Isolation of Prostate-derived Single Cells and Cell Clusters from Human Peripheral Blood

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Abstract

The detection of blood-borne prostate cancer (PCA) cells may help with clinical staging and the further understanding of PCA metastases. We discovered prostate-specific antigen (PSA)-positive stained but not PSA mRNA-expressing blood cells by means of cell sorting and PSA reverse transcription-PCR in patients. Therefore, we developed a cytokeratin immunomagnetic method to isolate PSA-positive epithelial cells from the circulating blood of PCA patients. We obtained blood-borne single cells from 6 of 10 PCA patients and clustered cells from 8 of 10 PCA patients. Patients with benign prostate hyperplasia tested negative for cell clusters. The reported isolation method yielded prostate-derived cells or clusters of them from PCA-diagnosed patients.

Patients and Methods

Patients. A volume of 40 ml of EDTA-treated peripheral blood from 8 PCA patients (3 with lymph node involvement), 4 patients with BPH, and 2 patients with bladder cancer and secondary PCA was taken at least 3 weeks after diagnostic fine-needle biopsy and subjected to the separation protocol. Women from the laboratory staff were chosen as negative controls.

FCM Analysis of PSA-positive Cells. A volume of 100 μl of EDTA-treated peripheral blood from the patients was incubated with 5 μl of each of the following monoclonal antibodies for 10 min at room temperature: anti-PSA-FITC (FS/26; IgG1; Dianova); anti-CD14-PE (RM052; IgG2a; Coulter-Immunotech); or anti-CD45-PE (1.33; IgG1; Coulter-Immunotech). The red blood cells were lysed in a Multi-Q-Prep system with the Coulter Immunoprep reagents (Coulter). Nonspecific staining was controlled by using mouse isotype control antibodies of the same IgG subclasses and concentrations.

Flow Cytometric Cell Sorting of Circulating PSA-positive Cells. Fluorescence-activated cell sorting was performed on a Coulter Epics Elite ESP flow cytometer (Coulter) adjusted for high-purity cell sorting. The data rate was less than 1,000 cells/sec. More than 30,000 cells positively stained for PSA and CD45 were sorted with a purity of >95%.

Combined Buoyant Density Gradient and Immunomagnetic Separation of Blood-borne Prostate-derived Cells and Cell Clusters from Freshly Obtained Peripheral Blood of Patients. The buoyant density gradient centrifugation was carried out as described by Griwatz et al. (15). In brief, 12 polystyrol tubes saturated with 1% FCS (Boehringer) in PBS were filled with 3 ml PolymorphPrep (Immuno) and overlaid with 3 ml NycoPrep 1.068 (Immuno). A volume of 5 ml of EDTA-treated blood drawn from patients was laid on top of the gradient. The centrifuge was centrifuged at 450 x g for 20 min at room temperature. The single band of cells between the interface of the platelet-enriched plasma and density of 1.068 g/ml was removed. The removed cells were pelleted by centrifugation at 200 x g. The harvested cells from the single band at the interface of the platelet-enriched plasma and the 1.068 NycoPrep density medium were fixed in 1 ml of 1% paraformaldehyde/PBS fixative (pH 7.4) for at least 2 h at room temperature, reaching a final concentration of 0.5% paraformaldehyde/PBS fixative. The preformation of the IPSC, comprising the biotinylated anti-cytokeratin antibody (K, 5+8.22/C 22; IgG1; Progen) and the rat-anti-mouse IgG2a+b antibody coupled to superparamagnetic microparticles (Miltenyi), was generated as described recently (15). The preformed IPSC was purified from nonbound mouse anti-cytokeratin antibody using a Mini-MACS column (Miltenyi). The harvested cell suspension was incubated with the preformed IPSC. The incubation medium contained 0.3% (v/v) saponin from saponaria species (Sigma-Aldrich) and 0.5% FCS in PBS. After an incubation time of 15 min at room temperature and 30 min at 4°C, the cell suspension was washed twice in 1 ml of 0.5% (v/v) saponin/PBS. An A1 column (Miltenyi) was prepared according to the literature (17). Briefly, the column was rinsed from bottom to top with −20°C 70% methanol then rinsed from top to bottom with 30 ml of 1% FCS/PBS and saturated with 1% FCS/PBS for 2 h. The separation experiments
were carried out under the following conditions; the putative IPSC-labeled cells were pipetted in a volume of 500 μl onto the top of the column and followed through by a 4-fold excess of 1% FCS/PBS. The IPSC-targeted magnetic cells were bound within the magnetic field of the steel wool fibers, whereas negative nonmagnetically stained cells passed the column and were collected in a tube as the "nonmagnetic fraction." This negative fraction was subsequently reapplied to the column four times. The column was then removed from the MACS stand and the cells were flushed sideways from bottom to top with a volume of 2 ml of 1% FCS/PBS using a syringe connected to the top of the column and then removed from the MACS stand. The column was rinsed with 2 ml of 1% FCS/PBS to remove the last nonspecifically attached cells. Therefore, the three-way stopcock was closed, and the cannula was removed. A 10-ml syringe filled with 1% FCS/PBS buffer was connected to the top of the column and then removed from the MACS stand.

The "positive magnetic fraction" was flushed from top to bottom into a 12-mm tube. Control experiments for nonspecific binding were performed with an isotype IPSC and rat anti-mouse IgG2a+b antibodies carrying microbeads (15).

**RT-PCR for the Detection of PSA mRNA in PSA-positive Stained Circulating Blood-Borne Prostate-derived Cells Separated from the Peripheral Blood of Patients.** The total RNA from the sorted cells was reverse-transcribed into cDNA using random hexamers and Super Script II reverse transcriptase (Life Technologies, Inc.). Therefore, 10 μl of the 40 μl of RNA eluted from the RNA extraction column were preincubated with 1 μl of random hexamers for 10 min at 70°C. After that, 2 μl of a 10-fold synthesis buffer (0.2 M Tris (pH 8.4), 0.5 M KCl, 0.025 M MgCl2, 1 μg/μl BSA, 2 μl of 0.1 M DTT, 1 μl of 0.01 M deoxynucleotide triphosphate mix, 1 μl Super ScriptII reverse transcriptase (200 units/μl), and 1.8 μl of nuclear-free water with 0.2 μl RNAsin (RNA inhibitor; 40 units/μl; Promega)) were added. The reaction mixture was incubated at room temperature for 10 min after further incubation for reverse transcription at 42°C for 50 min. The reaction was terminated by heating at 70°C. Finally, the chilled incubation mixture was incubated for 20 min at 70°C with 1 μl RNase H (Promega). For the first round and second (nested) round PCR, the reaction volume was 50 μl each. The reaction mixture contained 50 mM KCl, 10 mM Tris (pH 9.0; Promega), 200 μM deoxynucleotide triphosphate (Perkin Elmer Cetus), 1.5 mM MgCl2, 2 units Taq DNA polymerase (Promega), and 50 pmol of each primer as described by Jaakkola et al. (Ref. 12; outer sense, CACAGCCGACTTTCGAG; outer antisense, CCTTGTACCCTTCGGTAA; nested sense, TCCAATGAGGTGTGCGCA; nested antisense, GTGTACAGGGGAAGGCCCTC), and 31.6 μl diethylpyrocarbonate-treated water. In the first round, 10 μl of template cDNA from the reverse transcription were added to the PCR mixture, and in the second (nested) round, 10 μl of the PCR products from the first-round PCR were added to the reaction mixture. First- and second (nested)-round PCR cycling conditions were cycle 1, 4 min at 94°C, 1 min at 65°C, and 1.10 min at 72°C; and cycles 2—5, 1 min at 94°C, 1 min at 65°C, and 1.10 min at 72°C. The separation of the PCR products after the second-round (nested) PCR was performed on 4% agarose gel (agarose for nucleic acids < 500 bp; Biozym). The electrophoresis was performed at 60 mA and 90 V for 50—60 min at room temperature. The gels were stained with 0.5 μg/ml ethidium bromide. Fluorescent bands were visualized on an UV transilluminator and photographed by Polaroid camera equipment with Polaroid 667 film (3000 ASA). LNCaP cells expressing PSA mRNA were used as positive controls (ATCC 1740; DKFZ Tumorbank, Heidelberg, Germany). RNA integrity was proven by RT-PCR of β-actin mRNA with commercially available primers (β-Actin #302010; Stratagene).

**Immunocytchemistry of Blood-borne Prostate-derived Cells and Cell Clusters from the Peripheral Blood of Patients.** Cytocentrifuge preparations were made in a cytocentrifuge (Shandon) from a 150-μl cell suspension of the positive magnetic fraction (final MACS outlet). The slides were transferred to a moist chamber, and the cells were incubated for 10 min with diluted Blocking Solution in aqua dest. (1:10; KreaTech). The cytokeratin 8/18 antibody was visualized applying streptavidin-conjugated alkaline phosphatase (1.0 mg/ml; 1:1000 in Blocking Solution; Jackson ImmunoResearch) for 20 min. The reaction with alkaline phosphatase was developed using Newfuchsin (Carpenters). IPSC-separated epithelial cells from PCA patients were simultaneously stained for PSA using a FITC-labeled monoclonal antibody (F526; IgG1; Dionova). The fluorescence was monitored using a fluorescent microscope (Leitz Laborlux 12).

**Results**

**FCM of Sorted Circulating PSA-positive Stained Cells in the Peripheral Blood.** PSA- and CD45- or CD14-positive stained cells were gated out from the peripheral blood of the patients as shown in Fig. 1a—d. The peripheral blood cells were stained with an anti-PSA-FITC antibody, an anti-CD14-PE antibody, or a pan leukocyte marker, an anti-CD45-PE antibody, alternatively. In Fig. 1b, the dot plot for the two fluorescent dyes FITC and PE is shown, indicating a fraction of PSA-positive cells in gate R1 of the scatter plot (Fig. 1a), which surprisingly stained positive for CD45. FCM analysis using anti-PSA antibodies did not discriminate between circulating PSA-positive cells and blood-borne prostate-derived PSA-positive cells. The fluorescence of the remaining scatter-plot fractions R2—R4 (Fig. 1a) is demonstrated in Fig. 1c; less than 1% of the cells were double-stained for PSA and CD45. Fig. 1d represents the isotype control. The FCM analysis data corresponding to fraction R1 in Fig. 1b are visualized in Figs. 2A and B. Human peripheral blood cells sorted for anti-PSA-FITC (Fig. 2A) and CD45-FITC (Fig. 2B) substantiate the FCM data (Fig. 1b) that leukocytes can be stained positive for PSA.

**RT-PCR for the Detection of PSA mRNA from Sorted PSA-positive Stained Circulating Cells from the Peripheral Blood.** Fig. 3A—B shows the PCR products separated on 4% agarose electrophoresis gel stained with ethidium bromide. After 2 PCR rounds of 42 cycles, a PSA mRNA signal was obtained representing 194 bp from LNCaP cells as a positive control starting from a total RNA concentration of 10 pg (Fig. 3A, Lane 8) up to 1 ng (Fig. 3A, Lanes 9—11). The sensitivity of the PSA mRNA RT-PCR is demonstrated by comparing the band intensities of the specific PSA mRNA signal in Fig. 3A (Lane 8) and the very faint signal of β-actin in Fig. 3B (Lane 8). In the fraction of sorted PSA-positive stained circulating cells, there was no detectable PSA mRNA signal at 194 bp (Fig. 3A, Lanes 2—5). Female blood leukocytes were also negative for PSA mRNA (Fig. 3A, Lanes 6—7). RNA integrity was proven by β-actin mRNA RT-PCR (bottom, Lanes 2—11). The FCM analysis revealed PSA-positive stained leukocytes (CD45-positive) in the peripheral blood of patients, in which the mRNA could not be detected. The results of the RT-PCR for PSA mRNA suggest that the double-positive CD45- and PSA-stained cells do not express PCR-detectable levels of PSA mRNA.

**Combined Buoyant Density Gradient and Immunomagnetical Isolation of Prostate-derived Cells/Clusters from the Peripheral Blood.** The described isolation procedure was applied to 40 ml of peripheral blood from 14 patients. In Fig. 2C, an IPSC-captured single epithelial cell was stained red for cytokeratin. The same cell was labeled with an anti-PSA antibody conjugated with FITC (Fig. 2D). Similar results were obtained for cell clusters isolated from the peripheral blood of patients. A red-stained cell cluster for cytokeratin is shown in Fig. 2E, and the corresponding green fluorescence for anti-PSA-FITC is shown in Fig. 2F.

**Stage of the Disease and Isolated Single Cells or Cell Clusters from Patients with Prostate-related Illnesses.** The age, diagnosis, and clinical staging of the patients together with plasma PSA levels and isolated single cells and cell clusters are summarized in Table 1. In the blood of one patient (H. L.) suffering from BPH, a single cell could be discovered. In early PCA (T1N0M0), one of two patients (K. H.) exhibited both single cells and cell clusters in the peripheral blood. In advanced PCA, five of eight patients showed single cells, but seven of eight patients showed cell clusters when the isolation protocol was applied (Table 1). The plasma PSA level did not show
Fig. 1. FCM analysis of peripheral blood cells from a patient with BPH. a, forward-scatter/side-scatter plot. R1, large mononucleated cells; R2, granulocytes; R3, lymphocytes; R4, monocytes. b, fluorescent FITC (PSA-FITC)/fluorescent PE (CD45-PE) plot for the cell population R1. c, fluorescent FITC (PSA-FITC)/fluorescent PE (CD45-PE) plot for the three cell populations R2–R4. d, fluorescent FITC (ISO-FITC)/fluorescent PE (ISO-PE) plot for the isotype control.

Discussion

The key question at time of diagnosis of PCA is whether the disease is restricted to the prostate gland itself and hosts dormant cells or metastatic dissemination has already occurred. Before making therapeutic decisions, optimal staging criteria are required. At the time of diagnosis in about 60% of PCA patients, the extent of the disease is understaged (2, 9). Many patients who are treated surgically might have benefited from hormone therapy, and might have not suffered from the handicaps of radical prostatectomy.

The isolation of PSA and cytokeratin double-positive cells from the peripheral blood of PCA patients, which can be further genetically and biochemically processed for molecular markers of malignancy, would be a great help in identifying patients at metastatic risk. The rationale for this approach came from animal studies that suggest that about 0.01% of circulating cancer cells are able to lodge in other organs.

any correlation to the discovered PSA and cytokeratin double-positive cells.
ISOLATION OF BLOOD-BORNE PROSTATE-DERIVED CELLS

Fig. 2. PSA-positive stained cells from patients' peripheral blood. A, green fluorescence emission of leukocytes sorted from fraction R1 (Fig. 1a) by the anti-PSA-FITC stain; B, red fluorescence emission of the same cells simultaneously stained by CD45-PE. C, streptavidin alkaline phosphatase/Newfuchsin red-stained epithelial cells enriched from a T2bN1M0 PCA patient’s peripheral blood; D, green fluorescence counterstain of the same cell as shown on the opposite picture by anti-PSA-FITC. E, red-stained clustered cells for cytokeratin; F, green fluorescence counterstain of the same cluster by anti-PSA-FITC antibody.
Table 1 Prostate disease patients, staging, and cells discovered in the peripheral blood

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Age (yr)</th>
<th>PSA (ng/ml)</th>
<th>Single cells</th>
<th>Cell clusters (≥2 cells)</th>
</tr>
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<tr>
<td>W. A.</td>
<td>BPH</td>
<td>74</td>
<td>1.7</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A. V.</td>
<td>BPH</td>
<td>65</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>H. L.</td>
<td>BPH</td>
<td>72</td>
<td>2.4</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>G. H.</td>
<td>BPH</td>
<td>70</td>
<td>15.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>L. D.</td>
<td>BCA/PCA</td>
<td>64</td>
<td>T1N0M0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>K. H.</td>
<td>BCA/PCA</td>
<td>67</td>
<td>T1N0M0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>D. G.</td>
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<td>72</td>
<td>T2cN0M0</td>
<td>17.8</td>
<td>103</td>
</tr>
<tr>
<td>L. V.</td>
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<td>70</td>
<td>T3cN0M0</td>
<td>19.8</td>
<td>ND</td>
</tr>
<tr>
<td>D. H.</td>
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<td>63</td>
<td>T3aN0M0</td>
<td>30.0</td>
<td>ND</td>
</tr>
<tr>
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<td>42</td>
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<td>62.1</td>
<td>17</td>
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<tr>
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<td>13</td>
</tr>
<tr>
<td>K. H.</td>
<td>PCA</td>
<td>73</td>
<td>T2bN1M0</td>
<td>6.0</td>
<td>12</td>
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<tr>
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<td>76</td>
<td>T3cN1M0</td>
<td>51.3</td>
<td>3</td>
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Fig. 3. A, ethidium bromide-stained agarose gel (4%), demonstrating the results of the sensitivity PSA mRNA RT-PCR. Samples were subjected to the competitive RT-PCR procedure of 2 × 42 cycles. Lane 1, water blank; Lanes 2–5, RT-PCR products of the mRNA from fractions R1–R4, which were yielded by cell sorting; Lanes 6–7, RT-PCR products of the mRNA from leukocytes of healthy female volunteers. No specific PSA mRNA signal is depicted. Lanes 8–11, RT-PCR products of the mRNA from LNCaP cells. The lower band (194 bp), the specific PSA mRNA PCR product; upper bands, nonspecific and genomic amplification due to the high cycle number of PCR. Lane 12, DNA molecular weight markers. B, ethidium bromide-stained agarose gel (4%), demonstrating the results of the conventional β-actin mRNA RT-PCR. Samples were subjected to the competitive RT-PCR procedure of 2 × 42 cycles. In all samples, β-actin mRNA could be detected. Lane 1, water blank; Lanes 2–5, RT-PCR products of the mRNA from fractions R1–R4, which were yielded by cell sorting; Lanes 6–7, RT-PCR products of the mRNA from leukocytes of healthy female volunteers; Lanes 8–11, RT-PCR products of the mRNA from LNCaP cells; Lane 12, DNA molecular weight markers.

(18). This means that about 2 cells/ml of blood suffice for metastasis formation.

Staining with only an anti-PSA antibody is not sufficient to specifically detect PCA cells in the peripheral blood. The detection of a fraction of double-positive CD45+/PSA-stained cells clearly shows that leukocytes can also carry the PSA molecule or immunologically detectable PSA fragments. A sensitive RT-PCR procedure after cell sorting further revealed that the PSA-positive leukocytes did not express detectable amounts of PSA mRNA. The autofluorescence phenomenon could be neglected because the isotype control showed no positive cells (Fig. 1d).

However, for answering a well-addressed question, an FCM analysis under stringent multilabeling conditions can be an appropriate tool to sort out cancer cells from the peripheral blood, as recently shown for breast cancer cells (19). Apart from FCM sorting, Wang et al. (20), using only the magnetic microbead technique, were successful in detecting epithelial-derived cells from the peripheral blood of colon cancer patients.

We have shown in vitro that PCA cells can be isolated by simultaneously targeting PSA and cytokeratin 8/18 molecules (15). We extended the buoyant density gradient immunomagnetic method for the isolation of PSA- and cytokeratin 8/18-positive cells from patients suffering from BPH and PCA. First, the prostate-derived cells in the peripheral blood were separated from the bulk of blood cells by parameters of density and osmolality. Epithelial cells or clusters were banded (due to their equal mass:volume relation) around the interface between the density band of platelet-enriched plasma and the 1.068 g/ml density of the buoyant density gradient. These cells can be further enriched by the immunomagnetic method, which uses cytokeratin 8/18 as a target. The likelihood of prostate origin of the isolated cells can be determined by additional staining with anti-PSA-FITC antibody and RT-PCR analysis. These cells can be further character...
izered by immunological methods and also characterized semiquantitatively by molecular methods, as already shown for breast cancer cells (21). If, and only if, there is a benchmark of reliable molecular markers for a certain organ-specific cancer, it is likely that under the conditions of multipositive staining or signaling, the origin and identity of such cells could be determined.

Our methodological procedure can clearly discriminate benign and malignant disease of the prostate concerning the isolation of single cells or the obtained clustered cells (Table 1). PCA patients show clustered, likely prostate-derived cells. Cluster formation has been recently confirmed by culturing tumor explants in extracellular matrix (22). The occurrence of clustered cells only in PCA patients is curious and potentially useful in understanding the process of PCA metastasis formation.

For the immediate postbiopsy period (≤ 30 min), preliminary data indicate that iatrogenic factors might be involved in the conversion of a negative PSA RT-PCR to a positive one (23, 24), a result that might be due to the appearance of shed single or clustered cells in the circulation. However, there are some well-taken points to consider as to why our data suggest that the appearance of clustered cells is an unlikely result of iatrogenic factors.

Fidler summarized his work on human cancer metastasis in 1990, concluding that after 24 h, only <0.1% of initially delivered cancer cells remain in the circulation (18). In our study, blood samples were collected in all cases at least 3 weeks after prostate biopsy. Furthermore, the same fine-needle biopsy technique was applied to patients with BPH and PCA.

To meet the reliability criteria for the clinical diagnosis of PCA patients, the application of our technique is recommended for patients with presumed newly diagnosed PCA before the prostate is manipulated.

Riesenberg et al. (25) discovered PSA/cytokeratin double-positive cells with large morphological variations in bone marrow from 5 of 18 PCA patients. In 8 of 10 cancer patients, we found PSA/cytokeratin double-positive cells in the blood. Authors using RT-PCR for the detection of PSA expression in whole blood reported positive mRNA signals, which might indicate circulating PCA cells, considering the exclusion of nonspecific PCR signals (8–12).

In our study, the number of patients included is too small to show a correlation between the number of single cells or clusters isolated and the plasma PSA level. However, the method allowed us to separate blood-borne prostate-derived cells and clusters independently from the PSA level in the patients’ blood.

We conclude that the genetic and biochemical analysis of such isolated cells and cell clusters will give a clinical impetus contributing to the resolution of the question of PCA cell identity.

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References

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