for 1 h at 37°C and for 20 h at 4°C in a moist chamber, rinsed with TBS, incubated with the second antibody for 20 min, rinsed with TBS, incubated with the PAP for 20 min and rinsed with TBS. Diaminobenzidine was used as substrate for the peroxidase. The slides were counterstained with haemalaun (Merck, Darmstadt, F.R.G.). In control slides the primary antibody was omitted to exclude unspecific reactions.

Immunofluorescence staining

In double staining experiments an indirect immunofluorescence staining technique was employed. The cells were grown and fixed under the same conditions as described above. The slides were rinsed in Ca^{2+}-, Mg^{2+}-free phosphate buffered saline (PBS/O) and incubated for 20 min in FCS (1 : 20 v/v with PBS/O). Subsequently, the slides were incubated for 1 h in a moist chamber at 37°C with the previously determined optimal dilution of A 575 and anti-vimentin antibodies together, either combined, separately or not at all (for controls). After that, the slides were rinsed in PBS/O, incu-

Table 1. *Antibodies directed against cytoskeletal elements*

Antibody		Immunogen	MW (kD)	Source	Reference
Anti-cytokeratin 8*	(M)	Fraction from PtK1 cells	52.5	Amesham, U.K.	
Anti-cytokeratin 18	(M)	Fraction from HeLa cells	45	Boehringer, F.R.G.	[41]
Anti-cytokeratin 19	(M)	Fraction from human SV-40 transformed keratinocytes	40	Amersham, U.K.	
Anti-keratin A 575	(P)	Keratins from human stratum corneum of epidermis	68/64/58/56 50†	Dakopatts, Denmark	
Anti-TPA	(P)	Fraction of pooled human tumours	52.5/45/40	Sangtec, Switzerland	[27]
Anti-vimentin	(M)	Vimentin from porcine eye lens	57	Dakopatts, Denmark	[42]
Anti-actin	(P)	Actin from chicken back muscle	43	Bio-Yeda, IL	

(M) = monoclonal antibodies produced in the mouse; (P) = polyclonal antibodies produced in the rabbit; MW (kD) = molecular weight in kilodaltons of the proteins recognized by the antibody.
*Numbers of cytokeratins according to the catalogue of human cytokeratins of Moll [8].
†Spectrum of reactivity was indicated by the manufacturer.

bated with both fluorescein isothiocyante (FITC) conjugated sheep-anti-mouse immunoglobulin (Boehringer, Mannheim, F.R.G.) and a tetramethylrhodamine isothiocyanate (TRITC) conjugated swine-anti-rabbit immunoglobulin (Dakopatts, Denmark) as second antibodies.

The slides were analysed with a Zeiss microscope (IM 35), equipped with filters for FITC (450–490 nm) and TRITC (546 nm), respectively.

Evaluation of the slides

The percentage of stained cells was graded from +/− to +++, with +/− representing maximally 1%, + maximally 10%, ++ maximally 50% and +++ about 100% stained cells. Absolutely unstained cultures were marked −.

Pictures were taken on Kodak Ektachrome films (Kodak, Rochester, NY).

Immunohistochemistry on paraffin sections

From the surgical specimens, pieces were fixed in 4% buffered formalin, embedded in paraffin, sectioned at 5–7 μm and mounted on glass slides. Following deparaffinization and rehydration some slides were subjected to proteolytic enzyme treatment with pronase (protease type VII, Sigma, St. Louis, MO) for the staining experiments with the antibodies anti-TPA and A 575 [26]. For the other antibodies the enzymatic treatment could be omitted, since formalin fixation did not appear to mask relevant epitopes. The monoclonal antibodies against cytokeratins 8, 18 and 19 were not recommended for the use in routinely processed formalin fixed tissue. The anti-TPA antibody is known to react with cytokeratin proteins 8, 18 and 19 [27].

Table 2. *Staining patterns of cell cultures*

	VIM	CY 8	CY 18	CY 19	TPA	A 575
MCF-7	−	+++	+++	+++	+++	+/−
BT-20	−	+++	+++	+++	+++	+++
ZR-75-1	++*	++	+++	+++	+++	+++
T-47-D	+	+++	+++	+++	+++	+++
Hs578Bst	+++	−	+/−	+/−	+	++
MP-6	+++	++	+++	+++	+++	+++
MP-8	+++	++	++	+++	++	+++
MP-9	+++	++	++	+++	++	+++
MP-10	+++	++	++	++	++	+++
MP-11	+++	++	++	+++	+++	+++
MaCa-83	+++	+	+	++	+	+++
MaCa-84	+++	+	+	++	+	+++
McCa-85	+++	+	+	++	+	+++

+++ about 100% positive; ++ max. 50%; + max. 10%; +/− max. 1%; − negative. *Only patchy.
VIM = anti-vimentin; CY 8 = anti-cytokeratin 8; CY 18 = anti-cytokeratin 18; CY 19 = anti-cytokeratin 19; TPA = anti-TPA; A 575 = anti-keratin A 575.

RESULTS

Cytokeratin pattern (Table 2)

Cytokeratin filaments were analysed in outgrowing cells 3 days after initiation of the primary cultures (Fig. 1). The amount of positively stained cells varied from sample to sample, but within the sample the staining pattern was reproducible. This staining pattern was unchanged even after several days of culturing. With the two monoclonal antibodies against cytokeratins 8 and 18, typical for non-stratified simple and complex epithelia, it appeared that cultures derived from malignant tissues had a lower percentage of positively stained cells. This difference is less clear when the antibody against cytokeratin 19, also typical for non-stratified simple and complex epithelia, was used. Anti-TPA

showed the same reaction pattern as the monoclonal anti-cytokeratin antibodies (Fig. 2A, B). The polyclonal anti-cytokeratin antibody A 575 could not discriminate between cultures derived from benign or malignant tissue. Practically all cells reacted with this antiserum which proved the epithelial nature of cultured cells (Figs 1A, 2C, D).

The established mammary tumour cell lines showed a higher degree of heterogeneity. The staining pattern with the different antibodies against simple epithelia keratins resembled more the primary cultures derived from non-malignant samples than the carcinoma-derived cell cultures. On the other hand the myoepithelial cell line Hs 578 Bst had a staining pattern closer to the carcinoma-derived cell cultures (Fig. 3A). The A 575 antibody showed the same staining pattern as for primary cultures, with the exception of the MCF-7 line where only very few (max. 1%) of the cells were stained (Fig. 2E). The Hs 578 Bst cell line demonstrated only a weak reaction with this antibody.

In primary cultures the keratin filaments formed a fine network, in contrast to the established mammary carcinoma cell line where the staining product was always diffuse and patchy (Figs 1, 2E, F). The myoepithelial cell line demonstrated a similar network of keratin filaments as found in primary cultures (fig. 3A).

Vimentin pattern

All outgrowing cells in the primary cultures, 3 days after initiation, reacted with the vimentin antibody (Fig. 2H). This staining persisted throughout the observation period of 5 days and was unchanged after several transfers. Interestingly, the cells from malignant samples showed a much stronger staining than the cells from non-malignant samples at day 3 after culture initiation. After 5 days in culture the staining intensity was similar in both culture types.

In contrast to the primary cultures, staining in established cell lines was more heterogeneous. MCF-7 and BT-20 cells were completely negative, whereas in line ZR-75-1 up to 40% of the cells reacted in a patchy manner (Fig. 2G). In the cytoplasm of positive cells the colour precipitate was found in a distinct spot and no filaments were recognizable. In T 47-D cells less than 5% of the cells were positive with varying degrees of staining intensity and partially with a fine filamentous network. Hs 578 Bst cells were comparable to primary cultures with 100% positive cells and a well recognizable cytoskeleton (Fig. 3B).

To demonstrate the coexpression of vimentin and cytokeratin filaments in the same cell, cells were double-labelled with FITC and TRITC conjugated second antibodies. In primary cultures all cells which stained for cytokeratin, stained for vimentin as well.

Actin pattern

All cells in the primary cultures expressed well-structured actin filaments (Fig. 3D). No difference was seen between malignant and non-malignant cultures. The mammary carcinoma cell lines stained 100%, but only in BT-20 and T-47 D could rare filamentous structures be seen, whereas in MCF-7 and in ZR-75-1 no filaments were recognizable. The myoepithelial cell line showed well developed microfilaments which were similar to the primary cultures (Fig. 3C).

Staining pattern in paraffin sections

All carcinoma cells in the tumour specimens and all epithelial cells of tissue areas in the tumour periphery stained with both the A 575 and the anti-TPA antibodies. Myoepithelial cells remained unstained with the anti-TPA antibody. Epithelial cells were not decorated with the vimentin antibody, whereas fibroblasts and blood vessels were positively stained and served as a control. All basal cells in the non-tumoural tissue were stained, with the actin antibody, whereas all the apical (luminal) epithelial cells remained unstained. The actin antibody also stained blood vessels and connective tissue as expected. In the carcinomatous areas all tumour cells showed a weak staining for actin.

DISCUSSION

Normal mammary gland tissue is characterized as a complex epithelial tissue due to the fact that biochemically, both cytokeratins 7, 8, 18 and 19 of the simple epithelial subset and cytokeratins 5, 14, 15 and 17 of the stratified epithelial subset have been found [7, 8]. However, not all cytokeratin types detected by biochemical methods in a specific tissue are necessarily found in each cell. A complex cytokeratin pattern might suggest diverse and functionally different cell types or different states of differentiation.

The staining pattern for cytokeratins with the A 575 and anti-TPA antibodies in tissue sections was in agreement with previous reports [11, 12, 28]. It has been previously shown that monoclonal antibodies against cytokeratins 18 and 19 stained only luminal cells in normal tissue sections, whereas in carcinomas all cells were detected by these antibodies [5, 11, 12]. The relatively low percentage of stained cells in primary cultures with the three monoclonal antibodies against simple-epithelia cytokeratins 8, 18 and 19, particularly in cultures derived from malignant tissue, was unexpected. We interpreted this difference of *in vivo* and *in vitro* staining of cytokeratins in carcinoma cells as a

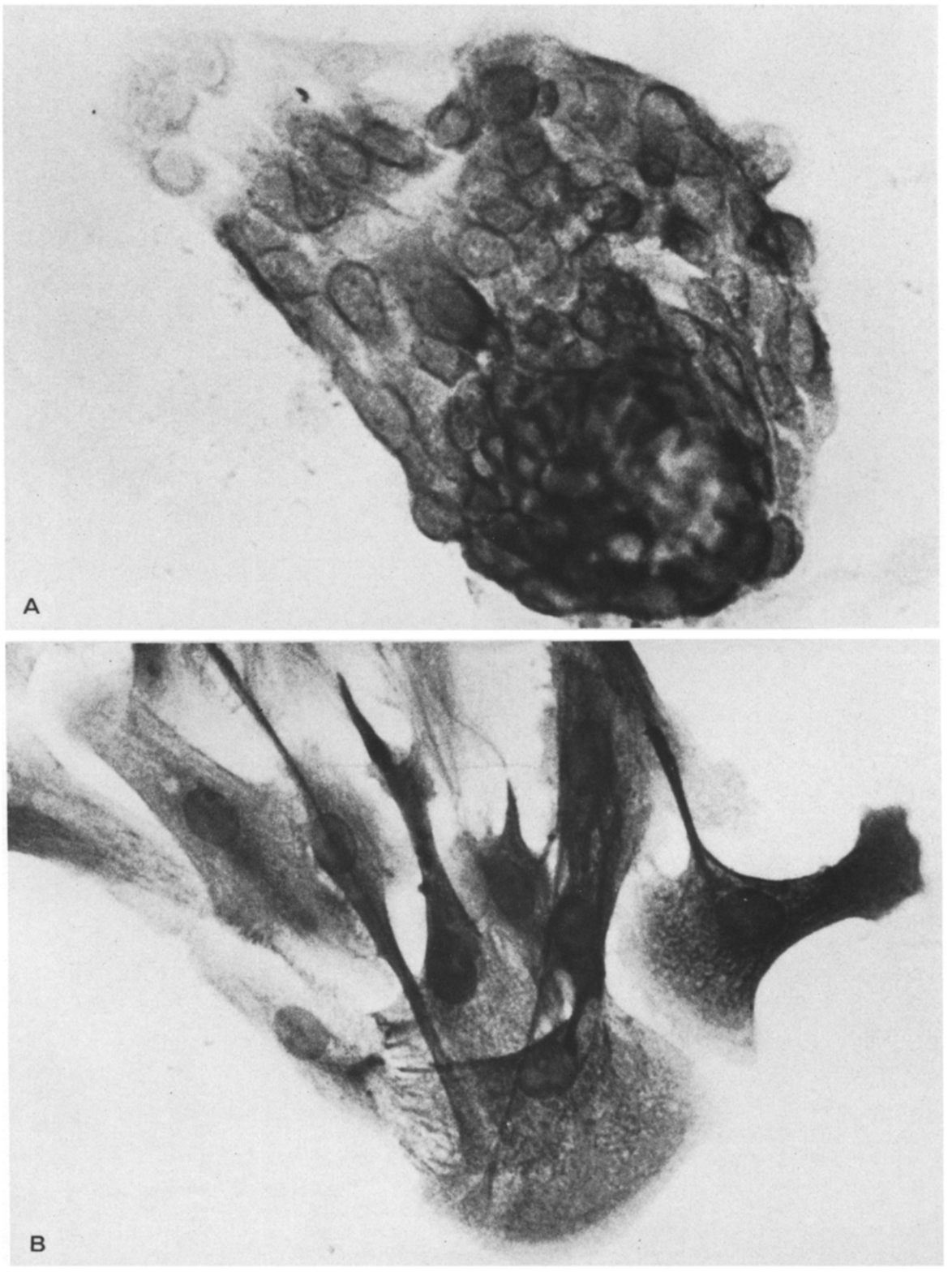

Fig. 1. (*A*) *MP*-9, 5 *days in culture, organoid with outgrowing cells. Stained with anti-cytokeratin* 19 *antibody, top left some negative cells.* (*B*) *MP*-6, 5 *days in culture, border of outgrowing cells. Stained with A* 575 *antibody, all cells positive with a fine network of filaments. A, B* × 630.

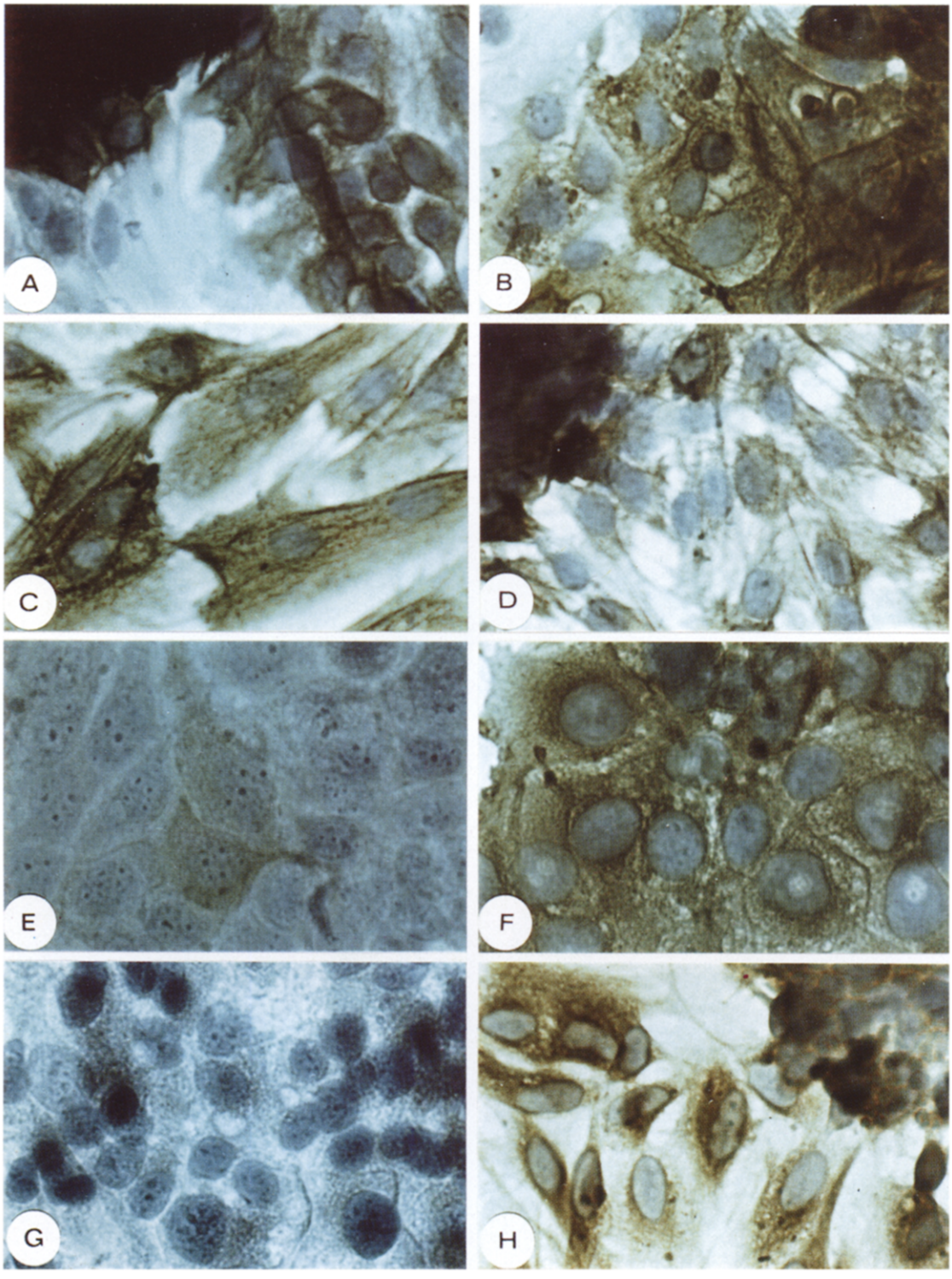

Fig. 2. (*A*) *MaCa* 84, 5 *days in culture, explant* (*upper left*), *stained with anti-TPA antibody some outgrowing cells stained positively.* (*B*) *MP*-10, 5 *days in culture, stained with anti-TPA antibody, compare to* (*A*) *for number of stained cells.* (*C*) *MaCa* 84, 5 *days in culture, stained with A* 575 *antibody, all cells were stained and showed a fine network.* (*D*) *MP*-10, 5 *days in culture, stained with A* 575 *antibody, organoid* (*upper left*) *with overall staining of outgrowing cells.* (*E*) *MCF*-7 *cell line, stained with A* 575 *antibody, only two cells show a weak reaction, without clear filamentous structures.* (*F*) *MCF*-7 *cell line, stained with anti-cytokeratin* 18 *antibody, all cells were stained but in a diffuse manner.* (*G*) *ZR*-75-1 *cell line, stained with anti-vimentin antibody, some cells demonstrated a patchy staining, others were completely negative.* (*H*) *MaCa* 84, 3 *days in culture, stained with antivimentin antibody, explant* (*upper right*) *with outgrowing cells, all cells showed a filamentous cytoskeleton. A–H* × 530.

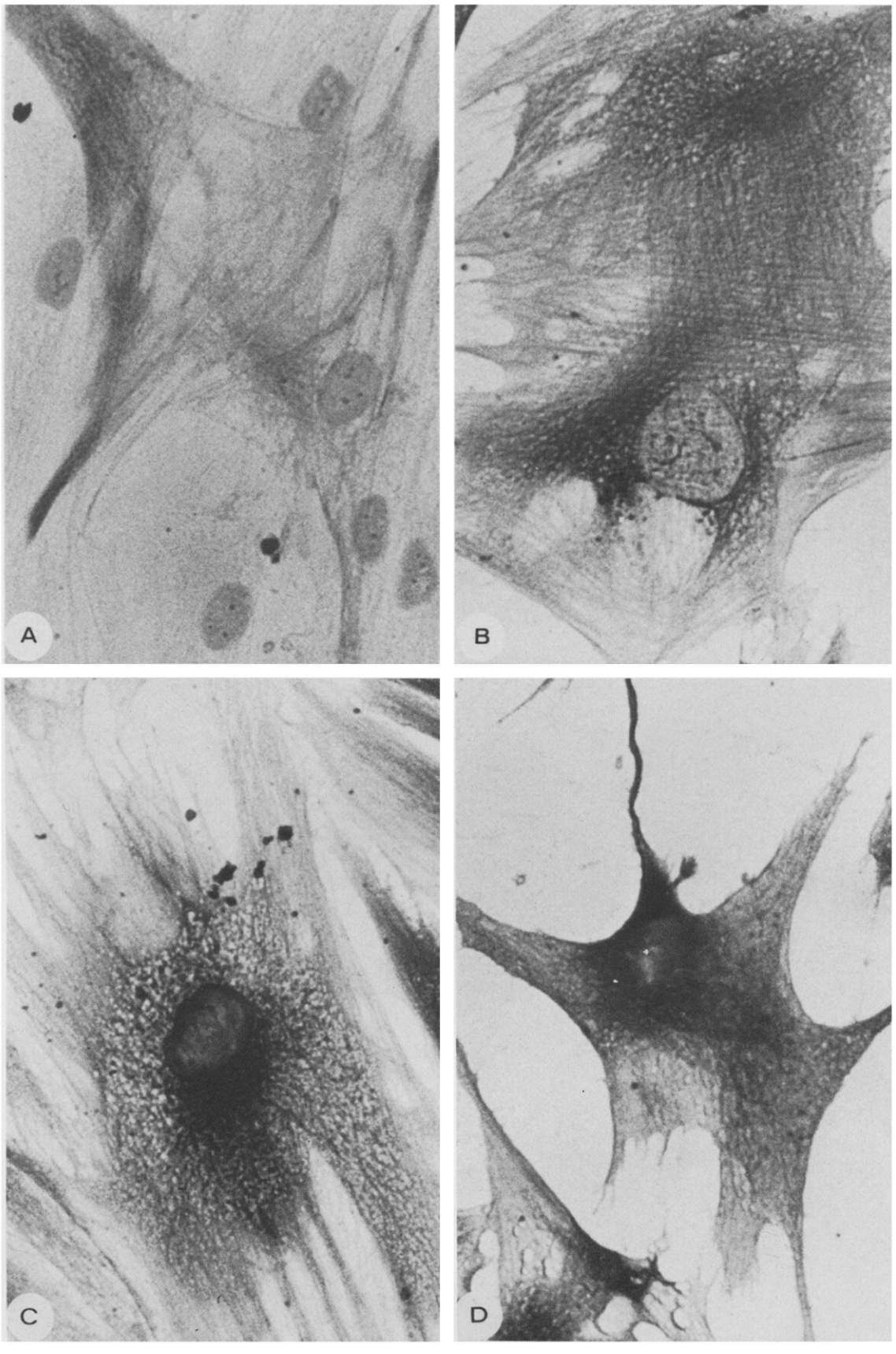

Fig. 3. (*A*) *Myoepithelial cell line HS* 578 *Bst, stained with anti-cytokeratin* 18 *antibody, few cells stained and displayed a fine network.* (*B*) *Hs* 578 *Bst cell line, stained with anti-vimentin antibody, all cells demonstrated a filigree network of vimentin.* (*C*) *Hs* 578 *Bst cell line, stained with anti-actin antibody, all cells showed fine actin filaments.* (*D*) *MaCa* 83 *stained with anti-actin antibody, all cells displayed fine filamentous actin structures.* A–*D* × 630.

change in cytokeratin expression or as a change in the accessibility of antigens induced by the *in vitro* culture conditions. The possibility of selective growth of cells preferentially expressing cytokeratins of the stratified subset cannot be excluded but seems unlikely.

The overall high percentage of stained cells in all primary cultures with the polyclonal A 575 antibody agreed with the staining pattern in tissue sections and confirmed the epithelial origin of all primary cultures. The A 575 antibody most likely reacted with all or some of the stratified epithelia cytokeratins 5, 14 and 15. This interpretation also agrees with the data of Nagle *et al.* [12] with a monoclonal antibody against cytokeratin 5 that stained only myoepithelial cells in normal tissues and with another monoclonal antibody that reacted with cytokeratins 14, 15, 16 and 19 and decorated all luminal cells in normal tissues and all tumor cells in carcinomas.

Established mammary carcinoma cell lines expressed higher amounts of simple epithelial cytokeratins in comparison to primary cultures. This agrees with the staining pattern found recently with a monoclonal anti-cytokeratin 19 antibody using the same and other mammary carcinoma lines [5]. The difference between established carcinoma cell lines and primary cultures suggests a lower degree of differentiation in primary cultures or a clonal selection after long-term *in vitro* growth in established cell lines. In MCF-7 cells less than 1% positively stained cells were found with the A 575 antibody. The biochemical analysis [7, 8] identified only cytokeratins 8, 18 and 19 in MCF-7 cells. Possibly the amount of other cytokeratins stained by A 575 antibody were too low to be detected with the biochemical method. Alternatively differences among various MCF-7 sublines containing few cells with a complex cytokeratin pattern are likely to exist. The Hs 578 Bst line, described as of myoepithelial origin [19], showed a low percentage of weakly stained cells when analysed with the A 575 antibody. This is in contrast to the staining found in our paraffin sections and by others [12]. This cell line was negative with the monoclonal anticytokeratin 8 antibody and only very few cells were decorated by the two antibodies against cytokeratins 18 and 19. This agrees with the staining pattern of myoepithelial cells from paraffin sections as demonstrated here and by others [11, 12]. This pattern argues for an abnormal cytokeratin expression *in vitro* or a selection during the *in vitro* establishment of the cell line. Similarly stained cells could also be seen in primary cultures and could represent the myoepithelial cell population. The early expression of vimentin in normal primary human mammary cultures [4] was confirmed. All cells were vimentin-positive independent of the source of tissue used for culturing. However, the intensity of staining was higher in cell cultures derived from malignant tissues. The reason for this difference is not understood as is the finding of vimentin in epithelial cells *in vitro*. Double staining experiments verified that both intermediate-sized filaments were produced in the same cell. Vimentin filaments in epithelial cells have been described previously [4, 29, 30] and interpreted as an *in vitro* adaptation phenomenon most likely because of the loss of the three-dimensional restriction imposed by the tissue of origin. This interpretation is supported by the findings of Ramaekers *et al.* [13, 31] and Summerhayes *et al.* [32] who demonstrated that cultured epithelial tumour cells when inoculated into nude mice stop vimentin expression when growing as solid tumours. Rheinwald *et al.* [33] observed that vimentin expression in human keratinocyte cultures remained undetectable or appeared at trace levels only when cultured on fibroblast feeder layers.

Established mammary carcinoma lines demonstrated low levels of vimentin. MCF-7 cells were completely negative. This would agree with the findings of Franke *et al.* [34] but is in contrast to the results of Sapino *et al.* [17] who demonstrated a fine network of vimentin filaments in MCF-7. ZR-75-1 cells showed the highest amount of vimentin positive cells of the mammary carcinoma cell lines tested. In these cell vimentin was found as condensed solitary spots without filamentous structures. In other permanent epithelial cells lines substantial vimentin expression has been found [29, 30]. Tissue of origin, time in culture or state of differentiation could influence the degree of vimentin expression *in vitro*.

Established mammary carcinoma cell lines showed a diffuse staining for actin with some filamentous structures in BT-20 and T 47-D. Primary cultures derived from benign and malignant breast tissue demonstrated a prominent network of fine actin fibres. The non-malignant, slow growing cell lines Hs 578 Bst showed a fine actin network similar to primary cultures. The culture substrate can influence the actin pattern [35]. We have grown all cells on a plastic substrate. Possibly the tumour lines deposit their own extracellular matrix which allows a change in the organization of actin filaments. Alternatively, a different form of actin polymerization has been described for fast growing cells [36, 37], which would include the tumour cell lines described here. The staining of actin filaments also depends upon the molecular structure and the organization of the filaments [37, 38]. The fact that luminal epithelial cells did not stain for actin *in vivo*, confirming the earlier report by Gabbiani *et al.* [39], might indicate that the *in vitro* conditions change the molecular organisation of the actin bundles. Differences in actin polymerization in established

carcinoma cell lines could also be due to the influence of oncogenes. Indeed, it has been described that actin filaments were destroyed by transformation of fibroblasts by SV 40 [40] or by microinjection of pp60 src into normal cells [36].

Our data show that the cells of primary cultures and permanent breast cancer cell lines are epithelial in nature but heterogeneous in the expression of intermediate filament proteins. The differential expression of IF indicates that tissue environment and culture conditions might enhance the degree of heterogeneity with respect to the occurrence of various intermediate filament proteins. The physiological significance of this finding with respect to proliferative potential and state of differentiation of the cells remains to be elucidated.

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