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Immunocytochemical Detection of Isolated Tumour Cells in Bone Marrow of Patients with Untreated Stage C Prostatic Cancer

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The micrometastatic spread of tumour cells is usually missed by conventional diagnostic techniques, although this spread largely determines the prognosis of patients with primary epithelial cancers. By use of the monoclonal antibody, CK2, to epithelial cytokeratin component number 18 (CK18), individual disseminated carcinoma cells present in bone marrow of cancer patients can now be identified. In the present study, this approach has been applied to patients with virginal stage C adenocarcinoma of the prostate. Double-sided aspirates of iliac bone marrow from 24 of 44 evaluable patients (54.5%) exhibited between one and 38 CK18-positive cells per sample of 2×10^6 mononuclear cells. In 13 of these 24 positive patients, CK-positive cells were only detected in one of the two aspirates analysed. There was no statistically significant correlation between this finding and established risk factors, such as the volume and histological grade of the primary tumour or the concentration of prostate specific antigen and prostatic acid phosphatase in serum. The follow-up time is too short to provide meaningful data on the prognostic significance of isolated CK18-positive cells in bone marrow, which, however, has been recently demonstrated in other types of primary epithelial cancers. In conclusion, the presence of prostatic tumour cells in bone marrow might be interpreted as an indicator of the metastatic capacity of an individual primary tumour. The immunocytochemical detection of these cells may, therefore, be useful for increasing the precision of current tumour staging, and to monitor minimal residual cancer in an individual patient.

Key words: prostatic cancer, bone marrow, micrometastasis, immunocytochemistry, monoclonal antibodies, cytokeratin

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INTRODUCTION

EARLY DISSEMINATION of malignant cells from small primary tumours is one of the main reasons for the failure to cure patients by an apparently radical operation [1]. Individual isolated tumour cells cannot be detected by current diagnostic procedures, and escape conventional histopathological examinations [2]. Immunocytochemical assays, based on monoclonal antibodies to cytokeratins, have been developed that allow the detection of individual epithelial tumour cells disseminated to mesenchymal organs, such as bone marrow (Table 1). The prognostic value of this new diagnostic approach has been demonstrated in clinical studies on primary breast, gastric, colorectal and lung cancer [3–9]. Prostatic cancer is one of the commonest fatal tumours in males in developed countries, for example the U.S.A. [10]. The annual incidence rate of clinical prostatic cancer has increased steadily over the past 50 years, and presently accounts for 22% of the total cancer incidence in males [10]. Although bone marrow is the preferential site of distant tumour cell dissemination in prostatic cancer, only a few studies on small numbers of patients have thus far been dedicated to the detection of micrometastatic prostate cancer cells in bone marrow [11–13].

The present study focused on patients with stage C disease because according to their clinical course, micrometastasis might occur early and would explain the limited therapeutic efficacy of radical local treatment [14, 15]. Our data provide the first direct evidence for a frequent micrometastatic involvement of bone marrow in stage C prostatic cancer. The approach presented here may be used to determine the metastatic capacity of an individual prostatic tumour with potential consequences for adjuvant systemic therapy.

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Table 1. Immunocytochemical detection of isolated tumour cells in patients with various types of epithelial cancer

Tumour origin	Marker	Prognostic value	References
Breast	EMA	+	[3]
	Cytokeratin	+	[4, 5]
	TAG12	+	[6]
Colorectum	Cytokeratin	+	[7]
	Ca-19-9	n.d.	[43]
Stomach	Cytokeratin	+	[8]
Pancreas	Cytokeratin and Ca-19-9	n.d.	[43]
Lung	Cytokeratin	+	[9]
	SM1	n.d.	[44]
	LCA 1-3	n.d.	[45]
Prostate	Cytokeratin	n.d.	[12, 13]
	Cytokeratin, PSA and EMA		[11]
Bladder	Cytokeratin	n.d.	[13]
Kidney	Cytokeratin	n.d.	[13]

EMA, epithelial membrane antigen; TAG12, tumour-associated glycoprotein 12; LCA, lung cancer-associated antigens; PSA, prostate specific antigen; n.d., not determined.

PATIENTS AND METHODS

Clinicopathological evaluation

In total, 44 untreated patients with clinical stage C (T_{3-4} N_0 M_0) adenocarcinoma of the prostate were studied. The histological diagnosis was performed on ultrasound guided transrectal core biopsies according to the Stanford technique [16], and tumours were graded according to the Gleason score [17]. All slides were reviewed by an expert pathologist. Organ spread of the tumour was diagnosed by digital rectal palpation. To determine the approximate volume of the tumour, hypoechoic area/s were measured (if present), the product of the greatest antero-posterior, transverse and superior-inferior dimensions were multiplied by 0.52 (prolated ellipse formula). The pelvic and abdominal lymph nodes were evaluated by computerised tomography (CT).

Overt metastases were excluded by chest X-rays, ultrasonography of the liver and bone scans. The serum concentrations of prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) were measured by the Tandem R monoclonal immunoradiometric assay (Hybritech), but the obtained results had no influence on tumour staging.

Immunocytochemical bone marrow analysis

After written informed consent, bone marrow was aspirated from both upper iliac crests under local anaesthesia. The volume aspirated varied from 6 to 10 ml, yielding between 6 to 10 $\times$ 10^7 cells. As described previously [2, 7–9], monoclonal antibody (MAb) CK2 (kindly provided by Dr M. Osborn, Max Planck Institut Göttingen, and later obtained from Dr H. Bodenmüller, Boehringer Mannheim, Tutzing, Germany) directed against the cytokeratin polypeptide No. 18 (CK18) was used as a reference antibody for tumour cell detection in bone marrow cytospin preparations. CK2 stains all "normal" (non-malignant) cells of simple epithelia and tumours derived thereof [18]. The MAb was used at an optimal concentration of 2.5 μg/ml. Appropriate dilutions of mouse myeloma proteins served as IgG_1 isotype control (MOPC21 from Sigma, Deisenhofen, Germany). The antibody reaction was developed with the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique, combined with the Neufuchsin method for visualising antibody binding [19]. Briefly, after incubation with the primary antibody, a polyvalent rabbit anti-mouse Ig antiserum (Z259, Dako, Hamburg, Germany) and preformed complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase antibodies (D651, Dako, Hamburg, Germany) were used at the dilutions recommended by the manufacturer (Dakopatts, Hamburg, Germany). The endogenous alkaline phosphatase activity was blocked by incubation with levamisole. To allow a fast screening for MAb-positive cells on the slides, no counterstaining was performed.

Statistical analysis

To evaluate significant differences between the incidences of cytokeratin positivity in bone marrow, comparing different subsets of patients, the χ^2-test was applied. The Student's t-test was used to test for significance of the differences between the mean values of the established risk parameters, comparing the CK18-negative and CK18-positive group. The Wilcoxon test for unrelated samples was used to test for a significant correlation between the number of CK18-positive cells in bone marrow and the values of the more established risk parameters analysed in this study. A statistical significance was denoted by P values of less than 0.05.

RESULTS

The use of the APAAP staining technique with MAb CK2 for the detection of single CK18-positive epithelial tumour cells resulted in a strong colour reaction without any background reaction (Figure 1). Thus, a cytological bone marrow preparation was scored positive when one or more stained cells were detected. Most of the positive cells appeared as isolated tumour cells while cell clusters were only detected in one patient.

As shown in Table 2, CK18-positive cells in bone marrow aspirates were found in 24 of 44 patients (54.5%). It is noteworthy that 13 of these 24 patients displayed CK18-positive cells in only one of the two double-sided marrow aspirates analysed for each patient, which emphasises the need for obtaining more than one aspirate from different sites. The relative frequency of positive cells varied from 1–38 per 2 $\times$ 10^6 mononuclear cells (MNC) with a mean value of 6 and a median value of 2. The majority of positive samples contained less than 10 CK18-positive cells per 2 $\times$ 10^6 MNC (Figure 2).

To evaluate potential associations between cytokeratin positivity and tumour characteristics or serum concentrations of prostate specific markers, we assessed the volume and the histological

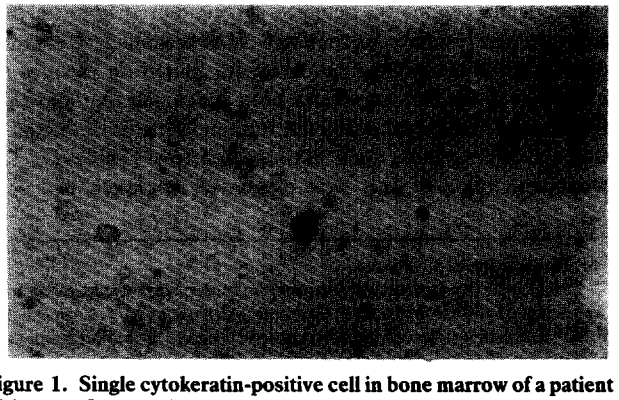

Figure 1. Single cytokeratin-positive cell in bone marrow of a patient with stage C prostatic cancer. The cytospin preparation was stained with MAb CK2, using the APAAP technique.

Table 2. Incidence of CK-positive cells in bone marrow of patients with stage C prostatic cancer

Risk factor	Number of patients per group	Cytokeratin positivity in bone marrow: Number of patients with ≥1 CK18+ cells per sample* (%)
Total group	44	24 (54.5)
A. Tumour volume		
<3 ml	23	13 (56.5)
≥3 ml	16	8 (50.0)
B. Histological grade		
Gleason score <7	16	9 (56.2)
Gleason score ≥7	24	14 (58.3)
C. PSA level in serum		
<40 ng/ml	34	20 (58.9)
≥40 ng/ml	7	3 (42.9)
D. PAP level in serum		
<5 ng/ml	25	14 (56.0)
≥5 ng/ml	9	5 (55.5)

*Samples of 2 × 10^6 mononuclear cells (MNC) aspirated from both sides of the upper iliac crest of each patient were stained with MAb CK2 using the APAAP technique.

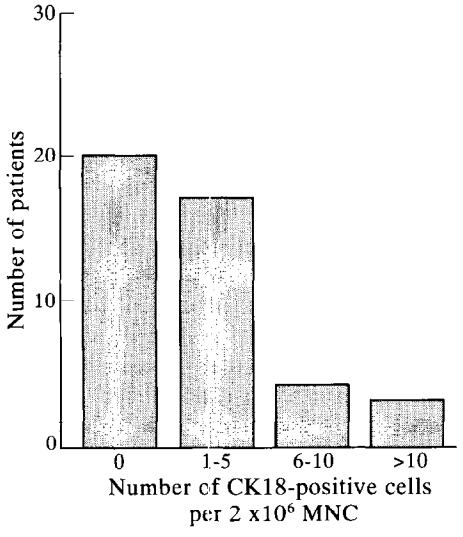

Figure 2. Frequency of cytokeratin-positive cells in bone marrow of patients with stage C prostatic cancer. For each patient, 2 × 10^6 nucleated cells were stained with MAb CK2, using the APAAP technique.

grade (Gleason score) of the primary carcinoma as well as the concentrations of PSA and PAP. As shown in Table 2, stratification of our patients into different risk groups according to these parameters failed to reveal significant differences in the incidence of cytokeratin positivity observed in the respective subgroups. Furthermore, the mean values of these parameters was not significantly different comparing patients with negative and positive bone marrow samples (Table 3). The same lack of correlation was observed if the relative frequencies of CK18-positive cells were taken into account (Figure 3). Thus, the presence of CK18-positive cells in bone marrow appeared to be an independent parameter in patients with stage C prostatic cancer.

Table 3. Correlation between cytokeratin positivity in bone marrow and primary tumour characteristics or serum marker proteins

Group	Tumour volume (ml)	Gleason score	PSA in serum (ng/ml)	PAP in serum (ng/ml)
Total	3.1 ± 0.2†	6.6 ± 0.2	40.4 ± 12.1	12.3 ± 6.5
CK-negative*	2.5 ± 0.4	6.6 ± 0.4	49.4 ± 23.6	22.9 ± 14.8
CK-positive*	3.5 ± 0.7	6.6 ± 0.3	31.7 ± 9.1	4.0 ± 0.8

*Samples of 2 × 10^6 mononuclear cells (MNC) aspirated from both sides of the upper iliac crest of each patient were stained with MAb CK2 using the APAAP technique. † The data shown represent mean values ±1 SE; the differences observed were not statistically significant (Student's *t*-test).

DISCUSSION

Recent studies have demonstrated the general feasibility of immunocytochemical and molecular approaches to the detection of individual disseminated prostatic cancer cells present in secondary organs such as bone marrow, lymph node or blood [11–13, 20–22]. However, the specificity and sensitivity of these approaches is largely affected by the intricacies of antigen expression and the lack of distinct morphological characteristics [23].

The present study shows that the bone marrow of patients with untreated stage C disease frequently have isolated tumour cells that can be easily detected by MAb CK2 to CK18, using the ultrasensitive immunocytochemical APAAP technique [18, 19]. Since haematopoietic cells produce endogenous peroxidase, alkaline phosphatase-based staining techniques are superior to immunoperoxidase methods [11]. Furthermore, the specificity of MAbs to cytokeratins is known to be higher than that of MAbs against mucin-like cell membrane proteins (e.g. epithelial membrane antigen) used by Mansi and associates [11], because these MAbs cross-react with haematopoietic cells [8, 24, 25]. In our experience, which is also shared by other investigators, morphology is rather misleading for the distinction between the normal and malignant nature of single cells. The immunocytochemical evidence for cytokeratin expression is observer-independent. Investigations by other groups, suggesting ectopic expression of cytokeratins in mesenchymal cells [26, 27], were not confirmed by our previous analyses, using MAb CK2 for tumour cell detection [2, 8, 9, 23]. The specificity of staining with MAb CK2 has been demonstrated in several studies by staining bone marrow samples obtained from large groups of non-carcinoma control patients [2, 8, 9, 23]. Cross-reactivity of MAb CK2 with haematopoietic cells has been further ruled out by double marker analysis, demonstrating exclusive positivity for either CK18 or the common leucocyte antigen, CD45, and vimentin, the mesenchymal intermediate filament protein [23]. The prostatic origin of CK18-positive cells in bone marrow was recently proven by double marker analysis, demonstrating co-expression of PSA [12].

Sampling the marrow at only one site may be insufficient, due to the low frequency of cells and their presumed heterogeneous distribution in the skeleton. Our recent methodological study indicated that the evaluation of two aspirates from both sites of the iliac crest is sufficient to detect approximately 90% of "positive" patients [23]. That more than one aspirate is required

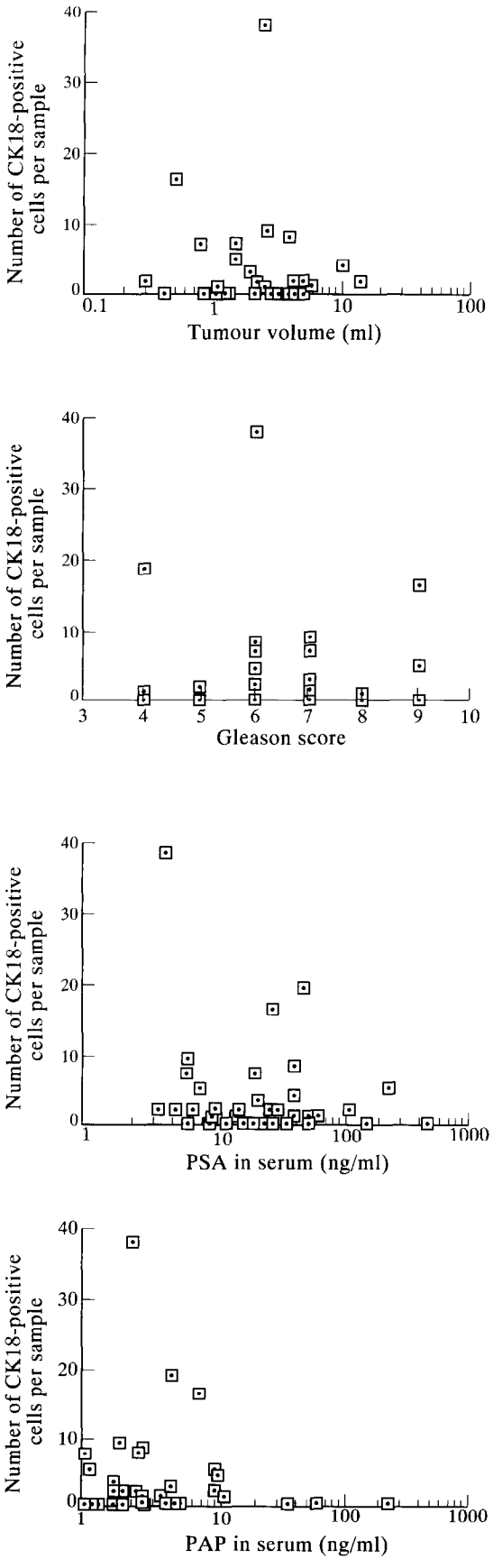

Figure 3. Quantitative correlation between the number of cytokeratin-positive cells in bone marrow and established risk factors. MNC, mononuclear cells; sample, 2 × 10^6 MNC.

confirms the earlier studies using similar methodology [28, 29]. Alternatively, more CK18$^+$ cells were revealed if the total number of cells analysed per sample was increased. The cumulative increase in the detection rate reached a plateau at approximately 2 × 10^6 marrow cells analysed [23]. Recent investigations by other groups [4, 6, 11] have been based on the evaluation of cell smears, a technique not permitting a reproducible quantitative transfer of cells to the slide surface. A quantitative estimate of residual tumour cells appears to provide additional information with regard to prognosis and monitoring of individual patients. The described cytospin device manufactured by the company Hettich (Tuttlingen, Germany) allows a reproducible quantitative transfer of up to 10^6 cells per slide [30]. In the present investigation, the frequency of CK18-positive cells in bone marrow ranged from one to 38 per 2 × 10^6 nucleated cells. Considering a total marrow cellularity in man of approximately 10^{10} nucleated cells per kg body weight [31], the estimated total load of residual tumour cells in bone marrow may vary between approximately 4 × 10^5 and 1.5 × 10^7.

The presence of CK18-positive tumour cells in bone marrow was not correlated to any of the analysed primary tumour characteristics and serum markers. This is rather surprising because all of these factors are prognostic indicators related to the metastatic capability of the primary tumour. Our results are consistent with the previous data of Mansi and associates [11], while Oberneder and colleagues reported a significant correlation with histological grade of the primary tumour in an unselected population of prostatic cancer patients [13]. One explanation for this discrepancy could be the fact that we evaluated only patients with stage C disease, in which the histological grade of the primary tumour varied only within relatively narrow margins.

Since most of the CK18-positive cells are isolated tumour cells, the term "micrometastasis" might be misleading because it rather implies the presence of a cell cluster. However, tumour cell clusters were only found in one of the 44 patients in the present study. Thus, the term "isolated tumour cells in bone marrow" appears to be more accurate. The fate of these cells might be quite heterogeneous. While a fraction of them may indeed represent precursors of overt metastases arising in the skeleton, others may leave the bone marrow and settle in a different secondary organ where they encounter a better "soil" [32]. The latter assumption is supported by the fact that the presence of CK18-positive tumour cells in bone marrow of patients with colorectal cancer is an independent predictor for metastatic relapse in other organs, such as lung and liver, while overt metastases in the skeleton rarely occurred [7]. The finding of isolated tumour cells in bone marrow is, therefore, primarily indicative of the metastatic potential of an individual tumour, and may not necessarily predict the site of metastatic growth. The incidence of CK18-positive tumour cells in bone marrow found in this study is similar to the frequency of lymph node metastases in stage C prostatic cancer reported in the literature [33–36]. In this context, it is noteworthy that most investigators also consider positive nodes as an expression of the general metastatic capacity of an individual tumour [14, 37–39].

Nevertheless, the prognostic impact of isolated tumour cells in bone marrow remains to be determined, because the follow-up time of the present study is too short to obtain meaningful data (less than 2 years). Indirect evidence for such an impact comes from prospective clinical studies on patients with other types of epithelial cancer, demonstrating the prognostic influence of individual disseminated tumour cells identified with MAbs to epithelial-specific marker proteins [3–9]. Furthermore,

the rate of metastatic relapse in the skeleton of patients with stage C prostatic cancer previously reported by Whitmore and associates [40], is identical to the incidence of cytokeratin positivity in marrow observed in our study. It is, therefore, tempting to speculate that the majority of these patients exhibited individual disseminated tumour cells in their bone marrow at the time of primary diagnosis and therapy.

We are aware of the previous work of Mansi and associates on the fate of isolated tumour cells in bone marrow of breast cancer patients, demonstrating that re-aspiration of bone marrow 6 months after initial evaluation showed the disappearance of these cells [41]. Our preliminary results from monitoring cytokeratin positive cells in patients with primary cancer of the urogenital tract (including prostate carcinomas) do not confirm Mansi's finding [13]. Tumour cells were revealed at similar rates in samples obtained either at primary surgery or 6–24 months later. These observations, together with the potential prognostic relevance of follow-up examinations [13], argue in favour of the use of marrow aspirates in the follow-up of patients.

In view of the still cumbersome microscopical screening, alternative methods permitting the processing of larger sample volumes, such as cellular enzyme immunoassays or molecular techniques [20, 42], are presently being validated in clinical studies. However, results obtained by these methods need to be compared to a gold standard to substantiate the claim of increased sensitivity over immunocytochemical approaches. Immunocytology applying mAbs to cytokeratins may serve as the gold standard for assessing new techniques.

In conclusion, the immunocytochemical technique applied in this study to patients with stage C prostatic cancer, enables us to assess the occult stage of minimal residual cancer. Provided that the future follow-up studies will confirm the prognostic relevance of this approach, the established cytokeratin immunoassay might be useful for increasing the diagnostic precision of current tumour staging. Since the bone marrow is an easily accessible organ, this assay may open an avenue to monitoring the course of an individual patient at the level of single residual tumour cells, which might also have important implications for adjuvant therapies.

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