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Comparison between cell kinetical and immunohistochemical studies on carcinoma and atypia/dysplasia of urinary bladder mucosa* **

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Summary. Results of cell kinetic analyses on transurethrally obtained material from urinary bladder are compared with parallel immunohistochemical tests on carcinoembryonic antigen (CEA) and tissue polypeptide antigen (TPA), performed on the same material. Labelling index increases from 1.4% in slight to 20% in marked urothelial atypia. CEA reaction in slight atypia is slight or moderate, slight, moderate or distinct in atypia, and moderate to distinct in carcinoma in situ. TPA always shows moderate to distinct reactions. Cell kinetically, urothelial carcinomas yield similar gradations. They were positive for CEA in 70% and for TPA in 100%. In G0 and GI carcinomas, negative and slightly positive reactions predominate, poorly differentiated lesions yield predominantly distinct reactions. In all grades, TPA ranges from slight to distinctly positive. As in cell kinetic analyses, there is a relationship between differentiation grade and stage for CEA expression. This does not apply for TPA.

The results permit us to draw conclusions on the different biological and histogenetical behavior of urothelial carcinomas. There are undoubtedly differences in the behavior of papillary-exophytical and solid invasive carcinomas in terms of both cell kinetics and immunohistochemistry.

Key words: Urothelial atypia – Carcinoma – Cell kinetics – Immunohistochemistry

Introduction

Detailed histological and cytological examinations on sections of urinary bladder carcinomas together with cytology of urinary bladder irrigation

* Dedicated to Professor Dr.Dr. h.c. mult. W. Doerr on the occasion of his 70th birthday

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fluid, DNA-cytophotometry, and cell kinetic analyses, have markedly improved the data used in grading and assessing the invasion tendency of these tumours (Helpap and Giesbert 1982; Helpap et al. 1983, 1984, 1985). Recently, immunocytological and -histological methods have been applied in differential diagnosis (Nelde and Bichler 1984; Steffens et al. 1984).

By use of polyclonal antisera, keratin was detected in more than 90% of urothelial carcinomas and cytokeratin was identified in the cytoplasm in 80%. For CEA, a positive reaction was found in 20 to 40% of the carcinomas. Prostate specific antigen, acid prostate phosphatase, and Ca 1 were always negative (Friedmann et al. 1984; Steffens et al. 1984). The tissue polypeptide antigen (TPA) was tested in the serum and morphologically to evaluate its usefulness as a tumour marker. Compared with CEA, TPA showed a remarkably higher sensitivity for urothelial carcinoma (90%:30%). Comparative histological and serological studies confirmed these patterns of distribution (Vogel et al. 1984). Recently extensive cell kinetic analyses have been performed on urinary bladder mucosa changes, and this material treated in vitro was available for further immunohistochemical examination. Therefore, combined proliferation kinetic and immunohistochemical studies were performed on identical slides of carcinomas and atypias of the urinary bladder mucosa. Those studies were designed to investigate a possible correlation between histological, immunohistochemical, and cell kinetic characteristics.

Material and methods

Cell kinetics. Fresh material from the urinary bladder with an average thickness of 1 mm was transurethrally resected and incubated in autologous plasma at 37° C and 2,2 atm carbogen pressure (95% O_2/5% CO_2) in a rocking apparatus, starting 5–10 min after biopsy. During the first hour, 3H-thymidine was added to the plasma, during the second hour, 14C-thymidine (5,0 µ Ci/ml 3H-thymidine; specific activity 20,0 Ci/mmol; 0.5 µ Ci/ml 14C-thymidine, specific activity 56 mCi/mmol; NEN Chemical, Boston, MA, USA).

At the end of incubation, the biopsy material was fixed in 4% neutral formalin and embedded in a routine procedere in paraplast. The histological sections were stained with haematoxylin eosin, PAS, and van Gieson. In addition, a number of slide preparations were coated with film emulsion (G5/K2 Emulsion Ilford) or with stripping films (Kodak AR 10). Autoradiographs were exposed at 4° C for 10 and 20 days.

In the autoradiographs stained with haematoxylin-eosin, the percentages of radioactively labelled tumour cell nuclei (labelling indices) were determined (Fig. 1). Using the double labelling method, the duration of DNA-synthesis phase was determined (for more detailed description of the methods of autoradiography see; Helpap 1980; Helpap et al. 1984). The urothelial carcinomas were classified, graded and assigned to different stages of extension, according to WHO criteria (Mostofi et al. 1973) and to the general directions of the UICC 1979). Also, the urothelial atypias were classified in 3 grades: mild, moderate, and severe. (Helpap et al. 1983, 1984, 1985).

Immunohistochemistry. 71 tissue specimens that had already been treated autoradiographically, were also examined immunohistochemically. The sections, which were about 3–5 µm thick, were applied to glue-coated slides and dried for at least 24 h at 37° C. The immunohistochemical examination was done using the indirect Pap-method.

Carcinoembryonic antigen (CEA) was stained with anti-CEA-antibodies from Dakopatts GmbH Hamburg (Art. Nr. A115). For mono-specification and elimination of non-specific

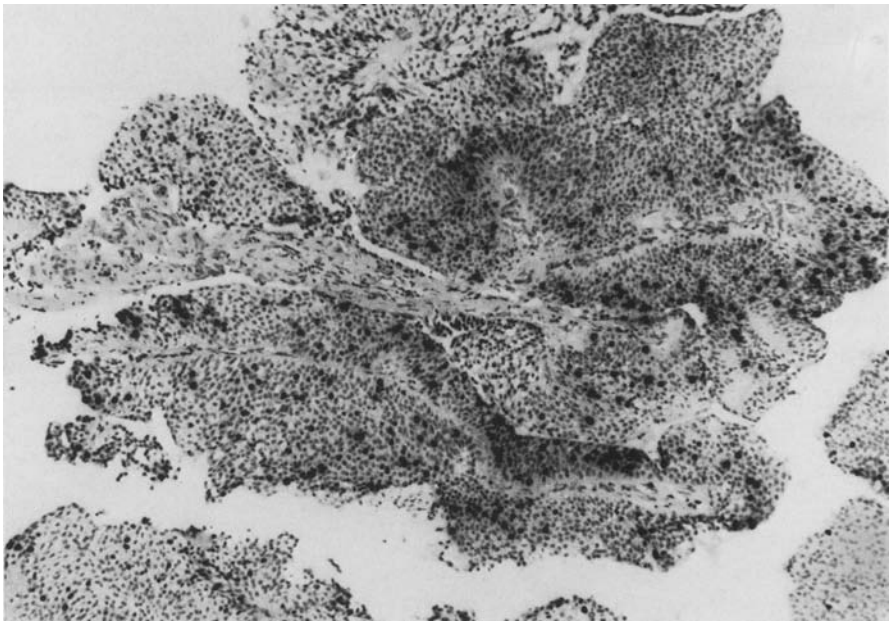


Fig. 1. 3H-TdR autoradiograph of urothelial carcinoma (stripping film AR 10, Kodak) ×112

cross-reacting antigens, the antibodies were absorbed in perchloric acid extracts of human spleen, complement-free plasma, and against purified CEA (Wagener et al. 1978; Nap et al. 1983; Berghäuser et al. 1984).

Tissue polypeptide antigen (TPA) was identified by purified monospecific rabbit-anti-TPA-antibodies from B. and V. Björklund (National Bacteriological Laboratory Stockholm, Sweden) and according to the staining methods described by V. Björklund (1980, 1984). For staining of TPA, the section slides were pretreated with protease. (Protease VIII, sigma at pH 7.2 in 0,1 ml PBS-buffer). The nuclei were counterstained with haemalaun.

For the *simultaneously performed control stainings*, the primary antibody was substituted by non-specific, non-immune pig respectively rabbit serum. Also, as a control, tumour-free urinary bladder tissue after tumour resection, and urinary bladder tissue from tumour-free individuals, which died from unnatural causes, were examined. As a positive control for CEA and TPA, an adeno-carcinoma of the lung was used.

Results

Autoradiographic studies

On 71 tissue specimens from the urinary bladder, cell kinetic analyses were performed which have already been published (Helpap et al. 1984, 1985). The labelling index of normal urothelium was 0,2%, in von Brunn cell nests it was up to 1,5%. Simple urothelial hyperplasia and urocystitis showed values of 3,1 and 4,1%. Mild atypia yielded a mitosis index of 0,01% and a labelling index of 1,4%. Moderate atypia showed a mitosis index of 0,1% and a mean labelling index of 8,2%, and severe urothelial atypia showed a mitosis index of 0,3% and a mean labelling index of 20%. The ranges

Table 1. Review of cell kinetics (mitosis and labelling indices) and immunohistochemical analyses of CEA and TPA on normal mucosal tissue of the urinary bladder and of urothelial atypia and carcinoma

Diagnosis	Mitosis index (%)	Labeling index (%)	CEA	TPA
Normal urothelium	0,01	0,6	∅ → (+)	∅ → +
von Brunn's nest	–	1,5	∅ → +	∅ → + + +
Urothelial hyperplasia	–	3,1	± → + +	+ → + + +
Urocystitis	–	4,1	+ → + + +	+ → + + +
Urothelial atypia/ dysplasia slight D 1	0,01	1,4	+ → + +	+ + → + + +
moderate D2	0,1	8,2 ± 3,8	+ → + + +	+ + → + + +
marked D3	0,3	20,0 ± 5,7	+ → + + +	+ + +
Urothelial carcinoma	0,3	20,0 ± 5,7	+ + → + + +	+ + +
G0	0,05	5,2	∅ → +	+ → + + +
GI	0,1	5,3 ± 4,9	∅ → + + +	+ + → ± + +
GII	0,6	19,3 ± 8,9	∅ → + + +	+ → + + +
GIII	1,1	26,2 ± 6,5	∅ → ± + +	+ → ± + +

are given in Table 1. The duration of the DNA-synthesis phase was 16,6 resp. 17,0 h for moderate and severe atypia.

With increasing grades of malignancy (G0-GIII), the urothelial carcinomas showed mean mitosis indices of 0,05 to 1,1%, and mean labelling indices of 5,2 to 26,2%. The duration of the DNA-synthesis phase decreased from 23 to 7,1 h (Table 1).

Immunohistochemical studies

On 71 tissue specimens of the material which had been examined histologically and cell-kinetically, an immunohistochemical analysis was performed on parallel sections for the demonstration of CEA and TPA. There were no differences in immunohistochemical results between incubated and immediately fixed biopsy material.

Out of the 71 paraplast blocks, only 47 contained parts of the carcinoma (66,2%). In the remaining blocks there was no remaining carcinoma, since the material had already been worked up in step sections for autoradiography and routine histology.

22 of the carcinomas corresponded to differentiation grade GI (46,8%), 12 carcinomas to GII (25,5%) and 13 carcinomas to GIII (27,7%). Out of the 22GI-carcinomas, 15 (68,2%) showed extension stage pTA and 7 (31,8%) stage pT 1. Out of the 12 GII-carcinomas, 5 (41,7%) showed extension stage pTA, another 5 (41,7%) stage pT 1, and 2 carcinomas corresponded to stage pT 2 (16,6%). The GIII-carcinomas showed 7 (53,8) in stage pT 1 and 6 (46,2%) in stage pT 2.

The evaluation of the 71 tissue specimens yielded 18 urothelial atypias. Those atypias were divided up in to 2 mild, 7 moderate, and 9 severe forms. Additionally, there were 2 carcinomata in situ which could not be distin-

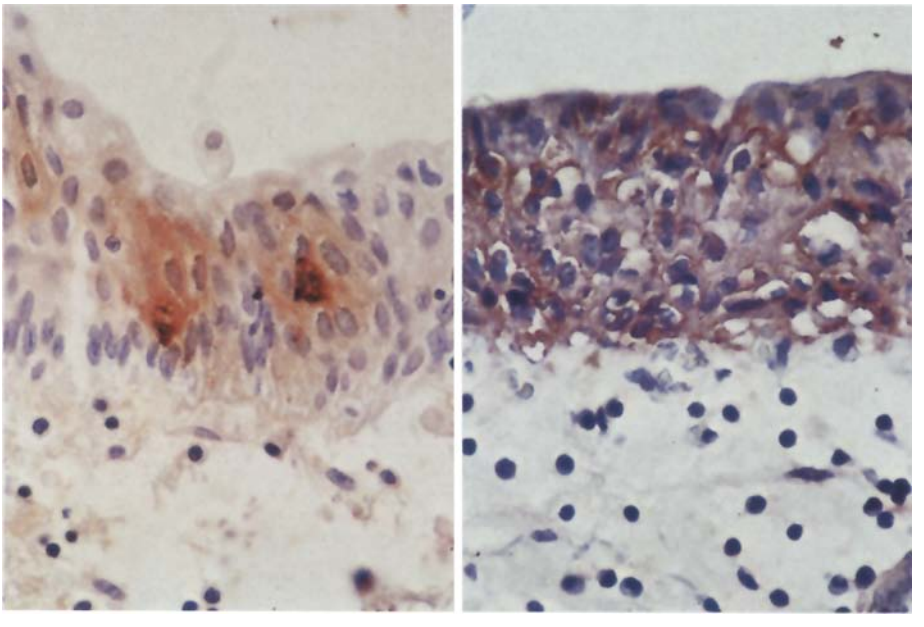


Fig. 2. CEA (**a**) und TPA (**b**) reactions in urothelial mucosa with moderate atypia. PAP ×280

guished from severe atypia on normal histological examination. Another 8 urothelial atypias (1×D1, 4×D2, 3×D3) were seen on section slides which contained no further cancer tissue and we assume that those atypias were present in the immediate vicinity of the carcinomas. Mild atypia was found together with a urothelial carcinoma, differentiation grade GII, there was one moderate atypia in a GI, GII, and GIII carcinoma respectively, and there were 4 severe atypias in a GII carcinoma and 2 severe atypias in a GII carcinoma.

For CEA and TPA, normal urothelium without inflammation showed either no or only a slightly positive reaction. In inflammation, the normal urothelium was always positive and showed a slight or distinct staining for CEA and TPA. v. Brunn cell nests were not stained or were slightly positive for CEA, for TPA they varied from unstained to distinctly positive.

Simple urothelial hyperplasia yielded a slight to moderate staining for both markers, with a range for TPA up to isolated distinctly positive stainings. (Table 1). Mild atypia (D1) yielded a slight to moderate staining for CEA, moderate to distinct for TPA. The moderate atypia (D2) was stained slightly or distinctly for CEA, and moderately to distinctly for TPA (Fig. 2a, b). The severe atypia (D3) showed a slight to distinct staining for CEA and an exclusively distinct staining for TPA. On TPA-staining, carcinomata in situ (WHO) showed the same staining as the severe atypia. The CEA-staining, however, yielded gradual differences between severe dysplasia and carcinoma in situ, the staining being moderate to distinct (Fig. 3a, b).

Urothelial carcinomas were CEA-positive in 70% and TPA-positive in

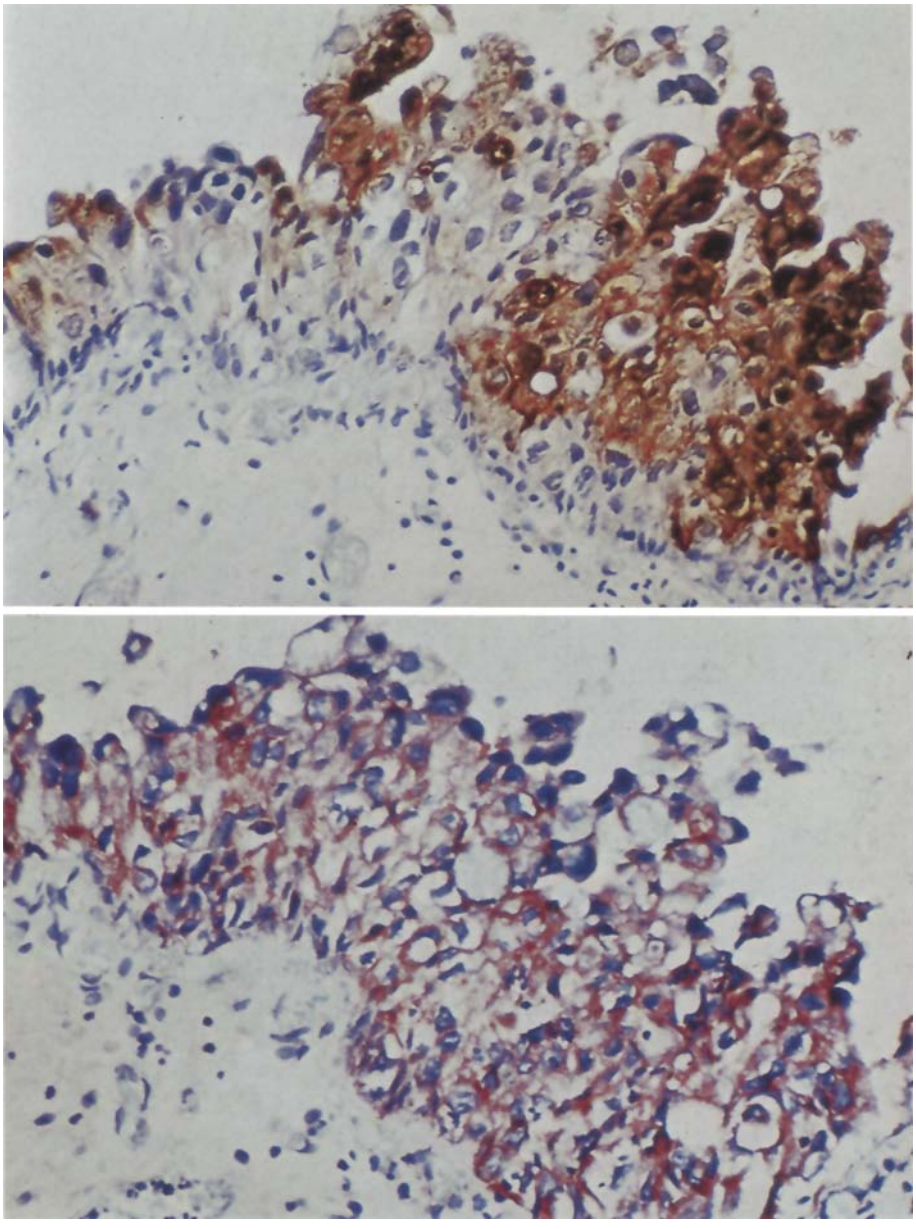


Fig. 3. CEA (**a**) and TPA (**b**) reactions in carcinoma in situ. PAP × 280

100%. The CEA-positivity distributed among the different grades of differentiation GI–GIII as follows: GI carcinomas were positive in 63%, GII carcinomas in 75%, and GIII carcinomas in 77%. The intensity of staining for CEA ranged from negative to distinctly positive, for TPA from slightly positive to distinctly positive (Table 1, Fig. 4).

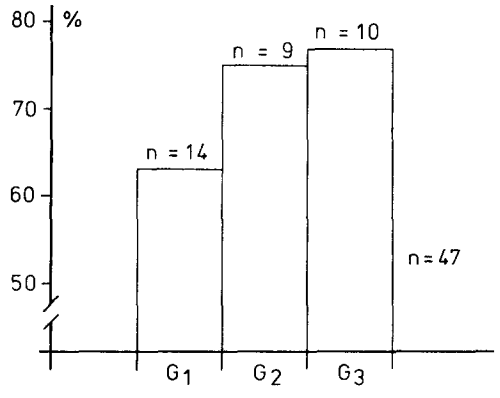


Fig. 4. Proportional distribution of CEA-reaction in urothelial GI to GIII carcinoma

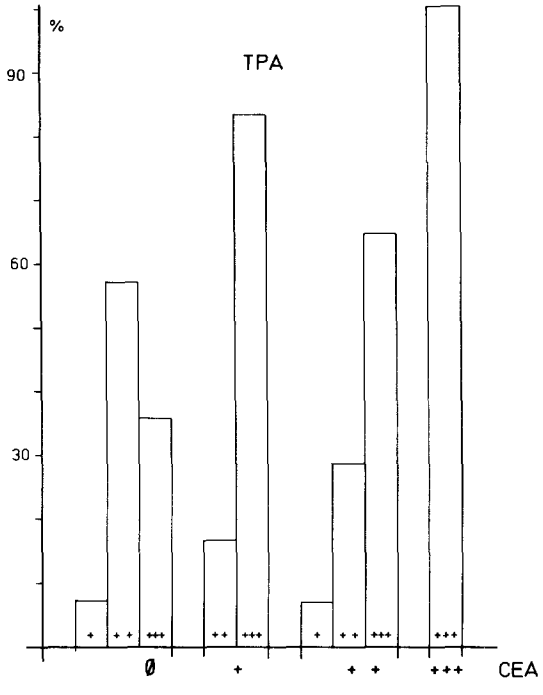


Fig. 5. Synopsis of CEA- und TPA-reactions in urothelial carcinoma; degree of staining intensity: mild (+), moderate (+ +) and distinct (+ + +)

Evaluation of CEA and TPA stainings on urothelial carcinomas of different stages showed the predominance of negative and slight stainings in the stages pTA and pT1. With carcinomas stage pT1, the proportional distribution was about equal between slight and moderate stainings. With stages pT2–3, moderate to distinct stainings predominated, the few cases stage pT4 were stained moderately and distinctly in about equal distribution.

Evaluation of TPA staining in the different stages of extension showed an evident predominance of moderate to distinct stainings in all stages from pTA to pT4.

Table 2. Percent distribution of different CEA positive tumour cell groups in urothelial carcinomas G0, I, II and III

Grading	Percent distribution of CEA positive tumour cell groups				
	Group 1 10%	Group 2 11–30%	Group 3 31–60%	Group 4 61–80%	Group 5 80%
G0	80%	–	–	–	–
GI	45%	27%	24%	3%	∅
GII	37%	30%	15%	9%	9%
GIII	24%	35%	24%	13%	2%

Comparison of CEA- and TPA-stainings in urothelial carcinomas showed following results. CEA immunoreaction being negative, there was a slight TPA staining in 7,1%, a moderate one in 57,2%, and a distinct one in 35,7%. With slight CEA staining, there was a moderate TPA staining in 16,7% and a distinct TPA staining in 83,3% on the same carcinomas. A moderate CEA staining showed a slight TPA staining in 7,1%, a moderate one in 28,6% and a distinct TPA staining in 67,3%. CEA staining being distinct, all examined carcinomas yielded distinct TPA staining as well (Fig. 5).

Semiquantitative evaluation of CEA reaction

1. In addition to classification in a grading scale, CEA reaction in tumour tissue was evaluated semiquantitatively. For this purpose, the proportion of CEA positive cells was determined by microscopical inspection in at least 10 fields of view using the 25 lens enlargement. The carcinomas were classified into groups of less than 10%, 11–30%, 41–60%, 61–80%, and over 80% of CEA positive tumour cells. A correlation with tumour size and tumour volume could not be sought, because we only used embedded material with the remaining parts of tumour tissue.

Grading. In papillomas (G0-carcinomas) although 80% of cases were CEA positive, tumours with less that 10% of CEA positive tumour cells clearly predominated (Table 2).

The percent distribution of the different CEA positive tumour cell groups of GI–GIII-carcinomas gave the following results (see Table 2). In GI-carcinomas, no case showed more than 80% of positive tumour cells.

Classification of all carcinomas into tumours with less than 10% and over 10% of CEA positive tumour cells gave the following data (made for comparison with results of Jautzke and Altenähr 1982).

	<10%	>10%
GI	45,5%	54,5%
GII	37,0%	63,0%
GIII	24,4%	75,6%

Thus, in GI carcinoma there is about an equal distribution. With a worse grading, there is a shift towards tumours with over 10% of positive tumour cells.

Tumour stage. Classification into less than 10% and more than 10% of CEA positive tumour cells in tumor tissue gave the following results when considered by stages:

	<10%	>10%
pTA	43,3%	56,7%
pT1	50,0%	50,0%
pT2/3	12,8%	87,2%
pT4	14,3%	85,7%

2. For TPA reaction, percentages can be ignored, since the entire tumour tissue yields a homogenously positive reaction. Figure 5 indicates only that on qualitative assessment of CEA reaction according to the grading scale, the TPA reaction in these cases is distributed in mild, moderate and distinct reactions.
3. For the atypias, a semiquantitative evaluation was not performed, because all typical cells also yielded strong immunohistochemical reactions, although in some cases, there was a focal reaction for CEA.

Discussion

Normal urothelium, urocystitis and simple hyperplasia

Normal urothelium shows a mitosis index of 0,01%, and a labelling index of 0,6%, urothelial hyperplasia shows a labelling index of 3,1%. With simultaneous urocystitis, the labelling index of the altered urothelium increases to 4,1% (Helpap et al. 1984). Thus, cell kinetic examinations show a considerable range of variation in the values given in the literature (Helpap et al. 1984; Blume et al. 1984; Dhlos et al. 1979) . In comparison, the immunohistochemical studies show a negative to slightly positive reaction for CEA and TPA on normal urothelium without inflammation, and a slight to distinct reaction with simultaneous urocystitis. In normal urothelium, CEA is slightly positive in superficial epithelium layers, and negative or very slightly positive in basal cell layers. Urothelial hyperplasias without considerable atypias are CEA-negative to moderately positive, and their immunohistochemical behavior with TPA is similar to that of normal urothelium with inflammation, from slightly to distinctly positive.

Urothelial atypias [dysplasias]

The labelling index of 1,4%, the cytological grade PAPII, and an euploidia in DNA-cytophotometry with mild atypia (Helpap et al. 1984), shows a

slight to moderate CEA staining and a moderate to distinct TPA staining in immunohistochemistry.

On TPA staining, the moderate and severe types of atypia do not show any differences gradually, they are all distinctly positive. The same applies to the carcinoma in situ. On CEA staining, the moderate and severe atypia are also similar with a slight to distinct staining, in contrast with a distinct difference in labelling indices, with a leap from $8{,}2 \pm 3{,}5\%$ to $20 \pm 5{,}7\%$.

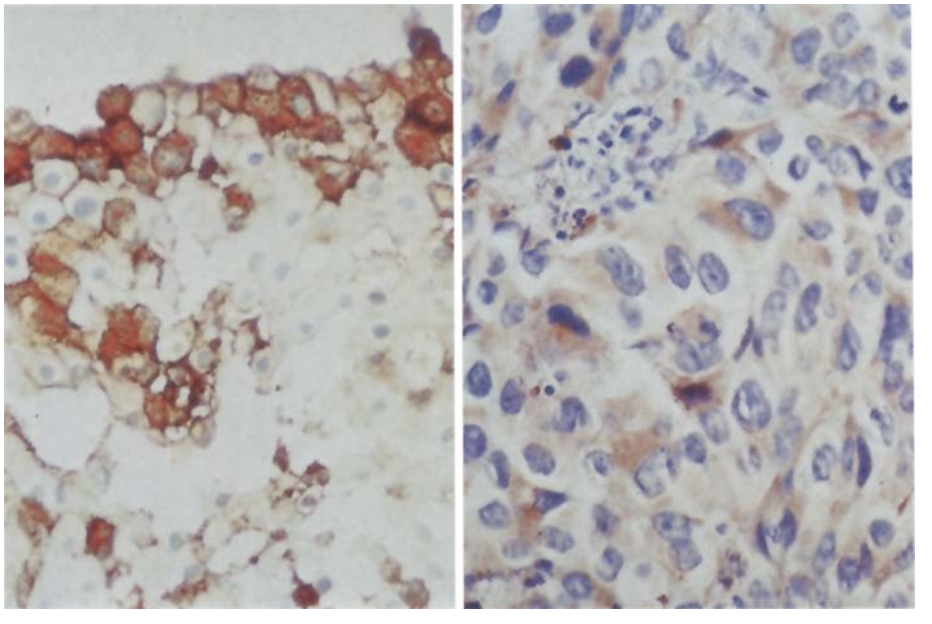
The carcinomata in situ, with equal percentages for mitosis index and labelling index, unlike the severe atypia, show some gradation, immunohistochemically being moderately to distinctly stained. With increasing cell atypia within the atypias, CEA in particular shows a distinct focal concentration of the antigen, partially on the cytoplasmic membranes, but also in the cytoplasm. On severe atypia resp. carcinoma in situ, DNA cytophotometry and cell kinetical parameters (labelling indices, duration of S-phases, eu- and aneuploidias) yield findings comparable to poorly differentiated carcinomas. Similarly the immunohistochemical stainings in severe atypia/dysplasia resp. in carcinoma in situ are comparable with a mainly solid invasive GII resp. GIII carcinoma.

Urothelial carcinomas. Whereas urothelial atypias/dysplasias were always positive for both markers, papillary and invasive urothelial carcinomas are partially CEA negative (see Fig. 2).

The main component of the CEA negative carcinomas distributes among G0-carcinomas (papillomas) and GI carcinomas. A subtile gradation, as for the labelling indices, the duration of S-phases, and the cytophotometrical findings, permits an attempt to divide the GI carcinomas into 2 subgroups (Helpap et al. 1983 and 1984) which cannot by repeated on the submitted immunohistochemical studies. This might not be expected, since the immunohistochemical staining reactions are evaluated qualitatively and semiquantitatively. On GI carcinomas, immunohistochemically negative and slightly positive reactions predominate, possibly these urothelial carcinomas correspond to the proposed group GIa with lower labelling index, a better cytological PAP grading and euploid DNA distribution. Immunocytological methods should be used in this instance as reported by Nelde and Bichler (1984) on first examinations. Further evidence for this hypothesis on the basis of the immunohistochemical studies, is the fact that about half of the GI carcinomas show less than 10% CEA positive tumour cells.

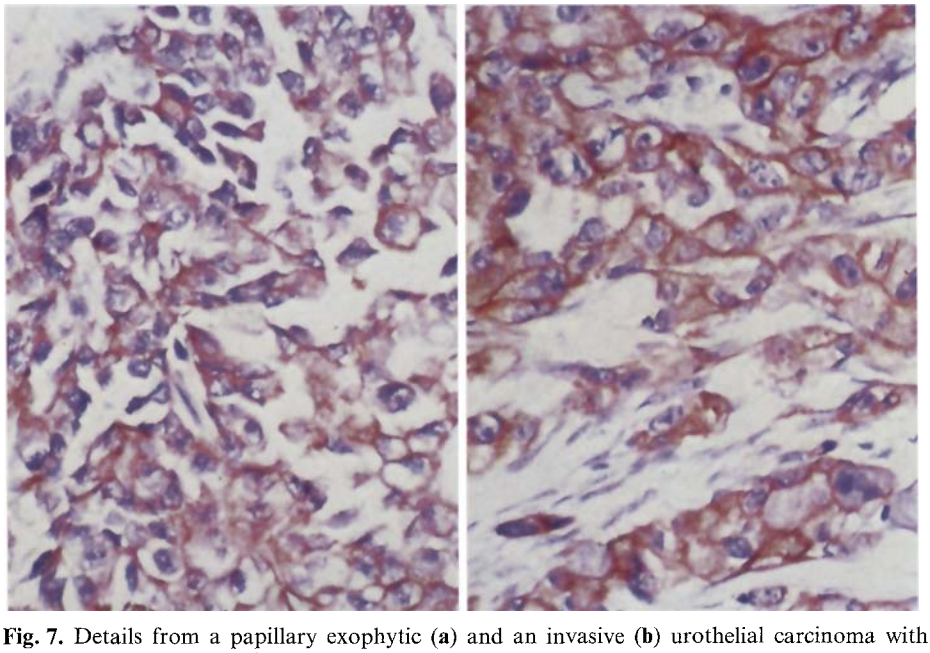
The GII and GIII carcinomas, whose values for cell kinetic analyses, cytology, and cytophotometry are close together, yield equal ranges of variation also on CEA and TPA stainings. In GIII carcinomas distinct stainings predominate for both markers.

Comparison of immunohistochemical behavior of CEA in papillary-exophytic and solid invasive urothelial carcinomas shows a clear difference. In the well to moderately differentiated exophytic carcinomas, the cell membrane accentuated localisation of the antigen predominates (Fig. 6a). In moderately to poorly differentiated solid invasive carcinomas, this localisation clearly stands back in favor of a diffuse, often fine grained intracellular



a b

Fig. 6. Section from a urothelial papillary exophytic carcinoma (**a**) with predominantly membrane linked CEA-reaction, and an invasive urothelial carcinoma (**b**) with fine granular intracellular CEA reaction PAP (**a**) ×112, (**b**) ×280



a b

Fig. 7. Details from a papillary exophytic (**a**) and an invasive (**b**) urothelial carcinoma with uniform TPA reaction in both patterns of growth. PAP ×112

distribution (Fig. 6b). The TPA reaction does not show such differences on exophytic and invasive carcinomas (Fig. 7a, b). Thus, the distribution of carcinoembryonic antigen is divergent for both types of tumour growth.

These observations support the thesis of KOSS (personal communication), that GI and GIII carcinomas represent two different types of carcinoma, possibly of different cell clones. Apparently, this is connected with different localisation and linkage of the antigen.

Cell kinetic analyses on urothelial carcinomas and urothelial atypias/dysplasias and benign mucosal changes have already been performed (review Helpap et al. 1984; Blume 1984).

Immunohistochemical studies on urinary bladder carcinomas have been carried out only with small case numbers (Steffens et al. 1984; Friedmann et al. 1984). An exception is the study of Jautzke and Altenähr (1982) with a systematic review on the demonstration of CEA in GI to GIII carcinomas. In this analysis, GI carcinomas are positive in 24%, all cases showing less than 10% positive tumour cells. GII carcinomas are positive in 72%, and GIII carcinomas in 76%. The proportion of cases with less than 10% CEA positive tumour cells is about the same in GII and GIII carcinomas (36% and 38%. The respectively cases with more than 10% positive tumour cells are 34% of GII carcinomas and 40% of GIII carcinomas. In Jautzke and Altenähr's study (1982), the CEA positive cases increase with the higher stage, in the stages pT 2/3 those cases predominate slightly, which show more than 10% positive tumour cells.

Steffens and Coworkers (1984), with a small case number, found CEA positivity of 34%. With increasing dedifferentiation in GII and GIII carcinomas, their cases show a stronger CEA reaction. Shevchuk et al. (1981), however, reported a decreasing CEA- expression with higher grade of differentiation.

Connections between TPA staining and grading with regard to staging do not exist. Generally, the TPA is described as a proliferation marker rather than an epithelial differentiation antigen (Björklund 1980; Nathrath and Heidenkummer 1983). At least on the base of the presented results on urinary bladder carcinomas, this concept has to be reconsidered. To what extent the description as an "epithelial filament protein" (Oehr 1984) does more justice to TPA, has to be determined from other immunocytological analyses.

Conclusions

On comparison of the results of cell kinetic analyses and immunohistochemistry, indications of comparable gradations, especially for carcinoembryonic antigen, are demonstrated. This applies to atypias/dysplasia as well as urothelial carcinomas. For proliferation kinetics we have reported that there are no differences between severe atypia dysplasia and carcinoma in situ. Immunohistochemically, the carcino embryonic antigen allows a gradation to be demonstrated, which corresponds with findings in gall bladder dysplasias (Albores-Saavedra, 1983). In exophytical and papillary tumour types,

the CEA staining is predominantly membrane-linked, in solid invasive tumours it is predominantly diffuse and cytoplasmatic. This is an apparent indication of a different biological behavior of the two tumour forms.

In carcinomas with higher grading strong CEA staining predominates, in advanced tumour stages distinct CEA stainings predominate. In all GI to GIII carcinomas, TPA staining is mainly moderate to distinct, TPA stainings are independent of the extension stages.

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