Expression of cytokeratins and vimentin in epithelial cells of normal and pathologic thyroid tissue

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Summary. The presence of intermediate filament proteins of the cytokeratin and vimentin type was evaluated in normal and pathologically changed thyroid tissue specimens. Using the indirect immunoperoxidase technique with 4 different cytokeratin monoclonal antibodies: RCK114 (broad spectred), K2080 (broad spectred), RGE53 (directed against component 18, present in simple epithelium) and RKSE60 (directed against component 10, associated with keratinization). Co-expression of cytokeratin and vimentin was evaluated with a double immunoenzyme staining technique.

The results indicate that normal and transformed cells express cytokeratins of the non-epidermal type. Cytokeratins of the epidermal type are sometimes present in carcinomas. They do not differentiate in tumour type (i.e. papillary, follicular, anaplastic or medullary carcinoma).

The co-expression of cytokeratins and vimentin is not restricted to carcinomas: in a small percentage of cases it is also present in normal epithelial cells of the thyroid gland. Moreover, the distribution pattern of cytokeratins and vimentin within the cell is changed in malignant transformed epithelial cells of the gland and seems to be inversely related to the degree of differentiation of these cells. The implications of our findings for the possible use of cytokeratins and vimentin in diagnostic pathology are discussed.

Key words: Thyroid gland – Thyroid cancer – Intermediate filaments – Immunohistochemistry

Introduction

Cytokeratins and vimentin are part of the intermediate filament proteins (IFP's) and as such are part

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of the cytoskeleton. They are characterized by specific biochemical and immunological properties (Bennett et al. 1982; Francke et al. 1978; Lazarides 1980). Their presence in specific cell types seems to be related to the embryonic origin with cytokeratins predominantly present in epithelial cells and vimentin in mesenchymal cells.

Cytokeratins are a complex family of 19 different polypeptides. They are expressed in different epithelia in different combinations of polypeptides ranging in their isoelectric pH value from 5 to 8 and in their molecular weight from 40 to 68 KD (Moll et al. 1982, 1984; Miettinen et al. 1984a). The finding that during malignant transformation this original cytokeratin pattern is largely maintained (Gabbiani et al. 1981, 1983; Ramaekers et al. 1983a, b; Rungger-Brändle et al. 1983) has allowed the use of cytokeratin subtype analysis in the characterization of metastatic carcinoma from an unknown primary.

Although the presence of vimentin in epithelial cells is considered to be rare, it has been reported in cultured carcinoma cells, mesotheliomas, renal cell carcinomas and in thyroid carcinomas (Virtanen et al. 1981; Herman et al. 1983; Ramaekers et al. 1983c, 1984a; Miettinen et al. 1984b; Jasani et al. 1985). With respect to the thyroid gland, Miettinen (1984) described the presence of vimentin, apart from in stromal elements, only in malignant epithelial tumour cells. In the same study a difference in cytokeratin expression between follicular and papillary carcinomas was reported. Both findings might be of potential help in notorious difficult diagnostic problems such as the differentiation between follicular adenomas and follicular carcinomas, and the differentiation of papillary structures that may be present in nodular goiters and hyperplastic thyroid tissues from papillary carcinomas. Therefore we studied the expression of several subsets of cytokeratins and the co-expression of cytokeratins and vimentin in normal and

pathologically changed thyroid tissues. Special attention was paid to possible changes in distribution pattern within the cell, related to the various pathological conditions.

Material and methods

Material. The material investigated comprised 3 cases of normal thyroid tissue, 4 of nodular goiter, 6 of auto-immune thyroiditis, 8 follicular carcinomas, 4 papillary carcinomas, 7 medullary carcinomas and 2 anaplastic carcinomas.

All thyroid tumours were classified according to the World Health Organisation international histological classification of tumours (Hedinger et al. 1974).

Tissue processing. Separate pieces of tissue were fixed in 4% neutral buffered formalin, processed overnight using graded ethanols and xylene and subsequently embedded in paraffin wax (melting point 52–54° C). In addition several pieces of tissue were snap frozen and stored in liquid nitrogen before use.

The diagnosis of each specimen was made on the histological picture of the sections prepared from paraffin blocks and stained with conventional techniques as previously described (Logmans et al. 1984).

Immunohistochemistry. From deep frozen tissue specimens, 6 μm thick serial sections were cut. These were air dried, and fixed for 10 min in acetone, after which the indirect immunoperoxidase technique was used as described before (van der Valk et al. 1983). The monoclonal antibodies (MoAbs) used in the first step and their specificity are described in Table 1. As second step antibody HRP-conjugated rabbit anti-mouse Ig (DAKO) was used. All immunoreagents were diluted in phosphate buffered saline (PBS), containing 1% bovine serum albumin (BSA). Working dilutions were previously found to give optimal staining results on normal control sections. After peroxidase staining with DAB-H2O2 and rinsing in PBS, sections were counterstained with haemalum and mounted with malinol. Control sections consisted of known positive specimens. In negative control sections the first layer antibody was changed into PBS or non-immune ascites fluid.

Double immunoenzyme staining. The co-expression of cytokeratins and vimentin within the same cell was demonstrated by double immunoenzyme staining. As MoAb's had to be used and to exclude cross-reactivity a special sequential staining technique was applied as described recently (Mullink et al. 1986). Briefly this procedure was as follows: dried and acetone fixed frozen sections were incubated for 60 min with the first MoAb (e.g. anti-vimentin), followed by an avidin-biotin peroxidase technique using an ABC staining kit (Vector Stain, Vector Labs, USA). The peroxidase label was developed with DAB-H2O2 with 0,07% imidazole for 10 min. After washing the sections were incubated for 60 min with the second MoAb (e.g. anti-cytokeratins MoAb K2080, see Table 1), and then incubated with respectively rabbit anti-mouse Ig (DAKO) (30 min) and monoclonal anti-alkaline phosphatase – alkaline phosphatase complex (APAAP) (DAKO) (60 min). The alkaline phosphatase reaction was performed using naphtol As-MX phosphate (Sigma) as substrate and Fast Blue BB salt (Sigma) as coupling agent in Tris-HCl buffer, pH 8.5, with 1 mM Levamisole (Sigma) for 30 min. All antibodies were diluted in PBS-BSA and all incubations were preceded by washing in PBS. After alkaline phosphatase staining, sections were washed in water and mounted with Kaisers' glycerin-gelatin. It appeared that when the indirect avidin-biotin-peroxidase method was used

Table 1. Monoclonal antibodies and their specificity

MoAb	Specificity	Source/Ref.
RCK 114*	Cytokeratins (broad spectred). All epithelial cells react.	a
RGE 53*	Cytokeratin component 18, mwt 45 KD. Simple epithelia react.	a, (Ramaekers et al. 1983 b)
RKSE 60*	Cytokeratin component 10, mwt 56.5 KD. karatinizing cells react.	a, (Ramaekers et al. 1983 b)
K2080*	Cytokeratins (broad spectred), mwt 38, 59, 66, 68 KD. Nearly all epithelial cells react.	b, (van Muyen et al. 1984)
Vim-9*	Vimentin, mwt 57 KD. Mesodermal cells react.	b

mwt = molecular weight
KD = kilodaltons
* MoAb: mouse IgG1 subclass
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[b] Obtained from Monosan, Sanbio, Uden, The Netherlands.
RGE 53 and RKSE 60 are now commercially available by Eurodiagnostics BV, Apeldoorn, The Netherlands.

to demonstrate the first antigen, the primary applied MoAb, although both of the mouse IgG1 subclass, was sufficiently masked by the bulky tree of antibodies with avidin-biotin complexes to prevent cross-reactivity with the anti-mouse Ig used in the second sequence (Mullink et al. 1986). As both cytoskeletal proteins were present in a beforehand unknown mutual proportion, it was necessary to use different dilutions of both MoAb's on each tissue specimen in order to obtain at least one section on which both components were optimally visible without masking one another. In this study it was sufficient to use anti-vimentin at a dilution of 1:10 and K2080 at a dilution fo 1:10 and 1:25, in both sequences. Control studies included single immuno-enzyme stainings for cytokeratins and vimentins in adjacent sections and changing of the primary antibody in the first sequence i.e. vimentin instead of keratin and the primary antibody in the second sequence keratin instead of vimentin. The results of single stianings for keratin and vimentin were always identical to the findings in the double immuno-enzyme staining irrespective of whether keratin or vimentin was used as primary antibody in the first sequence. Moreover, controls included serial sections on which the same procedure was performed, but with one of the subsequent incubation steps changed into PBS or normal immunoglobulin of the relevant species.

Scoring of the staining results. In each case and for each MoAb the positive cells were counted as mean percentages of the whole cell population of at least 10 high power fields (250 ×). Special attention was paid to the distribution pattern of cytokeratins and vimentin within the same cell in the double immunoenzyme staining.

Results

The results of the staining procedures are summarized in Tables 2 and 3.

Table 2 summarizes the cytokeratin patterns

Table 2. Staining results with the monoclonal antibodies against different types of cytokeratins

Tissue type (thyroid)	No	RCK114 (%)	K2080 (%)	RGE53 (%)	RKSE60 (%)	Type
normal	3	>95	>95	>95	0	1
Hashimoto's disease	2	>90	>90	>90	0	2
Graves disease	4	>95	>95	>95	0	1,2
nodular goiter	4	>95	>95	>95	0	1,2
adenoma	5	>95	>95	>95	0	2
follicular carcinoma	8	>95	>95	>95	0	2
papillary carcinoma	4	>95	>95	>95	0/25 [1*]	2
anaplastic carcinoma	2	>95	>95	>95	0/50 [1**]	2
medullary carcinoma	7	>95	>95	>95	0/70 [2***]	2

% the semi-quantitative estimated percentages of positive staining epithelial cells.
* 1 out of 4 papillary carcinomas stained positive with RKSE60 in 25% of the epithelial cells.
** 1 out of 2 anaplastic carcinomas stained positive with RKSE60 in 50% of the epithelial cells.
*** 2 out of 7 medullary carcinomas stained positive with RKSE60 in 70% of the epithelial cells.
type 1: a peripheral distribution of cytokeratins with sometimes more intense staining at the apical and basal ends of the epithelial cells (see also Figure 3).
type 2: a more diffuse cytoplasmic pattern with often a still stronger staining near the cell membranes (see also Figure 3).

Table 3. Staining results of epithelial cells in normal and pathological thyroid tissue with the double immunoenzyme staining technique using anti-vimentin and anti-cytokeratins monoclonal antibodies

tissue type (thyroid)	No	K (%)	V (%)	K + V (%)	type
normal	3	90	<5	5	A
Hashimoto's disease	2	70	10–20	10–20	A
Graves disease	4	75–90	<5	5–20	A
nodular goiter	4	20–50	<5	50–80	A
adenoma	5	50–90	<5	5–20	A
follicular carcinoma	8	20–50	<5	50–80	A/B
papillary carcinoma	4	10–20	<5	80	A
anaplastic carcinoma	2	5	<5	>90	B
medullary carcinoma	7	40–90	5–10	10–60	B

K = epithelial cells only staining for cytokeratins as demonstrated by MoAb K2080, in double immunoenzyme techniques.
V = epithelial cells only staining for vimentin as demonstrated by MoAb vim-9, in the double immunoenzyme techniques.
K + V = epithelial cells staining for both keratin and vimentin as demonstrated by the MoAb K2080 and vimentin in the double immunoenzyme technique.
%: The semi quantitative estimated percentages of positive staining cells
Type A: Vimentin orientated near the basal end and cytokeratins near the apical end of the cells (see also Fig. 3).
Type B: Vimentin with a perinuclear and cytokeratins with a peripheral orientation (see also Fig. 3).

observed in normal and pathologically changed thyroid tissue specimens. The normal thyroid gland specimens stained positive with RCK114, K2080, and RGE53 in more than 95% of the epithelial cells. This pattern was largely maintained in the various pathological conditions. A minority of the carcinomas (1 out of 4 papillary carcinomas, 1 out of 2 anaplastic carcinomas, 2 out of 7 medullary carcinomas) also showed a positive staining with RKSE60 in 25%, 50% and 70% respectively of the tumour cells (see also Figs. 1 and 2).

With regard to the distribution pattern of cytokeratins within the epithelial cells two patterns were observed: the normal follicular epithelial cells showed a typical peripheral distribution of cytokeratins with sometimes higher concentrations at

the apical and basal ends of the cells (celltype 1 of Fig. 3). In Hashimoto's disease, Graves disease, nodular goiter, adenomas, carcinomas we observed a shift to a more diffuse cytoplasmic distribution pattern. There was often a still stronger staining near the cell membranes (cell type 2 of Fig. 3).

Table 3 summarizes the results of the double immunoenzyme staining with anti-vimentin and K2080. This Table shows that all specimens examined contained cells which co-express cytokeratins and vimentin. Only the number of cells doing so differed. In normal thyroid gland specimens only a few cells showing this co-expression were present but in the various pathological conditions this number was increased and was highest in the carcinomas (see also Figs. 4; 5a, b; 6a, b and 7a, b).
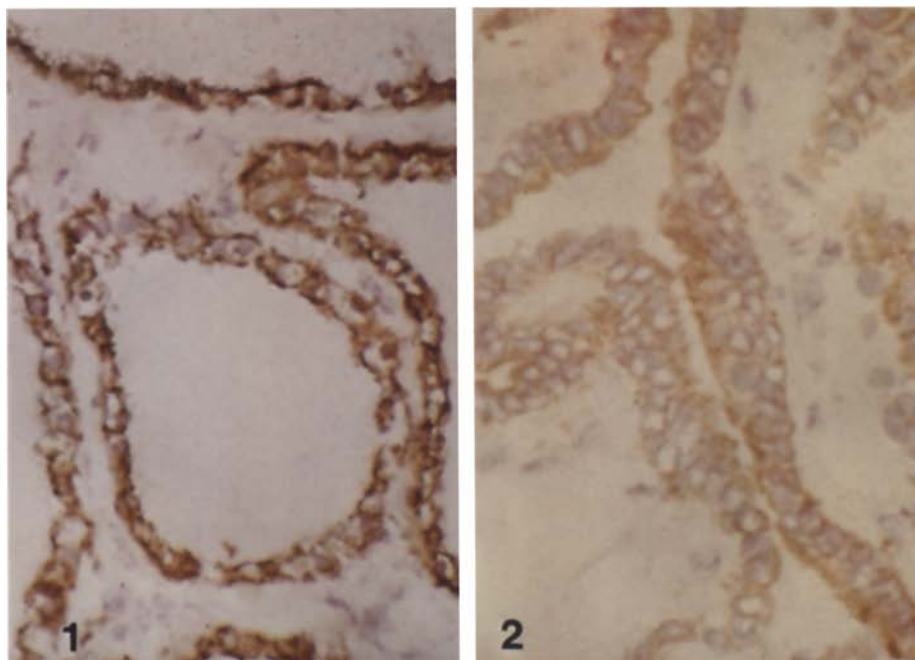
Fig. 1. Cytokeratin pattern as detected by RCK114 in follicular epithelial cells of the normal thyroid gland. The same pattern was observed by RGE53 and K2080 (not shown) ($\times$330, haematoxylin counterstained)
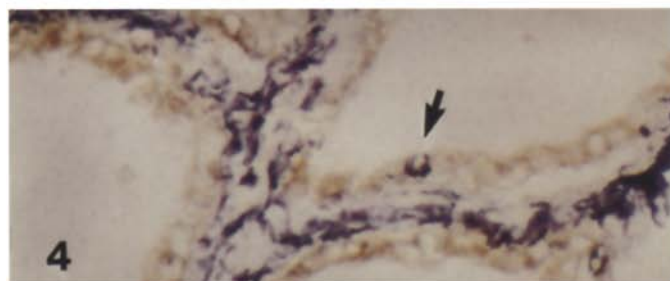
Fig. 2. Cytokeratin component 10 staining as detected by RKSE60 in a papillary carcinoma of the thyroid gland ($\times$330, haematoxylin counterstained)
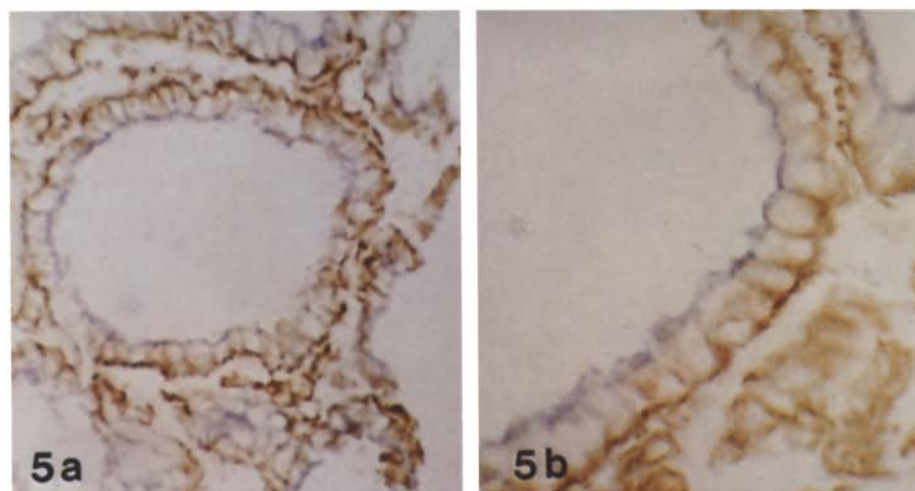
Fig. 4–7. Cytokeratins and vimentin as detected by the double immunoenzyme staining technique using K2080 and Vim-9

Fig. 4. Only a single follicular epithelial cell stains for both vimentin (blue) and cytokeratins (brown) in the normal thyroid gland. Distribution pattern of cell type A see arrow ($\times$330)

Fig. 5a, b. The number of double staining cells for vimentin (brown) and cytokeratins (blue) is significantly increased in nodular goiter. Distribution pattern of cell type A (**a** $\times$330, **b** $\times$528)

With regard to the distribution pattern of both cytokeratins and vimentin within the cell, two cell types were recognized: cell type A with vimentin orientated near the basal end and cytokeratins near the apical end of the cells, and cell type B, where vimentin showed a perinuclear and cytokeratins a peripheral orientation. Most specimens contained only cel type A. Cell type B was observed in both anaplastic carcinomas, the medullary carcinomas, and in the less well differentiated areas of
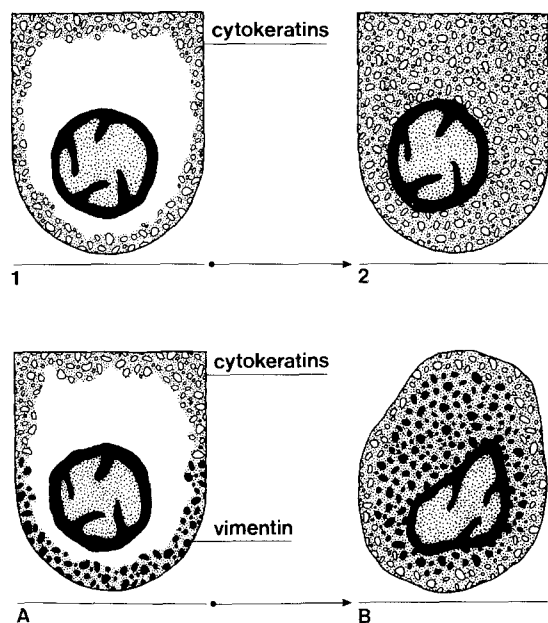
Fig. 3. Schematic drawing of the possible distribution patterns of cytokeratins and vimentin. The upper part of the figure shows the distribution pattern of cytokeratins as observed by monostaining with RCK114, RGE53 and K2080: cell type 1 with a peripheral distribution and cell type 2 with a more diffuse cytoplasmic distribution. The lower part of the figure shows the distribution pattern of cytokeratins and vimentin as detected by the double immunoenzyme staining technique using K2080 and vim-9. Cell type A shows vimentin orientated near the basal end and cytokeratins near the apical end of the cells, cell type B shows vimentin in a perinuclear and cytokeratins in a peripheral orientation

the follicular carcinomas. The four papillary carcinomas were well differentiated and contained only cell type A (see also Fig. 3).

Discussion

In this study we assessed the expression of several subsets of cytokeratins, and the co-expression of cytokeratins and vimentin in normal and pathologically changed thyroid tissue.

The staining results in Table 2 show that normal thyroid glands stain positive with RCK114, K2080 and RGE53 and not with RKSE60. As the thyroid gland is embryologically derived from the simple epithelium lining the foregut (Bodemer 1970) this cytokeratin pattern seems to be in line with its embryonic origin. As the original cytokeratin pattern is largely maintained during malignant transformation one might expect this pattern to persist in all pathological conditions. However, 1 out of 4 papillary carcinomas, 1 out of 2 anaplastic carcinomas and 2 out of 7 medullary carcinomas stained positive with RKSE60 as well. As RKSE60

is directed against component 10 of the cytokeratin polypeptides, an antigen known to be related to keratinization (Ramaekers et al. 1983 b), this represents an epithelial abnormality within the thyroid gland. This phenomenon might be explained by the occurence of squamous metaplasia, but this could not be observed in the haematoxylin-eosin stained parallel sections of the same specimens. The positive staining with RKSE60 was only observed in carcinomas, therefore its presence in thyroid specimens might be related to malignant transformation. The number of tumours examined, however, is too small to substantiate this. As the positive staining with RKSE60 was not restricted to a specific tumour type, its presence does not allow a differentiation in tumour types. In this respect our results are different from those of Miettinen et al. (1984) who reported a positive reactivity with his polyclonal anti-epidermal pre-keratin antibody in papillary carcinomas only.

As for histology and histogenesis, thyroid medullary carcinomas are generally considered as being different tumours distinct from papillary and follicular carcinomas. The origin of the medullary carcinomas is considered to be the calcitonin producing parafollicular cell. Our results show that medullary carcinomas do not differ fundamentally from carcinomas derived from the follicular epithelial cells, in their cytokeratin pattern. Since in the specimens examined no parafollicular cells were present, their normal cytokeratin pattern remains to be established. It is known that parafollicular cells are scarce and unevenly distributed in the normal thyroid gland.

Anaplastic carcinomas of the thyroid gland vary considerably in their histological picture. Sometimes differentiation from a sarcoma is impossible with conventional histological techniques. Our results show that their epithelial nature can easily be established by means of immunocytochemistry, using cytokeratin antibodies.

The changes in distribution pattern of the cytokeratins within the cell, observed in the various pathological conditions might reflect a degree of differentiation. They seem to be in line with the EM observation of Gabbianni et al. (1981): that in neoplastic epithelial cells tonofilaments are decreased and are located mainly in the perinuclear area, while in normal cells, they are more abundant and connected primarily to desmosomes.

The staining results of Table III show that all thyroid tissue specimens examined with the double immunoenzyme staining technique contain cells that co-express cytokeratins and vimentin. Only the number of cells doing so was significantly in-
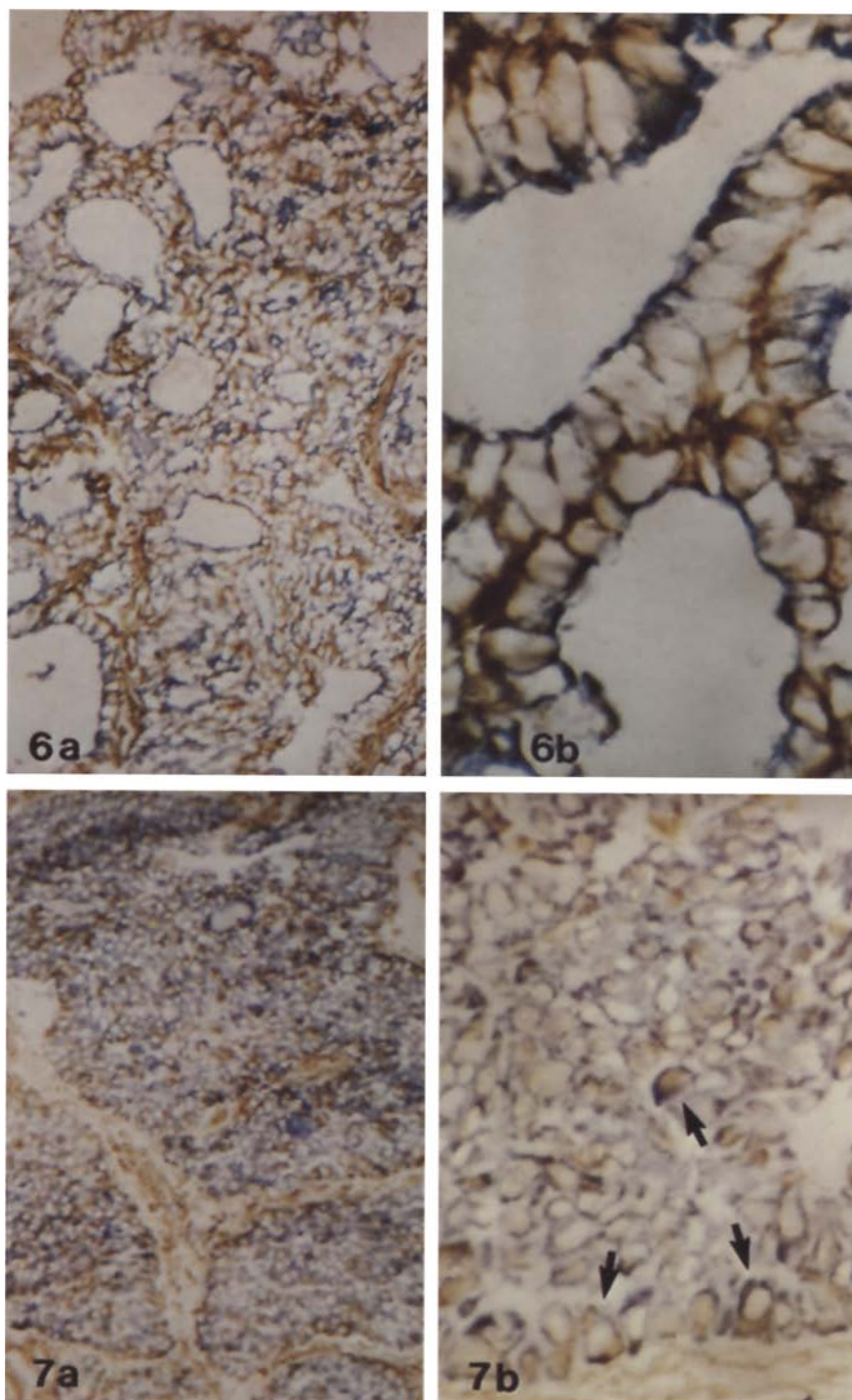
Fig. 6a, b. In a follicular carcinoma the cells show the distribution pattern of cell type A (vimentin, brown; cytokeratins, blue) (a ×208, b ×528). This pattern is also observed in papillary carcinomas (not shown)

Fig. 7a, b. In a medullary carcinoma the cells show the distribution pattern of cell type B see arrows (a ×208, b ×528). This pattern is also observed in anaplastic carcinoma and in the less well differentiated areas of the follicular carcinomas (not shown)

creased in pathological conditions. In this way it was not possible to make a distinction between proliferative and non-proliferative lesions, nor between follicular adenomas and follicular carcinomas. There was no significant difference in the number of cells co-expressing vimentin and cytokeratins in the various types of carcinomas. In this

respect these results differ from those of Miettinen et al. (1984) who found this co-expression only in thyroid carcinomas but not in the other lesions. A possible explanation might be the different technique used. With immunoenzyme methods morphological aspects can be studied better than with the immunofluorescence technique used by them,

thus allowing the detection of small numbers of double staining cells.

The co-expression of cytokeratins and vimentin is considered to be a rare phenomenon (Miettinen et al. 1984a, b; Ramaekers et al. 1983a, 1984c; Herman et al. 1983; Jasani et al. 1985). It has been described in particular as an adaptation to growth conditions when epithelial cells are brought into tissue culture (Virtanen et al. 1981; Ramaekers et al. 1984c; Franke et al. 1979, 1982). Ramaekers et al. (1984) have demonstrated the same phenomenon to occur, when epithelial cells are present in body fluids. These observations are in line with those of Rungger-Brändle et al. (1983), that many diseases affect the pattern of cell activity such as migration and proliferation rate, and that these changes may modify the organization of the cytoskeleton. In this respect, the observations made by several groups (Lazarides 1980; Jasani et al. 1985; Osborn et al. 1983; Erlandson 1984) are of particular interest: whenever co-expression of IFP's occurs, one of them is always vimentin. This may be because vimentin is of importance to the structural integrity of the cell nucleus (Lazarides 1980), the function of which comes into prominence during the proliferation phase of the cell cycle (Franke et al. 1979). The increase in epithelial cells that co-express vimentin and cytokeratins noticed under pathological conditions, as well as the changes in distribution pattern within the cell, noticed in this study seem to be in line with the observations stated above. In normal thyroid tissue and in non-malignant pathological conditions, cells co-expressing cytokeratins and vimentin showed a distribution pattern as depicted in cell type A of Fig. 3. In less well differentiated areas of the follicular carcinomas, the 2 anaplastic carcinomas and the medullary carcinomas vimentin showed a perinuclear and cytokeratin a peripheral orientation as depicted in cell type B of fig. 3. Possibly this shift from cell type A to B reflects an adaptation to pathological stimuli. No cytokeratins could be observed in the perinuclear areas, in contrast to the monostaining of these antigens, probably this is caused by masking effects of the greater amounts of vimentin present in this area.

In summary, the results of this study show that the expected potential value of the various cytokeratin polypeptides, for subtyping carcinoma metastasis of unknown primary origin is greatly hampered by the observation that changes in the cytoskeleton may occur under pathological conditions. The changes observed in this study can not be used as differentiation markers in diagnostic pathology such as the differentiation between: follicular ad-enomas and follicular carcinomas; papillary structures in nodular goiter or hyperplasia and papillary carcinomas; papillary carcinomas and follicular carcinomas.

However, they are indicative of pathological changes in general. The change from cell type A to cell type B observed in the double immunoenzyme staining and the positive reaction with RKSE60 might be indicative of malignancy. Whether they reflect an alteration in cell activity such as proliferation rate, motility and thus may lead to a more agressive behaviour remains to be established.

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