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Detection of Circulating Prostate-Specific Antigen–Secreting Cells in Prostate Cancer Patients, *Catherine Alix-Panabières,[1] Xavier Rebillard,[3] Jean-Paul Brouillet,[4,5] Eric Barbotte,[6] François Iborra,[7] Bruno Segui,[3] Thierry Maudelonde,[4,5] Colette Jolivet-Reynaud,[8] and Jean-Pierre Vendrell[1,2*]*
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The detection of circulating tumor cells in blood *(1–4)* requires highly sensitive, specific, and reproducible methods. During the last decade, immunocytochemistry *(5*, *6)*, reverse transcription-PCR *(7–9)*, flow cytometry *(10–13)*, and CellSearch and CellSpotter systems *(14)* have been assessed for the early detection of these rare circulating cells to predict tumor progression, survival in patients with metastatic cancer, and tumor dormancy *(15)*. The enzyme-linked immunosorbent spot (ELISPOT) assay has been validated for enumeration of cells secreting immunoglobulins and antibodies *(16*, *17)*, cytokines *(18)*, and viral antigens *(19)*. The 2-color ELISPOT assays allow enumeration of cells simultaneously secreting 2 cytokines *(20*, *21)*, IgG or IgA *(22)*, or monoclonal immunoglobulins into the blood of patients with multiple myeloma *(23)* and MUC-1/Cath-D–secreting cells in metastatic breast cancer patients *(24)*.

Here we describe a new ELISPOT assay for detection of human prostate-specific antigen (PSA)–secreting cells (SCs) in patients with prostatic carcinoma (PCa). This test was developed and optimized by use of LNCaP (ATCC; CRL-1740) and PC-3 (ATCC CRL-1435; provided by Pr. Pantel, Tumor Biology Institute, Hamburg, Germany) cell lines, which do and do not secrete PSA, respectively *(25)*; PSA-SCs were then assessed in the blood of 114 men who had given written informed consent. The patients were divided into 4 groups: (*a*) 24 patients (median age, 73.5 years; range, 58–90 years) diagnosed with clinically localized PCa (LPCa), (*b*) 24 patients with metastatic PCa (MPCa), (*c*) 31 patients with benign prostatic hyperplasia (BPH; n = 27; median age, 69 years; range, 52–82 years) or acute prostatitis (AP; n = 4; median age, 60 years; range, 50–63 years), and (*d*) 35 patients (median age, 67 years; range, 22–96 years) with nonprostatic disease (NPD) and 8 healthy controls (median age, 67 years; range, 49–81 years) with serum PSA <4 μg/L. Among the patients with LPCa, 12 (patients 1–12; median age, 74 years; range, 65–90 years) were studied before treatment and 12 others (patients 13–24; median age, 74 years; range, 58–86 years)

were studied after treatment by radical prostatectomy (RP; n = 4; 4–6 weeks after RP), transurethral resection of the prostate (n = 5), radiotherapy (n = 2), or hormone therapy (n = 1). The characteristics of these groups are given in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/. Among the patients with MPCa, 8 patients were studied at the diagnosis of PCa (median age, 75.5 years; range, 66–88 years) and 16 patients had metastatic localizations 2–12 years after RP (median age, 66.5 years; range, 55–81 years). Peripheral blood mononuclear cells (PBMCs), including cancer cells, were isolated by lysis of erythrocytes in blood samples collected in EDTA tubes (5–28 mL). Prostate-derived cells were enriched by the depletion of blood-derived cells by use of an anti-CD45 monoclonal antibody (mAb) together with a magnetic separation procedure (Dynal), and PSA was measured by a PSA immunometric assay (TRACE technology; B.R.A.H.M.S.).

The PSA ELISPOT assay was performed as described recently for MUC-1/Cath-D *(24)*. Briefly, Immobilon-P membranes were coated with mAb 11E5C6 (0.9 mg/L; bioMerieux) directed against total PSA *(26)*, and 10^5 to 1 LNCaP cells and 5×10^4 to 2×10^5 of enriched $CD45^-$ cells from patients with PCa were seeded in 4 different wells. After 24–48 h of cell culture, alkaline phosphatase- or phycoerythrin-labeled anti-free PSA mAb 6C8D8 (0.3 and 0.9 mg/L, respectively; bioMerieux) was added *(26)*. The percentages of cytokeratin-negative (CK^-) and PSA-positive LNCaP and PC-3 cells were determined by flow cytometry (FC 500 apparatus; Beckman-Coulter) by intracytoplasmic staining by isothiocyanate of fluorescein-conjugated anti-human CK mAbs (DakoCytomation), phycoerythrin-conjugated anti-human PSA mAbs (bioMerieux), and the IntraPrep™ permeabilization reagents (Beckman-Coulter). LNCaP, PC-3, and $CD45^-$ cells (6×10^4) from 2 MPCa patients (patients 29 and 44) and from 2 healthy controls were seeded on glass slides and immunostained for PSA and CK.

LNCaP cells taken between doubling times 1 and 20 were incubated for 24 h on the ELISPOT plate (n = 6); a mean (SD) of 29 (4.9)% of the cells secreted PSA, whereas PC-3 cells did not. Flow cytometric analysis (n = 6) showed that 100% of the cells of the 2 lines secreted intracytoplasmic CK; 27 (2.1)% of the LNCaP cells but none of the PC-3 cells were PSA-positive. Finally, similar results were obtained with immunocytochemistry experiments (n = 6; Fig. 1 of the online Data Supplement). The threshold for detection of the PSA-secreting cells by the PSA ELISPOT assay was estimated by serial dilutions of the LNCaP cells (10^5 to 1 cell/well). The PSA ELISPOT assay detected cells at concentrations 4 orders of magnitude lower than did the PSA immunoassay in culture supernatants (Table 1). To increase the sensitivity of the PSA ELISPOT assay, erythrocyte lysis and $CD45^+$ cell depletion were performed before the remaining cells were tested for PSA secretion by dispersion of 10^3, 10^2, 10, and 1 LNCaP cells in 10 mL of control blood (Table 1).

In patients without prostate cancer, no PSA-SCs were detected in the blood of 27 patients with BPH, 4 patients

Table 1. Detection threshold and recovery of the PSA ELISPOT assay.

A. Detection thresholds of the PSA ELISPOT assay and the PSA immunometric assay

	PSA ELISPOT assay,[a] spots/well		PSA immunometric assay,[b] μg/L in supernatant	
LNCaP cells/well	LNCaP cells	PC-3 cells	LNCaP cells	PC-3 cells
100 000	Uncountable	0	1.29 (0.17)[c]	0
10 000	Uncountable	0	0.03 (0.02)	0
1000	259.0 (24.7)	0	0	0
100	24.0 (2.1)	0	0	0
10	3.7 (1.2)	0	0	0
1	0.3 (0.4)	0	0	0

B. Recovery of the PSA-ELISPOT assay

LNCaP cells		LNCaP cells in control blood		
Total, n	PSA-SCs among untreated LNCaP cells, n	Cells added, n	PSA-SCs found in 10 mL of blood,[d] n	Recovery of LNCaP cells after processing,[e] %
1000	310.6 (67.0)	1000	116.0 (58.4)	37.3 (26.0)
100	32.0 (8.0)	100	13.2 (5.3)	41.3 (21.5)
10	3.8 (0.7)	10	3.4 (0.8)	89.5 (12.5)
1	1.0 (0.0)	1	1.0 (0.0)	100.0 (0.0)

[a] Cells were cultured for 48 h on nitrocellulose membranes of the ELISPOT plates.

[b] Supernatants of the cultured cells were tested by the PSA immunometric assay.

[c] Mean (SD) of 4 wells.

[d] Four concentrations of LNCaP cells were dispersed in 10 mL of control blood; the erythrocytes were lysed, $CD45^+$ cells were depleted, and the remaining cells were tested by the PSA ELISPOT assay.

[e] The recovery of LNCaP cells after processing was determined as the ratio (× 100) of the number of PSA-SCs obtained after dispersion of the LNCaP cells in 10 mL of control blood to the number of PSA-SCs enumerated among untreated LNCaP cells. Mean (SD) of 6 experiments.

with AP (Table 2 of the online Data Supplement), and 35 with NPD (Table 3 of the online Data Supplement) or in 6 healthy controls (data not shown), although most had abnormally increased serum PSA. Among 12 PCa patients tested before treatment, 5 (42%) had detectable PSA-SCs (median, 9 cells; range, 2–172 cells), whereas for the others no PSA-SCs were detected (Table 1 of the online Data Supplement). These results could be explained by the absence of cell shedding by the primary tumor or by the random distribution of a small number of PSA-SCs *(27)*. Serum PSA (median, 34 μg/L; range, 22–5300 μg/L) was significantly higher ($P = 0.001$) in patients with PSA-SCs than in those without circulating cells (median, 15 μg/L; range, 4.5–20 μg/L), but the cell numbers were not correlated with the serum PSA concentration ($r = 0.31$; $P = 0.12$). For 12 patients eligible as responders to treatments because all were asymptomatic, bone-scan negative, and without biochemical recurrence, serum PSA (median, 1.12 μg/L; range, 0–4 μg/L) was significantly lower than in patients tested before treatment (median, 19.5 μg/L; range, 4.5–5300 μg/L; $P = 0.0001$), and no PSA-SCs were observed. The number of patients who had PSA-SCs before treatment was significantly higher than the number of patients after efficient treatment (5 of 12 vs 0 of 12; $P = 0.04$). This observation suggests that elimination or therapeutic control of the prostate tumor process stopped the shedding of cancer cells into the bloodstream. Among MPCa patients, 20 (83.3%) had PSA-SCs in the blood (Table 1 of the online Data Supplement; median PSA-SCs, 21.5; range, 0–684; median serum PSA, 92.5 μg/L; range, 0.3–8400 μg/L). The percentage of MPCa patients with PSA-SCs was significantly higher than the percentage of patients with LPCa (20 of 24 vs 5 of 12; $P = 0.02$). For 2 MPCa patients, positive cells were observed by CK/PSA immunocytochemistry (Fig. 2 of the online Data Supplement). In contrast, no CK- or PSA-positive cells were detected in the blood of the healthy controls, suggesting that the PSA-SCs in MPCa patients probably represented cells derived from the primary tumor. The specificity and sensitivity of the PSA ELISPOT assay were 100% (95 confidence interval, 94.6%–100%) and 69.4 (54.4–84.5)%, respectively, and the positive and the negative predictive values were 100 (86.3–100)% and 85.7 (77.9–93.5)%, respectively.

The ELISPOT assay offers advantages compared with other methods: (*a*) It has a resolution orders of magnitude greater than that achieved by flow cytometry and PSA immunometric assays because the secreted PSA proteins are immunocaptured by the membrane in the immediate vicinity of the cells before being diluted in the culture supernatants. (*b*) In contrast to the real-time reverse transcription-PCR technique, the ELISPOT assay is based on the identification of only viable functional cells targeted by the proteins they secrete. (*c*) The anti-PSA antibodies used in the PSA ELISPOT assay have been carefully selected for their high specificity, which eliminates false positives. The PSA ELISPOT assay is, however, time-consuming because 48 h of cell-culture is necessary for immunocapture of the secreted PSA by the ELISPOT support.

The combination of the PSA ELISPOT assay with a cell-enrichment procedure and cell cryopreservation allowed the detection of as few as 1 PSA-secreting LNCaP cell disseminated in 10 mL of control blood. In addition, control PBMCs did not secrete PSA, suggesting that residual PBMCs remaining after immunomagnetic enrichment do not interfere with the PSA ELISPOT assay. Consequently, this ELISPOT assay appears to be able to detect a single PSA-SC in 20 mL of blood from patients with MPCa or LPCa. The number of circulating prostate-derived cells was not significantly correlated with the serum PSA concentration. PSA-SCs were enumerated in the majority of the PCa patients, whereas they were not detected in patients with BPH, AP, or NPD or in healthy controls, in agreement with previous reports *(28)*. This observation strongly suggests that circulating PSA-SCs were shed only by the malignant prostate tumors. The presence of these cells was more frequent in PCa patients before rather than after treatment and in patients with MPCa rather than LPCa, suggesting that detection of these cells could be used as a marker to measure therapeutic efficacy and tumor progression in PCa patients. We recommend that PSA ELISPOT assays be performed on more patients with PSA values between 4 and 10 μg/L because the results of those experiments may be of interest for clinicians to differentiate patients with LPCa from patients with benign disease.

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Plasma 8-Isoprostane Concentrations in Patients with Age-Related Cataracts, *Bin Wang,[1*] Huaijun Zhu,[1] Hong Sun,[2] Jinshun Pan,[1] Zhilan Yuan,[2] and Rongbin Yu[3]* ([1] Department of Pharmacology, School of Basic Medical Science, Nanjing Medical University, Nanjing, China; [2] Department of Ophthalmology, First Affiliated Hospital of Nanjing Medical University, Nanjing, China; [3] Department of Epidemiology and Biostatistics, School of Public Health, Nanjing Medical University, Nanjing, China; * address correspondence to this author at: Department of Pharmacology, School of Basic Medical Science, Nanjing Medical University, 140 Hanzhong Road, Nanjing 210029, China; fax 86-25-86862884, e-mail binwang@njmu.edu.cn)

Cataracts are the most common cause of blindness and visual dysfunction in the world. Cataractogenesis is a highly complex, multifactorial process. Epidemiologic studies have shown that potential risk factors include age, sex female, exposure to ultraviolet light, smoking, diabetes, and oxidative stress *(1–4)*. Opacification of the ocular lens may be initiated or promoted by oxidative damage, and data in the literature support an important role of oxidative damage in cataract formation *(5, 6)*. Although animal experiments show evidence for a protective role of antioxidants *(7, 8)*, the association between low concentrations of antioxidants and increased risk of cataracts remains controversial. Whereas some studies have demonstrated such associations, others have not *(9, 10)*.

Isoprostanes are a complex family of compounds produced from arachidonic acid. One of the isoprostanes, 8-isoprostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), belongs to a family of eicosanoids of nonenzymatic origin *(11)*. 8-Iso-$PGF_{2\alpha}$ has been widely used as a valid marker of oxidative stress *(12, 13)*. Several methods are currently used to quantify 8-iso-$PGF_{2\alpha}$, including gas chromatography–mass spectrometry, gas chromatography–tandem mass spectrometry, and liquid chromatography–tandem mass spectrometry *(14, 15)*, but their cost and technologic requirements limit their routine use. Recently, immunoassays have also been developed to measure 8-iso-$PGF_{2\alpha}$ *(16, 17)*.

Koliakos et al. *(18)* found that the mean concentration of 8-iso-$PGF_{2\alpha}$ in the aqueous humor from cataract patients with exfoliation syndrome was higher than in patients with cataracts only. However, no study has evaluated plasma 8-iso-$PGF_{2\alpha}$ concentrations in cataract patients.

In this study, we used an enzyme immunoassay to measure the plasma concentrations of 8-iso-$PGF_{2\alpha}$ in patients with age-related cataracts and in age/sex frequency-matched controls to explore the potential role of systemic oxidative status in the development of cataracts.

Unrelated patients older than 50 years of Chinese nationality with age-related cataracts were recruited at the Nanjing Medical University Affiliated Hospital. Control patients were frequency-matched for age and gender. For each case, a control was matched within the same 5-year age group. All participants had a complete ophthalmologic examination. Cataract status was determined by lens examination with a biomicroscope and ophthal-