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Immunocytochemical detection and phenotypic characterization of micrometastatic tumour cells in bone marrow of patients with prostate cancer

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Abstract Monoclonal antibodies (mAbs) specific for cytokeratins are potent probes for the identification of disseminated individual epithelial tumour cells in mesenchymal organs such as bone marrow. We have used a monoclonal antibody (mAB) against cytokeratin 18 (CK18) for the detection of individual metastatic tumour cells in bone marrow aspirates from 84 patients with carcinoma of the prostate. CK18+ cells were detected in a sensitivity of 1 per 8×10^5 marrow cells using the alkaline phosphatase anti-alkaline phosphatase (APAAP) system for staining. We were able to detect CK18+ tumour cells in the marrow of 33% of patients with stage N0M0 prostate cancers. The incidence of CK18+ cells showed a significant correlation with established risk factors, such as local tumour extent, distant metastases and tumour differentiation. For further characterization of such cells in patients with prostate cancer, we developed an immunocytochemical procedure for simultaneous labelling of cytokeratin component no. 18 (CK18) and prostate-specific antigen (PSA). In a first step, cells were incubated with a murine mAb against PSA, followed by gold-conjugated goat anti-mouse antibodies. In a second step, a biotinylated mAb to CK18 was applied as primary antibody and subsequently incubated with complexes of streptavidin-conjugated alkaline phosphatase, which were developed with Newfuchsin substrate. The binding of gold-labelled antibodies was visualized by silver enhancement. CK18+ cells co-expressing PSA were found in bone marrow aspirates from 5 out of 14 patients with carcinomas of the prostate. The specificity of CK18 for epithelial tumour cells in bone marrow was supported by negative staining of 12 control aspirates from patients with benign prostatic hyperplasia (BPH). Thus the prostatic origin of CK+ cells in bone marrow of patients with prostate cancer has been directly demonstrated for the first time in this work. In conclusion, the approaches presented appear to be reliable methods of identifying and phenotyping individual prostatic carcinoma cells and may help to identify those patients with prostate cancer who are at high risk of relapse.

Key words Prostate cancer · Micrometastasis · Bone marrow · Double immunocytochemistry · Cytokeratin · Prostate-specific antigen

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Carcinoma of the prostate gland is, or is on the way to being, the most commonly diagnosed cancer in the western world. The leading cause of death from most types of cancer is early metastasic spread of tumour cells. Up to now, current tumour staging cannot account for the presence or absence of distant micrometastases in patients with small primary tumours. As a result, one of the most critical prognostic determinants for the subsequent clinical course is missed in many patients who undergo a potentially curative resection of a small primary tumour. Over the past 10 years several attempts have been made to detect individual disseminated tumour cells by immunocytological techniques based on phenotypic cellular characteristics of varying degrees of specificity.

More recently, monoclonal antibodies (mAbs) against cytokeratins specifically expressed by epithelial cells [4, 8, 9, 22, 23, 38] proved to be sensitive and specific probes for the detection of individual epithelial tumour cells disseminated to mesenchymal organs such as bone marrow [27, 31]. The presence of such cells is associated with an unfavourable prognosis in breast and gastrointestinal cancer [6, 20, 32, 33].

However, little is known about the biological properties of cytokeratin-positive (CK+) micrometastatic tumour cells. Using a double labelling technique based on two immunoenzymatic methods, Pantel et al. recently demonstrated that CK+ cells in the marrow of patients

with breast and gastrointestinal cancer exhibit tumour-associated characteristics such as a deficient expression of MHC class I molecules [28]. Interference of two colour reactions, however, can be a serious limitation of double staining methods relying exclusively on enzymatic staining techniques. Therefore, we developed a staining protocol combining the immunogold procedure with an alkaline phosphatase immunoenzymatic technique, allowing the simultaneous detection of two antigens localized in the same subcellular compartment. Using mAbs recognizing prostate-specific antigen (PSA), which is exclusively secreted by prostatic tissue [11, 19, 24, 34, 37], we achieved direct demonstration of the tissue-specific origin of CK+ cells in bone marrow of patients with primary adenocarcinomas of the prostate [29]. The double staining method presented in this paper is a reliable approach that can be used for any immunophenotyping of individual tumour cells.

Materials and methods

1. Detection of micrometastasis by cytokeratin staining

Patients and bone marrows aspirates

As described previously [1, 17, 18, 25–29, 31–33], bone marrow aspirates were taken from the iliac crests of patients with either prostate cancer ($n = 84$) or BPH (control group, $n = 12$) at the time of primary surgery with the patients under general anaesthesia. A mean volume of 6 ml of bone marrow was obtained per aspiration, yielding an average of about 10^7 nucleated cells. After density centrifugation through Ficoll-Hypaque (Pharmacia) [21], interphase cells were cytocentrifuged on glass slides at 1000 rpm for 5 min (8×10^4 cells/slide). Following overnight air-drying, slides were either stained immediately or stored at −80°C. Per patient 10 slides bearing 8×10^5 cells were stained and analysed (4×10^5 per aspiration).

Immunocytochemistry

According to the previous work of Schlimok's group [31–33], mAb CK2 (Boehringer Mannheim, Germany) directed to the cytokeratin polypeptide no. 18 (CK18) was used at 2.5 µg/ml as the primary antibody for staining the cytospin preparations as well as cryostat sections of primary tumours. CK18 is expressed on cells of simple epithelia and tumours derived from them [8, 9]. Appropriate dilutions of mouse myeloma proteins served as IgG1 isotype control (MOPC21; Sigma, Deisenhofen, Germany).

The antibody reaction was developed with the alkaline phosphate anti-alkaline phosphatase (APAAP) technique combined with the Neufuchsin method for visualizing antibody binding [5].

2. Phenotypic characterization of individual tumour cells by double staining

Bone marrow aspirates and tissue samples

We further analysed marrow aspirates of 14 patients with histologically proven carcinoma of the prostate and immunocytochemically detected CK+ cells in their bone marrow. Aspirates from 12 patients with BPH served as controls.

Double immunocytochemistry

Cytocentrifuge preparations and cryostat sections were thawed, air-dried, fixed in acetone and washed in a phosphate-buffered saline. Slides were transferred in a moist chamber and preparations were incubated horizontal for 20 min in PBS containing 10% AB serum (Biotest) to reduce non-specific binding. Mouse monoclonal antibody ER-PR8 (Dako, diluted 1:5 in PBS/10% AB serum) against PSA [13] was applied for 45 min. Each incubation step was followed by three washings in PBS to ensure complete removal of unbound antibodies. Goat anti-mouse antibodies coupled to 1 nm gold particles [Auroprobe one reagent (Amersham, U.K.), diluted 1:50 in PBS/10% AB serum] were applied for 30 min.

The second part of the staining procedure was initiated with the incubation of samples in PBS/5% mouse serum (Dako) for 20 min to avoid binding of antibodies to murine antigens. Mouse monoclonal biotinilated antibody CK2 (Boehringer Mannheim) to CK18 was then applied for 45 min in a concentration of 8 µg/ml in PBS/10% AB serum. The samples were incubated in alkaline phosphatase conjugated streptavidin (Jackson Immuno Research, 1:200 in PBS/10% AB serum) for 30 min, and the cells were subsequently postfixed with buffered glutaraldehyde (2% in PBS, 10 min).

The alkaline phosphatase reaction was developed by Newfuchsin and naphthol AS-BI phosphate (Sigma) according to the instructions of Cordell et al. [5].

The silver enhancement of the colloidal gold particles was performed with a silver enhancement kit following the prescription of the manufacturer (Amersham). After washes in distilled water, the preparations were mounted with Kaiser's glycerol gelatin (Merck).

Controls

Appropriate dilutions of murine IgG1 (MOPC21 from Sigma) served as isotype control to exclude unspecific staining. To further exclude crossreactivity between the two antibody systems, HT29 colon carcinoma cells (American Type Culture Collection, Bethesda, Md) known to express CK18 but not PSA were placed onto glass slides by cytocentrifugation and stained simultaneously. As positive control we used the prostate carcinoma cell line LNCaP, which expresses both CK18 and PSA [15].

Results

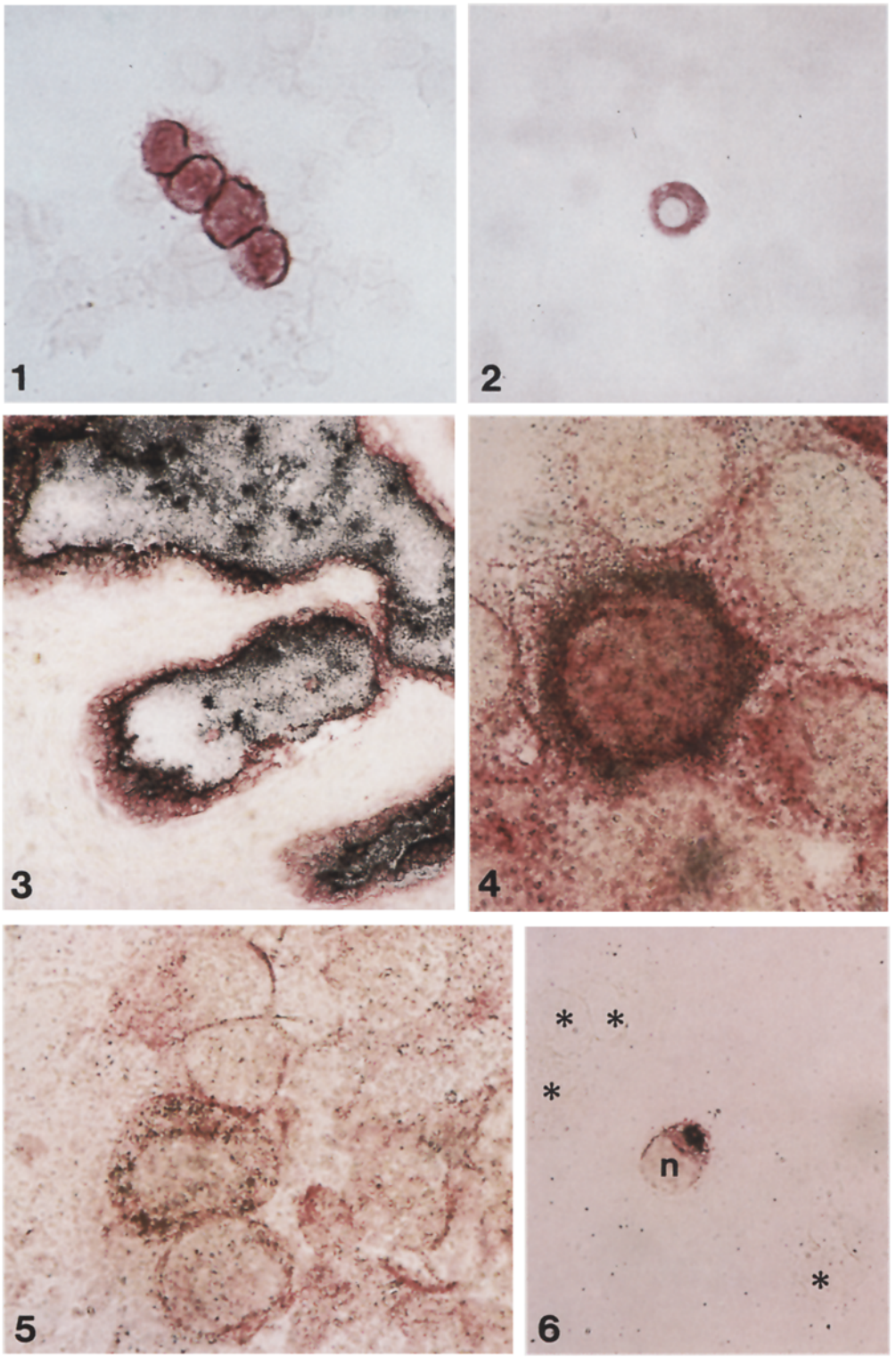
Detection of CK18-positive micrometastatic tumour cells in bone marrow aspirates

Bone marrow samples aspirated from the iliac crests of 84 patients with prostate cancer and 12 patients with BPH at the time of primary surgery were examined with mAb CK2. We found that 30 of the 84 (36%) marrow samples from patients with histologically proven prostate cancer were positive for epithelial cells (Table 1). The samples of all 12 patients in the BPH control group were negative.

Most commonly we found small numbers of single CK18+ cells, but clusters were also demonstrated (Figs. 1, 2). The average number of CK18+ cells was 6 per 8×10^5 analysed mononucleated marrow cells.

Correlation with established risk factors

The incidence of positive findings was significantly influenced by local tumour extent, distant metastases and tumour differentiation ranging from 18% in T1/2N0M0 in



Figs. 1, 2 Cytokeratin-positive (CK+) cells (*red*) in bone marrow aspirates of patients with prostate cancer are mostly found as single cells (*2*). Cell clusters of variable size can also be observed (*1*). Myeloic cells can be seen as unstained shadows

Fig. 3 In cryostat sections of hyperplastic prostatic epithelium, prostate-specific antigen (PSA; *black*) is localized in the tubulo-alveolar secretory epithelium and glandular lumina

Fig. 4 Single expression of CK18 (*red*) and co-expression of CK18 (*red*) and PSA (*black*) in LNCaP cells

Figs. 5, 6 Double-labelled micrometastatic cells (CK18, *red* and PSA, *black*) in bone marrow of patients with prostate cancer. Haematopoietic cells remain unstained (*asterisks*). The morphology of the double-positive tumour cells differs considerably. *n,* nucleus

well-differentiated tumours to 57% in metastasized tumours (Table 1). In well and moderately differentiated tumours we also demonstrated a significant correlation with the lymph node status. In the presence of distant metastases the incidence of CK18+ cells was increased to 57%, as against 33% in stage M0 (Table 1). We found no correlation with PSA serum levels.

Phenotypic characterization of individual tumour cells by double staining

Tissue

The cytoplasmic cytokeratin staining was restricted to the epithelium of prostatic glands and ducts (Fig. 3), while

Table 1 Epithelial tumour cells in bone marrow of patients with prostatic carcinoma – correlation with established risk factors

		Cytokeratin-positive cells
Tumour stage		
T1/2	$P<0.001$	8/41 (19,5%)
T3/4		22/43 (51,2%)
N0	n.s.	23/60 (38,3%)
N>0		7/20 (35,0%)
M0	$P=0.007$	22/67 (32,8%)
M1		8/14 (57,2%)
Tumour grade		
G1/2	$P=0.006$	16/61 (26,2%)
G3		12/23 (52,2%)
Total		30/84 (35,7%)

stromal cells were consistently negative. Anti-PSA staining was confined to the secretory part of the epithelial lining of prostatic glands and ducts and to the glandular lumina (Fig. 3). The supporting basal epithelial cells and the prostatic stroma were unlabelled. The specificity of the double labelling was further supported by the consistent absence of staining with isotype (IgG1) control antibodies.

Cell culture

Approximately 60% of the LNCaP cells displayed CK18+ staining, and about 30% of those CK positive cells were also labelled with anti-PSA antibody (Fig. 4). PSA+ cells displayed a heterogenous staining pattern.

To exclude cross reactivity between both detection systems as a possible source of false-positive findings, HT29 colon carcinoma cells were used as controls. HT29 cells were only labelled with mAb CK2 while not binding anti-PSA antibody.

To evaluate whether PSA expression is linked to the cell cycle of LNCaP cells, we performed double labelling with mAbs to PSA and Ki-67, known to be a marker of cell proliferation [14]. Our results demonstrate that PSA expression is not associated with the proliferative state of LNCaP cells, since we observed PSA+/Ki-67− as well as PSA−/Ki-67+ or PSA+/Ki-67+ labelled cells.

Bone marrow

In 5 out of 14 patients with primary adenocarcinomas of the prostate, co-expression of PSA on CK 18+ cells was demonstrated (Figs. 5, 6). The size and overall morphology of these double-positive cells was variable. In general they were morphologically indistinguishable from haematopoietic bone marrow cells. In the remaining 9 patients, no PSA antigen could be identified in CK18+ cells by either conventional light microscopy or epipolarization illumination.

PSA expression on CK18+ cells has so far not shown a correlation with PSA serum levels or differentiation of the primary tumours.

As a control for the specificity of the two markers for the detection of tumour cells, 12 bone marrow aspirates from patients with BPH were double stained under identical conditions. None of these specimens contained any single or double positive cells.

We were especially interested in 2 patients included in this study who had second carcinomas (1 had transitional cell carcinoma of the bladder and the other male breast cancer). In one of these cases the prostatic origin of micrometastatic CK18+ cells in the bone marrow was clearly demonstrated by co-expression of PSA.

Discussion

The detection, phenotypic characterization and elimination of occult micrometastatic tumour cells is one of the most important challenges of modern oncology.

We have demonstrated that the method described, using mAb CK2 and the APAAP staining technique, makes the identification of micrometastatic tumour cells in bone marrow of patients with prostate cancer possible, allowing the early diagnosis of occult tumour cell dissemination [1, 18, 25–27, 29].

Other authors have described similar results for gastric, colorectal, breast and non-small-cell lung cancer before [6, 20, 28, 31, 32, 33]. Follow-up studies have already demonstrated the prognostic relevance of CK18+ cells in breast and gastrointestinal cancers [20, 32, 33].

The specificity of the described method is underlined by the absence of CK-positive bone marrow findings in control-patients with non-malignant diseases. In our control group (12 patients with BPH) no CK-positive marrow was observed. Schlimok et al. and Pantel et al. [31–33] also reported no false-positive results in larger control series ($n=117$). The sensitivity of the assay could not be increased by the use of mAB against other cytokeratins (e.g., CK19) or pancytokeratin mAB in comparative analyses.

The correlation of CK18+ bone marrow findings with established risk factors such as local tumour extent, lymph node involvement and tumour differentiation (Fig. 1), and the impact on the prognosis already shown for other tumours, gives suggestive evidence for a prognostic significance of CK+ cells in bone marrow of patients with prostate cancer. However, the median observation period is too short to allow a definitive answer to this question by multivariate analysis.

The detection rate of CK18+ micrometastatic cells in low-stage respectively low-risk patients groups still ranges between 18% and 33%. Established staging examinations failed to reveal metastatic dissemination in these patients. A further analysis of the proliferative and metastatic potential respectively of molecules involved in tumour recognition and tumour specific lysis on micrometastatic tumour cells is essential for the discrimination of their individual biological behaviour.

Double immunocytochemistry

The simultaneous demonstration of two different antigens in the same cell is often required for identification of individual cells and assessment of in situ association of different cellular characteristics. We were interested in developing a double staining technique without interference of colours. With double enzyme methods false-negative results often result from overlapping of two colours, particularly when the levels of expression of the involved antigens differ. We therefore developed a combined immunoenzymatic and immunogold method in which co-localization of antibody binding is easily visualized by the presence of black grains on the background of a red-coloured product [29].

False-positive results caused by non-specific antibody binding can be excluded by the absence of positive staining after incubation of specimens with unrelated isotypic IgG1 antibodies. The specificity was further supported by negative staining of mesenchymal cells such as stromal cells of prostatic carcinomas or haematopoietic bone marrow cells known to lack both CK18 and PSA.

Besides non-specific binding of the primary antibodies, possible cross-reactivity between the secondary reagents of the two detection systems can limit the specificity of double staining methods. No cross-reactivity was demonstrated by consistent single staining of CK18+/PSA− HT29 colon carcinoma cells.

Besides peroxidase and phosphatase techniques, colloidal gold markers have proved to be the most effective probes. Recently, smaller gold particles with better penetration into intact cells have become available, which have better labelling efficacy [10]. Furthermore, the size of the gold particles can be increased by silver enhancement [7], thus leading to a better visualization of antibody binding.

Taken together, the excellent signal-to-noise ratio of the immunogold labelling (by the contrast of the black silver precipitate on the red-coloured background of the alkaline phosphatase substrate) allowed a clear visual assessment of individual cells.

In a first attempt, we used this technique for phenotyping of individual epithelial tumour cells in bone marrow of 14 patients with adenocarcinomas of the prostate. The prostatic origin of these CK+ cells could be verified by demonstrating co-expression of PSA in 5 of the 14 patients analysed. This proportion is consistent with the heterogenous expression of PSA on the LNCaP tumour cell line (Fig. 6), as well as on tumour cells in prostate cancer [29, 35]. The size and morphology of CK18+/PSA+ tumour cells in bone marrow varied considerably, supporting thus the view that by morphological criteria single tumour cells cannot be identified in bone marrow aspirates [31].

However, the CK+ cells lacking PSA that were found in the remaining bone marrow aspirates of 9 patients may still also be derived from primary tumours.

CK18+ cells were not present in 102 control marrows from patients with no evidence of malignant epithelial disease [33], and they do not express the CD45 antigen [31]. Thus, expression of CK18 protein in mesenchymal cells in vivo, as indicated by previous investigations [12], appears to be a very rare event, at least at levels detectable by our immunoenzymatic staining techniques. Furthermore, CK18+ cells display phenotypic changes that are characteristic for tumour cells, such as a deficient expression of MHC class I antigens [28] or an enhanced expression of growth factor receptors [32]. Finally, several investigators have recently proven the prognostic relevance of a cytokeratin-positive marrow finding in breast and gastrointestinal cancers [6, 20, 32, 33].

In conclusion, the double staining method described in this paper allows the phenotyping of individual tumour cells, detected in the bone marrow of cancer patients.

We think that the correlation of CK+ bone marrow findings with established prognostic factors, their absence in non-malignant diseases, their demonstrated prognostic relevance for other tumours and the preliminary data from phenotypic analyses by double immunocytochemistry give suggestive evidence that prognostic relevance can also be expected for prostate cancer. Therefore, routine bone marrow screening and consecutive adjuvant therapy of patients with CK+ marrow must be considered as a possible future perspective for prostate cancer and other genitourinary tract malignancies.

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