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Short Communication

Epithelial Tumour Cells in Bone Marrow of Patients with Pancreatic Carcinoma Detected by Immunocytochemical Staining

S. Thorban,[1] J.D. Roder,[1] K. Pantel[2] and J.R. Siewert[1]

[1]Department of Surgery, Technische Universität München, Ismaningerstr. 22, 81675 München; [2]Department of Immunology, Ludwig-Maximilians-Universität München, Goethestr. 31, 80336 München, Germany

In the present study, epithelial cells in the bone marrow of 42 patients with pancreatic carcinoma were identified immunocytochemically with monoclonal antibodies (MAbs) CK2, KL1 and A45-B/B3 directed to epithelial cytokeratins (CK), using the alkaline phosphatase anti-alkaline phosphatase method. The specificity of the MAbs was demonstrated by negative staining of marrow from 25 non-carcinoma age-matched control patients. Analysis of bone marrow aspirates from cancer patients revealed CK-positive cells in 14 (58.3%) of 24 cancer patients treated with curative intent and 10 (55.6%) of 18 patients with extended disease. After a median follow-up of 15.6 months (range 3–31 months), 5 (35.7%) out of 14 patients who underwent complete surgical resection but had tumour cells in bone marrow presented with distant metastasis and 6 (42.9%) with local relapse as compared to none of 10 corresponding patients without such cells ($P < 0.05$). The described technique may help to identify patients with pancreatic cancer and potential high risk of early metastatic relapse. The results promise to be of important assistance in determining prognosis and consequences in therapy of early stage pancreatic cancer.

Key words: pancreatic carcinoma, micrometastasis, immunocytochemistry, monoclonal antibodies, cytokeratins

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INTRODUCTION

In spite of recent improvements in operative techniques and adjuvant therapies, the 5-year survival after resection for pancreatic carcinoma is still 5–20% [1].

Following the UICC-classification [2], local tumour growth (T-stage), lymph node involvement (N-stage) and distant metastasis (M-stage) are decisive events in the natural history of the cancer. Both univariate and multivariate analysis confirmed the prognostic importance of defining the radicalness of the resection. Patients with postoperative residual cancer (R-stage) have a significantly poorer survival than patients undergoing a radical resection [1, 3, 4]. Morphological biological parameters such as ploidy, proliferation rate and growth factors have not been able to improve the prognostic reliability so far [5]. According to our recent multivariate analysis, the most important prognostic factors are tumour grading (G), histological type of the tumour, R-classification (curative resection rate) and tumour diameter (T-stage) [6].

However, new prognostic predictors are still needed, especially in early stages of tumour development, in order to select patients with epithelial micrometastasis as immunotherapeutic targets or for regional pre-operative chemotherapy. Early tumour stages or minimal residual disease might be more accessible for these therapies than gross metastases.

The development of monoclonal antibodies (MAbs) directed against epithelial antigens has enabled the immunocytochemical detection of even single disseminated tumour cells in the bone marrow of patients with various types epithelial tumours [7–10]. The sensitivity of this diagnostic approach represents a widening of clinical staging and has proved to be superior to conventional staining techniques [11]. Several groups have recently demonstrated the potential prognostic relevance of epithelial tumour cells in bone marrow of patients with breast, colorectal, gastric and lung cancer [8–10].

Correspondence to S. Thorban.
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In the present prospective study, the bone marrow of pancreatic carcinoma patients, subjected to primary curative or palliative surgery was examined for the presence of disseminated epithelial tumour cells. The data presented may be useful for determining the exact tumour stage and for better stratification for pre-operative regional chemotherapy or cancer therapy with MAbs in pancreatic cancer.

PATIENTS AND METHODS

A total of 42 patients with histologically proven adenocarcinoma of the pancreas and 25 patients without neoplastic primary disease from the Department of Surgery was included in this prospective trial, dating from November 1992 to May 1995. The cancer patients were subjected to our usual preoperative staging procedure by computer tomography, abdominal and endoscopic ultrasonography, chest radiography, radionuclide bone scanning and endoscopic retrograde choledocho-pancreaticoscopy.

After written informed consent, all the patients were subjected to aspiration of 2–5 ml bone marrow and additional removal of bone biopsy from both iliac crests. The bone marrow was obtained by direct puncture under anticoagulation using sodium citrate. The bone marrow aspirates from the various aspiration sites were combined before further processing. Part of the material was smeared on a slide, stained according to May–Grünwald–Giemsa and examined by a reference cytologist. The remaining material was used to produce cytocentrifuge preparations following gradient centrifugation and purification steps. The interface cell layer obtained from this separation procedure was released as a cytocentrifuge preparation with a cell density of 100 000 cells per slide. Epithelial cells in the bone marrow were identified immunocytologically (alkaline phosphatase anti-alkaline phosphatase, APAAP) by incubation with monoclonal antibodies (MAbs) CK2 against cytokeratin 18 (Boehringer/Mannheim; M. Osborne, Max Planck-Institut Göttingen), KL1 against pan-cytokeratin 56000 kD (Dianova, Hamburg) and A45-B/B3 (IgG1), which detects a common epitope of a variety of cytokeratin components including CK 8, 18 and 19. Endogenic formation of phosphatase was prevented by pre-incubation with levamisol. The relative proportion of positive cells was recorded semiquantitatively, differentiating between single cells and cell clusters. Six slides per patient, comprising 5×10^5 mononuclear cells, were evaluated.

Positive cells could be distinguished from unstained cells due to their intense red staining, without any background reaction observed. Thus, staining of one or more cells within the cytospin was rated as a positive finding. Bone marrow from 25 patients without known malignant diseases served as control specimens to test for the specifity of immunostaining. The follow-up examinations were conducted 3, 6, 12 and 15 months after bone marrow puncture in the outpatient tumour department of our hospital or by private practitioners. These examinations included the evaluation of the patients clinical symptoms (weight loss, ileus, jaundice) as well as radiological examinations (computerised tomography, skeletal scintigraphy) and any possible histological verification of foci, suspected as local recurrence or distant metastases.

Statistical analysis

The χ^2-test was used for evaluation of statistically significant differences between the subgroups analysed. $P < 0.05$ was regarded as statistically significant.

RESULTS

Single CK-positive cells were detected in 20 (47.6%) of 42 marrow samples while clusters of positive cells were found in 4 (9.5%). Only 4 (9.5%) cancer patients were CK2-positive as compared to 12 (28.6%) patients who displayed KL1-positive cells. Eight (19.0%) of the bone marrow samples analysed with MAb A45-B/B3 were positive, whereas the control specimens had no stained cells (Table 1).

In contrast to recent investigations evaluating a poorly defined number of mononuclear cells smeared on glass slides, we screened cytospin preparations which allow a more precise quantitation of stained cells. The relative frequency of CK-positive cells in cancer patients was 1–66 per 5×10^5 nucleated cells analysed.

During the median follow-up period of 15.6 (3–31 months), the occurrence of distant tumour relapse was significantly associated with the outcome of the immunocytochemical screening at the time of primary surgery. Distant metastases were detected in 5 (35.7%) out of 14 patients with CK-positive bone marrow findings after curative resection. One of these patients exhibited skeletal metastases. Primary sites of non-skeletal metastases were the liver ($n = 2$) and peritoneum ($n = 2$). Local relapse was diagnosed in 6 (42.9%) of the 14 patients with positive bone marrow findings (mean follow-up: 13.9 (range 3–29) months).

In contrast, none of the 10 patients without epithelial cells in the bone marrow developed distant metastases nor exhibited local relapse (mean follow up: 17.3 (3–36) months; Table 2).

DISCUSSION

In the last 10 years, attempts have been made to detect individual disseminated tumour cells by immunocytological techniques based on more or less specific phenotypic cellular characteristics. Assays for the detection of single tumour cells in bone marrow have been previously developed for various forms of epithelial cancers to identify micrometastic disease. As reliable markers for normal and malignant epithelial cells, especially carcinoma cells disseminated to bone marrow, MAbs directed against cytokeratins were used which could identify single cells. The specifity and prognostic significance of this immunocytochemical approach have been supported by extensive investigations in patients with breast, colorectal, stomach and lung cancer [8–10].

Although our evaluation of the clinical relevance of this minimal tumour load is still preliminary, the positive correlation to the recurrence of early metastatic relapse suggests a prognostic significance as an indicator for systemic tumour cell dissemintion. At present, the number of patients and

Table 1. Incidence of CK-positive cells in bone marrow of pancreatic cancer and non-carcinoma control patients

No. of patients with >1 immunostained cell per sample (%)*				
	CK2+	KL1+	A45-B/B3+	Total
Cancer patients	4/42 (9.6%)	12/42 (28.6%)	8/42 (19.0%)	24/42 (57.1%)
Non-carcinoma control patients	0/25	0/25	0/25	0/25

*Based on the evaluation of 4×10^5 nucleated cells with MAbs KL1, CK2 and A45-B/B3.

Table 2. Rate and type of tumour relapse depending on the presence or absence of CK-positive cells in bone marrow

Immunoreactivity*	No. of patients per group†	Metastatic relapse‡	Local relapse
Positive	14	5 (35.7%)§	6 (42.9%)§
Negative	10	0 (0.0%)§	0 (0.0%)§
Total	24	5 (20.8%)	6 (25.0%)

*As defined with anti-CK MAbs CK2, KL1 and A45-B/B3. †Only patients with R_0 resection and no postoperative mortality were eligible; mean follow-up 15.6 (3–31) months. ‡Distant metastases in the liver ($n = 2$), peritoneum ($n = 2$) and skeleton. §$P < 0.05$ (chi-square test) as compared to CK-positive patients.

follow-up period (mean: 15.6 months) are too small to perform a multivariate analysis with established risk factors in patients with cancer of the pancreas.

However, tumour cells in the bone marrow may not necessarily have the potential to form clinically detectable metastases and may remain dormant. This assumption is supported by the relatively small proportion of overt bone metastases observed in our study. The major sites of tumour relapse in pancreas cancer are regional lymph nodes, local and diffuse peritoneal spread and the liver [3, 4].

Normally epithelial cells do not occur in bone marrow. Thus, tumour cells in the bone marrow are suggestive of a generalised malignancy. The percentage of detection by conventional staining techniques is lower than by immunocytochemical methods [12, 13]. The APAAP procedure in combination with the cytokeratin specific MAbs CK2, KL1 and A45-B/B3 improved the detection of tumour cells in bone marrow as compared to conventional cytology and histology [14].

In our study, bone marrow was aspirated from two sites of the upper iliac crest of 42 patients with adenocarcinoma of the pancreas before surgery. The two-sided aspiration was recommended because the distribution of metastasising cells in the marrow might not be homogenous. When unilateral and bilateral aspirates were compared, the frequency of tumour cells increased with the analysed sites [10].

Juhl and associates [15], using immunocytology to investigate the peritoneal cavity and the bone marrow of 147 patients with gastric, colorectal and pancreatic cancer, detected positive tumour cells in the bone marrow of 29% of colorectal, 25% of gastric and 58% of pancreatic cancer patients. Twenty per cent of the pancreatic cancer patients with stage I/II disease and 65% with stage III/IV disease and even 45% of the patients after curative resection had positive bone marrow findings. No correlation could be observed concerning the histological grading.

In conclusion, our data suggest that the presence of CK-positive cells in bone marrow of patients with cancer of the pancreas indicates the disseminative capacity of the primary tumour. Such cells could reach the peritoneal cavity or the liver, but no local outgrowth in bone marrow is necessary. The detection of micrometastasis might be important to assist in the decision of whether systemic treatment is required in addition to surgical removal of the primary tumour.

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