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Detection of circulating prostatic cells during radical prostatectomy

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Abstract The detection of micrometastasis of prostate cancer could help to decide more appropriate therapeutic strategies in an individual patient. We have developed a flow cytometric method for detecting cytokeratin-positive cells in the peripheral blood before, during and after radical prostatectomy in patients with prostatic carcinoma. By means of this technique we were able to detect a higher number of cytokeratin-positive cells in the intraoperative blood sample than in the pre- and postoperative blood sample in 15 patients with prostate cancer ($P < 0.05$). Our results show an increase in the number of cytokeratin-positive cells with increasing tumor stage and grade, as well a good correlation of prostate-specific antigen (PSA) value with the number of cytokeratin-positive cells ($r > 0.6$). Our results underline the importance of no-touch techniques at prostatectomy to minimize release of tumor cells into the circulation during surgery. In the light of our results we consider that the indication for cell savers during radical prostatectomy should be reevaluated. The possibility of detecting single metastatic cells in peripheral blood will enable better individual patient management, and open up new modalities for diagnosing early prostate cancer and enhancing patient monitoring in relapse and tumor progression.

Key words Prostate cancer · Flow cytometry · Circulating prostatic cells

Introduction

The metastatic process is incompletely understood, but involves separation of groups of clonogenic cells from the primary, entry into and transportation by lymphatic or vascular channels, arrest in the target organ, and proliferation. Suspicious cells have been found in the systemic circulation after surgical manipulation of prostate and colon cancer, but the identification of these cells as metastatic cancer cells has not been possible [7, 9, 11]. Presently, the biological importance of these prostate cells in the circulation is not known. Is intraoperative tumor cell dissemination, with the attendant risk of possible micrometastasization, detectable?

In recent years increasing attention has been directed towards the use of monoclonal antibodies against cytokeratins to detect micrometastatic carcinoma cells [8, 15]. Cytokeratins belong to the group of intermediate filaments, which are one of the main components making up the cytoskeleton of epithelial cells. Keratin immunoreactivity varies in the human prostate, probably due to expression of different keratin proteins [1]. More than 20 different antibodies against cytokeratin are currently available for the detection and classification of normal and neoplastic cells of epithelial origin [5, 13, 17]. It has not yet been possible to analyze and sort these cells by flow cytometry. In this study we used a DNA-binding fluorescent liquid and an anti-cytokeratin antibody to detect these cells in the blood by flow cytometry, before, during and after radical prostatectomy in patients with prostatic carcinoma. This is a feasibility study, in which the evidence and possible nature of these cells are discussed.

Materials and methods

Patients and tissue

Blood samples, fresh frozen and paraffin-embedded tumor tissue from 15 patients with prostatic carcinoma and two patients with benign prostatic hyperplasia were investigated by a new flow cytometric method before, during and after radical prostatectomy. Three patients underwent a radical retropubic and a 12 patients radical perineal prostatectomy (Table 1). On the basis of the histopathology there were nine pT3, five pT2 and one pT1 prostatic carcinomas. Patients were examined for metastases by bone scans,

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Table 1 Number of patients (*n*), age, operative method (*OP*) [perineal radical prostatectomy (*p*) or retropubic radical prostatectomy (*r*)], weight of prostate (*WP*), tumor stage (TNM classification) and grade of malignancy, preoperative prostate-specific antigen (*PSA*), and numbers of cytokeratin-positive cells per 10 000 cells measured in each blood sample obtained preoperatively (*Preop*), from the operation site (*Intraop*) the central line (*CL*) and postoperatively (*Postop*)

n	Age (years)	OP	WP (g)	TNM stage/Grade	PSA (μg/ml)	Nos. of cytokeratin-positive cells per 10 000			
						Preop	Intraop	CL	Postop
1	59	p	40	pT3 N0 M0 G2	3.4	1	21	34	0
2	65	r	40	pT3 N0 M0 G2	8.3	0	4	1	3
3	64	p	28	pT2 N0 M0 G2	1.4	2	0	1	3
4	73	r	–	pT2b N0 M0 G3	14.3	0	12	2	5
5	64	r	–	pT1b N0 M0 G2	3.4	0	0	4	2
6	65	p	28	pT2b N2 M0 G2	28	4	51	2	11
7	70	p	49	pT3 N1 M0 G3	8.6	4	16	28	3
8	61	p	37.5	pT3 N0 M0 G3	18.2	3	17	2	4
9	61	p	40.8	pT2b N0 M0 G3	2.9	0	26	2	1
10	66	p	46	pT3 N0 M0 G3	26.9	6	21	23	25
11	54	p	38.8	pT2 N0 M0 G2	5.6	2	3	6	3
12	71	p	45	pT3 N0 M0 G2	11	3	11	9	3
13	65	p	24.7	pT3 N0 M0 G3	4.2	0	2	30	1
14	61	p	72	pT3 N0 M0 G2	3.1	0	3	1	1
15	60	p	–	pT3 N0 M0 G3	20	0	98	0	6

chest radiography and pelvic lymphadenectomy. No evidence of metastases was found in any of the patients. The postoperative histopathological examination showed lymph node metastases in two patients. Blood samples from five healthy females and five healthy males served as a control group.

Experimental protocol

In each patient 4-ml EDTA blood samples were obtained immediately before the operation, intraoperatively from the central line and the operation site, and postoperatively on the day following the operation. Table 2 shows the individual steps of the density gradient separation, immunofluorescence staining and flow cytometry. In a preliminary experiment we compared the lysis method and the Ficoll method of density gradient separation. Various dilutions of a mesothelial suspension with a cell density of 1×10^6 were added to 4 ml of EDTA blood and analyzed comparatively by the lysis method and the Ficoll method of density gradient separation, followed by immunofluorescence staining and flow cytometric detection. In the lysis method the erythrocytes are lysed in ammonium chloride solution and the remaining cells washed three times for removal of the cell remains, fixed in 70% alcohol and washed again.

Before flow cytometric measurement DNA staining with propidium iodide was carried out in addition to the immunofluorescence staining. DNA staining served as a control, as the cytokeratin-positive cells were additionally identified as cells on the basis of their DNA content. Cell debris and cell aggregates could thus be excluded from measurement.

For this, after the hematoxylin-eosin (HE) sections had been evaluated and marked by the pathologist, the carcinomatous material from the freshly obtained prostatic tissue was mechanically separated in RPMI 1640 medium with L-glutamine and collagenase (type CLS III, 184 U/mg). During an incubation period of 90 min the cells were released from the tissue block and, after filtering, the prostate cells were present as a single-cell suspension. The single-cell suspension was then DNA-stained with propidium iodide. After immunofluorescence staining the mechanically separated prostate tissue was measured as positive control. Gating criteria were established using all negative controls of 15 neoplastic prostates and two benign prostates. Then the second decade of fluorescence intensity (10E2) was taken as the borderline between positive and negative cells. To test the intraspecific and interspecific reproducibility, blood samples from five patients with prostate cancer were measured twice by the same and a second operator.

Statistics

Linear regression analysis and Student's *t*-test were used for statistical analysis. Values of $P < 0.05$ were considered to be statistically significant.

Table 2 Methods of density gradient separation, immunofluorescence staining and flow cytometry

Density gradient separation by Ficoll
Dilute each EDTA blood sample with phosphate-buffered saline (PBS) in a ratio of 1:1
Pipette 4 ml Ficoll into a centrifuge tube, overlay with the pre-diluted EDTA blood sample without mixing, and centrifuge for 30 min (923 *g*, room temperature)
Transfer a white cell ring (lymphocytes and cells of non-hematological origin) to a new tube using a Pasteur pipette and centrifuge the new tube for 15 min (410 *g*, room temperature)
Pour off the supernatant and shake up the white pellet with 70% alcohol (incubation time 30 min) and wash twice

Direct immunofluorescence staining
Transfer the fixed cells from the density gradient separation into Eppendorf microtubes
Incubate with 30 μl fluorescein isothiocyanate (FITC)-conjugated anti-human cytokeratin (DAKO, MNF 116) antibody and 30 μl PBS
Add 1 ml PBS and centrifuge for 10 min (2000 rpm, room temperature)
Pour off supernatant and resuspend in 1 ml PBS

Flow cytometry
Flow cytometric detection by FACStar plus (Becton Dickinson, USA)
Cell sorting of the cytokeratin-positive cells and cytological evaluation of these by light and electron microscopy

Results

Mesothelial cells were positively stained with the cytokeratin antibody used. Distinctly more cytokeratin-positive cells were detected by the Ficoll method than by the lysis method. The Ficoll method was therefore chosen for our investigations. Before immunofluorescence staining with cytokeratin antibodies the mechanically separated prostate cancer tissue served as a negative control (Fig. 1). The corresponding dot plot after staining with cytokeratin antibodies served as a positive control (Fig. 2). In all negative controls of 15 neoplastic prostates and two benign prostates no cells with a fluorescence intensity greater than the second decade (10E2) were found in any of the measurements. The second decade (10E2) was therefore taken as the borderline between positive and negative cells.

No false positive detection was observed in the control group. Evaluation of the interspecific and intraspecific reproducibility in five patients with prostate cancer revealed no differences. Table 1 shows the number of cytokeratin-positive cells per 10 000 cells measured as a function of site of origin, time and tumor stage. The largest numbers of cytokeratin-positive cells were detected in the intraoperative blood samples ($P < 0.05$; Figs. 3, 4). Comparison of the mean numbers of cytokeratin-positive cells in the blood samples of all 15 patients with prostatic carcinomas shows that the highest values were in the intraoperative blood samples and the lowest in the preoperative samples ($P < 0.05$). Comparison of the mean values of the individual blood

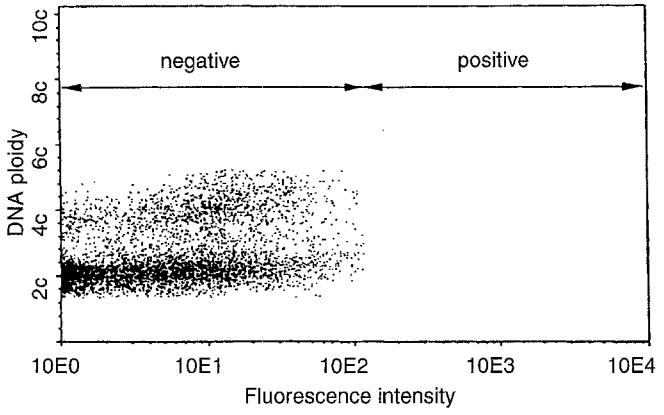

Fig. 1 Dot plot of mechanically separated prostate tissue after cytokeratin staining (patient 8)

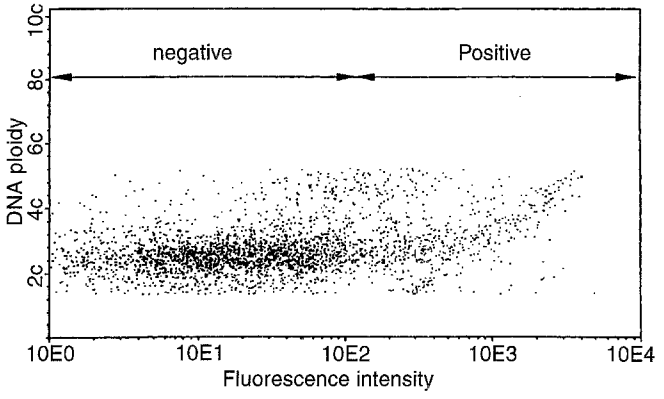

Fig. 2 Dot plot of mechanically separated prostate tissue without cytokeratin staining (patient 8)

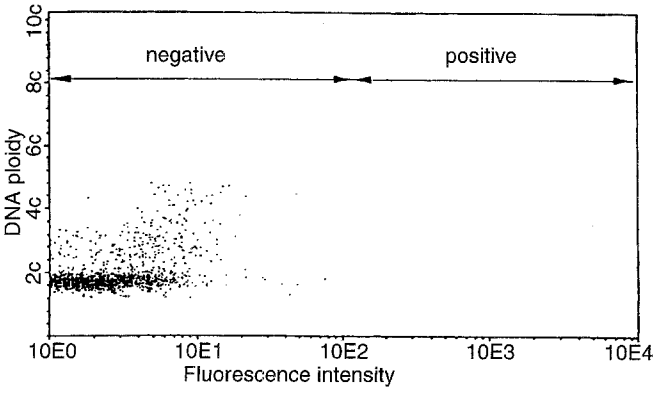

Fig. 3 Dot plot of prostate cancer (pT3G3) before cytokeratin staining (patient 8)

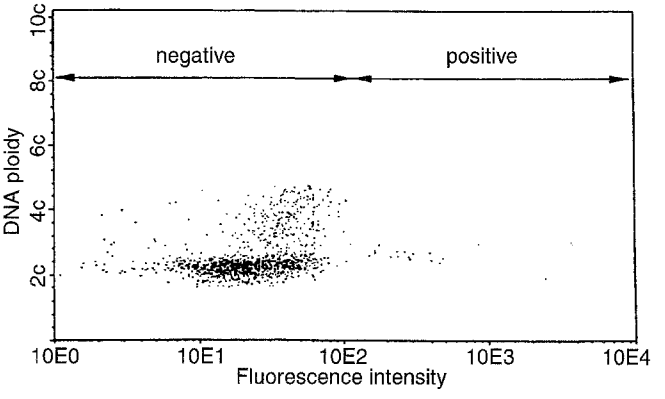

Fig. 4 Dot plot of prostate cancer (pT3G3) showing the results obtained in the intraoperative blood sample after cytokeratin staining (patient 8). Seventeen cytokeratin-positive cells were detected

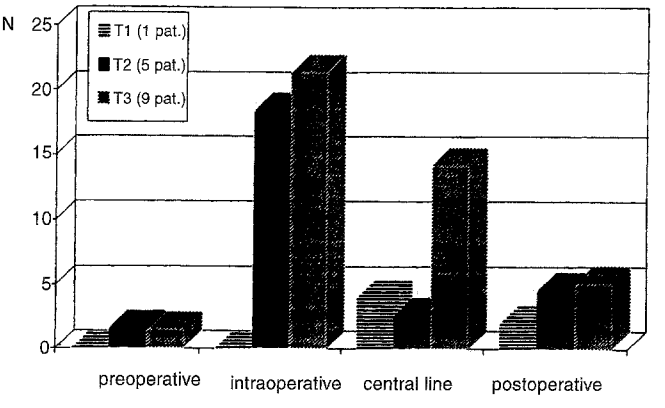

Fig. 5 Correlation between number of cytokeratin-positive cells (N) and tumor stage (T1–T3) in the pre-, intra- and postoperative blood samples and the central line

samples shows an increase in the number of cytokeratin-positive cells with increasing tumor stage and grade, as well as a good correlation of PSA value with the number of cytokeratin-positive cells ($r > 0.6$ in all blood samples; Table 1, Fig. 5).

Discussion

The cytoskeleton contains a number of different intermediate filaments made up of fibrillary proteins which show cell-specific variations in molecular size. The following types of intermediate filament are found: keratin, neurofilaments, vimentin and desmin. Keratin filaments consist of various keratin proteins, the cytokeratins, with molecular weights of 40 000 to 65 000, and occur in epithelial cells. They are important for stabilization of the cell shape. On the basis of antibody staining of the cytokeratins it is possible to draw conclusions regarding the primary tumor tissue [5, 17, 22].

We used a fluorescein isothiocyanate (FITC)-labeled monoclonal mouse anti-human cytokeratin antibody which is directed against a number of cytokeratins of epithelial cells of size 45–56.5 kDa. These include cytokeratins 10, 17 and 18 and react with a large number of benign and malignant epithelial lesions [13, 17]. We were able to demonstrate cytokeratin-positive cells in the peripheral blood and at the operation site before, during and after radical prostatectomy in all 15 prostatic carcinomas. Ghossein et al. [8]. were able to detect circulating tumor cells in the peripheral blood of 27% (15/55) of their patients with advanced prostatic carcinomas using reverse transcriptase polymerase chain reaction (RT-PCR) to amplify the mRNA of prostate-specific antigen (PSA). Moreno et al. [14] were able to detect PSA-synthesizing cells in the peripheral circulation of metastatic prostate cancer patients by PCR amplification. Cama et al. [2] found a positive RT-PCR for PSA and prostate-specific membrane antigen respectively in the peripheral blood of 16 and 10 of 20 patients with metastatic prostate cancer. Prostate-specific membrane antigen is a novel cloned prostatic marker which is, like PSA, almost prostate specific [10]. Hamdy et al. [9] detected PSA-positive cells in the peripheral blood of all 25 patients with metastatic prostate cancer using a monoclonal antibody against PSA and flow cytometry.

On the assumption that we would be able to detect cytokeratin-positive cells in prostatic carcinomas by flow cytometry we tried to isolate these cells by cell sorting. Cell sorting requires low cell concentrations in order to ensure a sufficiently large distance between the cells [19]. On account of the low detection rate of cytokeratin-positive cells isolation of these cells by cell sorting was not possible. Nussbaum et al. [15] were able to isolate cytokeratin-positive cells by cell sorting in two of four cases of prostatic carcinoma. The tissue of origin of the tumors is not mentioned by the authors. It is possible that these were advanced carcinomas with a strong tendency towards metastasization. These authors took the maximum green fluorescence (FITC) in the preoperative sample as the lower limit for the distinction between cytokeratin-positive and cytokeratin-negative cells. In our study, however, we were able to show that cytokeratin-positive cells are already detectable in the preoperative blood samples. We therefore consider it more accurate to determine cytokeratin positivity on the basis of the negative controls, the prostate biopsy as positive control and the preliminary mesothelial cell experiments.

Nussbaum et al. [15] were not able to detect any cytokeratin-positive cells in the postoperative blood samples of any of their 11 patients, which included four with advanced prostatic carcinomas. In our group of patients, 14 of 15 with prostatic carcinoma had cytokeratin-positive cells in their postoperative blood samples. Nussbaum et al. [15] used the lysis method for separation of the circulating tumor cells. In our preliminary experiments with mesothelial cells we were able to detect a higher rate of cytokeratin-positive cells by the Ficoll method than by the lysis method. Ficoll is a hydrophilic polymer with molecular weight of 400 000. The different cell populations are separated by Ficoll after centrifugation by means of their different cell densities. The higher cell losses using the lysis method are probably due to the greater number of washing steps and greater lysis of mesothelial cells in addition to the erythrocytes.

Flow cytometric detection of cytokeratin-positive cells using the cytokeratin antibody MNF 116 detects epithelial cells from a prostatic carcinoma and the normal prostate. This antibody also reacts by immunohistochemical staining with prostate cancer [13]. The value of cytokeratin-positive cells needs to be investigated further. Serum PSA levels of 20 ng/ml were found to be a good predictor of bone scan metastasis [3]. Our results show that there is a correlation between PSA and cytokeratin-positive cells. The highest number of cytokeratin-positive cells was detected in patients with serum PSA values of more than 20 ng/ml. The good correlation between cytokeratin positivity and tumor stage, grade, serum PSA and positive lymph nodes in our patient sample may be an indication of dissemination of prostatic carcinoma cells during and after radical prostatectomy. These results have to be considered with caution because of the small number of patients in this study. As the greatest numbers of cytokeratin-positive cells were found in the intraoperative blood samples, intraoperative dissemination of cytokeratin-positive cells is very likely.

Our results underline the importance of no-touch techniques to minimize release of tumor cells into the circulation during surgery. The no-touch technique described by Turnbull et al. [21] is based on early ligation of lymphovascular channels. The 5-year survival rate in 588 patients with colonic carcinoma was 68.85% in the no-touch group and 52.13% in the control group [21]. Robson et al. [18] proposed a reduction in tumor cell seeding by early ligation of the renal artery.

The dissemination of cytokeratin-positive cells must be regarded as a warning signal in surgical oncology. For the surgeon this means strict adherence to no-touch techniques in order to prevent the release of cytokeratin-positive cells. The detection of an increased number of circulating cytokeratin-positive cells during radical prostatectomy for prostatic carcinoma would support the use of neoadjuvant or perioperative chemotherapy or hormone therapy [12, 20]. Autologous blood transfusion using a cell saver during radical prostatectomy carries the risk of reinfusion of cytokeratin-positive cells. This is corroborated by the investigations of Dale et al. [4], who were not able to show sufficient elimination of malignant cells from the blood by the cell saver. Other authors consider that cell savers should only be used during certain phases of radical prostatectomy [6]. In the light of our results we are of the opinion that the indication for the use of cell savers during radical prostatectomy should be reevaluated.

With the help of double DNA/cytokeratin labeling of intact cells to eliminate interfering subpopulations we were able to perform flow cytometric analysis of fresh prostatic carcinoma material. In agreement with Park and Kimler [16] this permits more precise analysis of the tumor's DNA content and helps to exclude cell debris and cell aggregates. The proposed technique is an accurate method of detecting cytokeratin-positive cells during radical prostatectomy. Further research is required to establish whether prostatic carcinomas with a significant number of cytokeratin-positive cells in the peripheral blood have a higher risk for development of metastasis, relapse or progression. The potential role of cytokeratin-positive cells in the evaluation of metastatic prostate cancer remains to be established. The use of immunodetection by PSA or androgen receptor and flow-cytometry, an enhanced RT-PCR for PSA, and a prostate-specific membrane antigen PCR may help to characterize these cytokeratin-positive cells and identify prostate cancer with a metastatic potential.

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