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Comparison between NK cell activity and prostate cancer stage and grade in untreated patients: correlation with tumor markers and hormonal serotest data

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Summary. NK cell activity was measured in 24 patients with untreated prostate cancer (11 subjects with localized disease, D0, and 13 patients with stage D tumor) and 10 healthy controls. In these same subjects serum prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), testosterone, prolactin and cortisol concentrations were assessed. The data obtained were correlated with both tumor spread (localized vs disseminated disease) and grade (well-differentiated cancer, G1, vs moderately and poorly differentiated carcinoma, G2 and G3). In patients with stage D0 cancer mean NK activity (33.0 ± 10.6) was virtually identical with the mean value recorded in healthy men (34.5 ± 7.1), while in subjects with stage D1–D2 disease NK activity was significantly reduced (11.9 ± 7.1). These findings correspond with our data on treated subjects, in whom NK activity level was found to correlate well with the presence of tumor cells in the circulation. In subjects free of malignant tumors but with a chronic disease (diabetes, arthritis, severe rheumatic disorders) mean NK activity was clearly reduced (5.7 ± 1.5). The use of NK activity data as a probe for tumor metastases was found to be statistically as reliable as was the application of the PSA serotest (but not serum PAP concentrations). None of the measured hormonal parameters correlated well with tumor stage. Both testosterone and prolactin serum concentrations were found to be lower in the G2 and G3 cancer group than in well-differentiated (G1) tumors, in accordance with the published literature. In conclusion, NK lytic activity measured in untreated prostate cancer patients has been found to be insensitive to differences in prostate cancer grades ($P = 0.86$) but readily differentiates tumor dissemination from localized disease ($P < 0.01$). From the results of this preliminary study we advocate the assessment of NK activity as potentially useful supplement to prostate cancer staging protocols.

Key words: Hormone levels – NK activity – Prostate cancer – Serum PSA – Serum PAP

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Clinical approaches used in prostate cancer diagnosis and monitoring include protocols that analyze (1) primary tumor volume (digitorectal examination and ultrasonography), (2) cytological, histological and immunohistochemical characteristics of both primary tumor tissue specimens and regional lymph nodes, (3) indirect evidences relating to distant metastases (radiography and scintigraphy) and (4) circulating gene products that may denote tumor load and genotype [3, 10]. More detailed description of tumor genotype may be gained by applying molecular genetic techniques such as the polymerase chain reaction assay [15]. Metastases are a cause of death and hence novel clinical tools aimed at an earlier and more accurate detection of tumor dissemination processes are of the utmost importance.

The role of NK spontaneous lytic activity has been recognized as one of the body's defences against tumor dissemination [20]. The mechanism of such non-MHC-restricted action, although simpler than mechanisms related to the activity of other parts of the immune system, is far from being understood [8, 9]. NK cell activity was recently studied in treated prostate cancer patients and found to be (1) strongly dependent on the presence of prostate cancer cells in the circulation and (2) fully independent of the differentiation grade of those same cells, of the antineoplastic drug administered (diethylstilbestrol, estramustine phosphate, cyproterone acetate, flutamide) and of changes in plasma hormone concentrations (cortisol, prolactin, estradiol) provoked by these same agents [4].

Untreated prostate cancer is usually a heterogeneous tumor composed of androgen-sensitive and hormone-insensitive cell subpopulations. During initial anti-androgen treatment androgen-dependent tumor cells undergo apoptosis while hormone-independent cells eventually become the major cell subpopulation through selective growth. Accordingly, treated and untreated prostatic carcinomas may differ dramatically in their gene products which in turn may play a role in the regulation of NK lytic activity. In treated prostate cancer patients NK activity assay was found to be a sensitive probe for the presence

of tumor cells in the circulation [4]. The correlation between NK cell activity, tumor marker serotests (prostate-specific antigen, PSA, and prostatic acid phosphatase, PAP) and blood hormonal parameters (testosterone, prolactin and cortisol) is reported here. This study was undertaken to delineate the possible use of NK activity data in (1) prostate cancer staging protocols and (2) the recognition of early steps in the cascade of events leading to cancer dissemination.

Materials and methods

Subjects

A total of 24 patients with cytologically and/or histologically proven prostate cancer were studied. The extent of their disease was estimated by means of digitorectal examination, ultrasound, abdominal computed tomography, radiographs and scintigraphic images. In some patients lymph node involvement was detected during transvesical prostatectomy. Tumor grades were recorded as well-differentiated cancer (G1, 14 patients), moderately differentiated tumor (G2, 3 patients) or poorly differentiated disease (G3, 7 patients). The mean age of the patients was 71.3 years.

The control group consisted of 10 healthy men not fully age-matched with the cancer patients (average age 62.4 years). Additionally, equivalent data were assessed from 6 subjects free of any malignant tumor but suffering from a long-term chronic disease (arthritis, 2 patients; diabetes, 2 patients; arthritis with severe rheumatic disorders, 1 patient; rheumatic disorders with chronic lung diseases, 1 patient) (Table 2).

In all subjects serum PSA, PAP, testosterone, prolactin and cortisol concentrations were measured together with the NK activity data.

Assay for NK activity

The activity of peripheral blood NK cells was measured by an in vitro ^{51}Cr release assay with K-562 target cells [4].

Preparation of effector cells

Human peripheral blood mononuclear cells were separated from heparinized venous blood by centrifugation on a Ficoll-Triosil density gradient. This procedure provides mononuclear cells with a yield of more than 90% and a viability higher than 95%. Cells at the interface were collected, washed twice in medium 199 (without serum), resuspended in 10% human AB serum-RPMI medium, and adjusted to a cell concentration of 10×10^6/ml.

Preparation of target cells

K-562 target cells (2×10^6 to 4×10^6 in 0.5 ml 10% human AB serum-RPMI medium) were labeled with 150 µCi ^{51}Cr (as sodium chromate; Amersham, England) for 60 min. Cells were washed three times in 10% human AB serum-RPMI medium, suspended in the same medium and adjusted to a cell concentration of 2×10^5/ml.

Cytotoxic assay

Target cells (0.1 ml) were mixed with effector cells (0.1 ml) at different effector to target ratios (12.5:1, 25:1, 50:1) in triplicate. The results from the 50:1 ratio are reported here. Controls with K-562 target cells were incubated either with medium (spontaneous release, mean 10.6%, range 8.3%–12.2%) or with 0.05% Triton X-100 (maximum release, mean 83.7%, range 82%–90.1%). After 4 h at 37°C in 5% CO_2 samples were diluted with 1 ml RPMI. After centrifugation at 800 *g* for 5 min 0.8 ml aliquots of each supernatant were counted using a gamma counter.

The percentage of the isotope released from target cells and the corresponding specific cytotoxicity were calculated from:

$$\text{Percentage release} = \frac{[(\text{experimental } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}) \times 100]}{\text{maximum } ^{51}Cr \text{ release} - \text{spontaneous } ^{51}Cr \text{ release}}$$

Serotest measurements

Blood samples for the assessment of serum tumor markers (PSA, PAP) and hormonal parameters (testosterone, prolactin and cortisol) were taken prior to any manipulation of the prostate or alternatively 8 days after minor prostatic manipulations and/or 30 days after either transrectal biopsy or transurethral resection of the prostate (TURP). Serum was frozen at −20°C until assessed.

Serotest values were measured using radioimmunodetection kits from Yang Laboratories, Washington, USA (PSA), BSM Diagnostica, Vienna, Austria (PAP and testosterone), Institute for Immunology, Zagreb, Croatia (cortisol), and Vinca Institute, Belgrade, Serbia (prolactin). The normal ranges of these tests were 0–2.5 ng/ml (PSA), 0–2 ng/ml (PAP), 3–14 ng/ml (testosterone), 6–25 µg/100 ml (cortisol) and 125–600 mIU/ml (prolactin). Normal values for the Yang polyclonal PSA assay were found to range from 0 to 10 ng/ml [17].

Tumor evaluation

Tumors were staged according to WHO standards, taking into account changes in local tumor burdens, lymph node involvement and bony (and soft tissue) dissemination. Stage D0 denotes localized tumor (stage A–C) while stages D1 and D2 indicate involvement of lymph nodes and distant structures, respectively.

Statistical methods

The mean ± SD is given in both tables. Radioimmunological values and NK activity data were analyzed using Macintosh software, applying a *t*-test for paired samples.

Results

Mean values of NK activity and blood PSA, PAP, testosterone, prolactin and cortisol concentrations measured in 24 untreated prostate cancer patients are presented in Table 1. These data were statistically correlated on the basis of different tumor grades (G1 tumors vs G2+G3 tumors) regardless of the stage and spread of the disease ($P > 0.05$). The table includes the data from 10 normal men.

Cross-correlation of these same data with the stage of the disease is presented in Table 2. Patients were divided in two groups: subjects with localized tumor (11 patients) and patients with Stage D cancer (13 subjects). For comparison the equivalent results from healthy men and

Table 1. NK cell activity, serum PSA, PAP, testosterone, prolactin and cortisol concentrations in untreated prostate cancer patients: correlation with tumor grade (G1 vs G2+G3) regardless of tumor stage[a,b]

Patients	No.	NK activity (%)	PSA (ng/ml)	PAP (ng/ml)	Testosterone (ng/ml)	Prolactin (mIU/l)	Cortisol (μg/100 ml)
Healthy men	10	34.5 ± 7.1 (18.9 – 42.6)[c]	2.1 ± 1.4 (1.0 – 3.7)	1.0 ± 0.3 (0.7 – 1.4)	4.9 ± 2.8 (2.2 – 8.4)	300 ± 105 (185 – 515)	24.8 ± 11.2 (16.7 – 48.8)
Prostate cancer grade 1 stage A1–D2	14	20.6 ± 14.2 (4.7 – 45.4)	19.6 ± 21.0 (4.4 – 88.0)	8.4 ± 7.1 (0.6 – 25.6)	7.2 ± 2.4 (2.8 – 10.9)	635 ± 291 (150 – 910)	20.1 ± 7.6 (12.1 – 36.2)
Prostate cancer grade 2 + 3, stage A1–D2	10	23.8 ± 11.8 (7.7 – 30.5)	49.2 ± 27.1 (22.4 – 80.0)	20.9 ± 21.5 (0.9 – 61.5)	5.9 ± 2.4 (2.3 – 9.2)	360 ± 170 (170 – 1235)	20.8 ± 5.0 (12.2 – 30.0)
t-test (2 vs 3)		$P = 0.86$	$P = 0.05$	$P = 0.10$	$P = 0.23$	$P = 0.04$	$P = 0.74$

[a] Normal ranges of serum concentrations are: PSA 0–10 ng/ml; PAP 0–2 ng/ml; testosterone 3–14 ng/ml; prolactin 125–600 mIU/l; cortisol 6–25 μg/100 ml
[b] Mean values ± SD are given
[c] Values in parentheses are ranges

Table 2. NK cell activity, serum PSA, PAP, testosterone, prolactin and cortisol values in untreated prostate cancer patients: correlation with tumor stage (stage D0 vs stage D1 + D2) regardless of prostate cancer differentiation grade. Equivalent values in patients with chronic diseases are also given[a,b,c]

Patients	No.	NK activity (%)	PSA (ng/ml)	PAP (ng/ml)	Testosterone (ng/ml)	Prolactin (mIU/l)	Cortisol (μg/100 ml)
Healthy men	10	34.5 ± 7.1 (18.9 – 42.6)[a]	2.1 ± 1.4 (1.0 – 3.7)	1.0 ± 0.3 0.7 – 1.4	4.9 ± 2.8 2.2 – 8.4	300 ± 105 185 – 515	24.8 ± 11.2 16.7 – 48.8
Prostate cancer stage D1 + D2, G1–G3	13	11.9 ± 7.1 (4.7 – 30.5)	51.1 ± 16.0 (9.2 – 103.0)	16.1 ± 22.5 (1.5 – 61.5)	6.3 ± 2.3 (2.8 – 9.2)	570 ± 380 (170 – 1235)	20.7 ± 6.6 (12.1 – 37.4)
Prostate cancer stage D0, G1–G3	11	33.0 ± 10.6 (18.2 – 51.2)	24.5 ± 14.1 (5.1 – 80.0)	7.5 ± 2.9 (0.9 – 49.8)	6.1 ± 2.7 (2.3 – 10.9)	540 ± 275 (150 – 910)	22.9 ± 6.6 (14.2 – 30.0)
t-test (2 vs 3)		$P < 0.01$	$P < 0.01$	$P > 0.05$	$P > 0.05$	$P > 0.05$	$P > 0.05$
Chronic diseases[d]	6	5.7 ± 1.5 (2.9 – 7.6)	2.2 ± 2.6 (0.1 – 7.8)	1.6 ± 0.8 (0.1 – 2.5)	5.1 ± 2.0 (2.3 – 8.3)	205 ± 80 (90 – 315)	18.4 ± 4.6 (12.7 – 24.4)

[a] Normal ranges of serum values are: PSA 0–10 ng/ml, PAP 0–2 ng/ml, testosterone 3–14 ng/ml, prolactin 125–600 mIU/l, cortisol 6–25 μg/100 ml
[b] Mean values ± SD are given
[c] Values in parentheses are ranges
[d] Men free of malignant tumors but suffering from diabetes (2), arthritis (2), arthritis and rheumatic disorders (1), rheumatic disorders and chronic lung diseases (1)

subjects with chronic diseases are also given in Table 2. Testosterone level is (incidentally and most probably also trivially) found to be higher in prostate cancer patients than in controls, despite almost identical ranges in serotest values within all groups of subjects. However, these differences were statistically insignificant ($P > 0.05$).

During the course of the investigation 15 patients initially presenting with prostate cancer were excluded from the study because they had a chronic systemic disease (diabetes, arthritis, severe rheumatic disorders, chronic lung diseases) already known to influence NK activity level [4]. In 6 of these 15 subjects NK activity was measured (Table 2); the mean value (5.7 ± 1.5) was similar to the NK activity recorded in 9 corresponding prostate cancer patients [4] referred for androgen deprivation therapy (5.3 ± 6.2). In the remaining 9 patients excluded from the study on the basis of chronic disease NK activity was not measured. In the aforementioned report [4] the decline in NK activity in all subjects with diabetes and arthritis was recorded regardless of their prostate status (normal, benign or malignant). In some of these subjects excluded from either our previous study [4] or this one both chronic and acute inflammation of the prostate was found. The correlation between prostatic inflammation and NK cell activity has not been investigated.

The PSA test was more sensitive than PAP serotests data in defining tumor load (Table 2). An statistically insignificant correlation was found between hormone serotests and NK activity data regardless of the stage and grade of the disease.

Discussion

In treated cancer patients NK-mediated cytotoxic activity has been proven to be inversely related to tumor load and spread [4–6, 18]. Our previous study revealed an obvious lack of positive correlation between prostate cancer differentiation grade and the efficiency of immunosurveillance in hormonally treated patients [4]. However, antiandrogen therapy is characterized by both androgen withdrawal and substantial changes in basal levels of several circulating hormones, such as estradiol, prolactin and cortisol [14]. The possible impact of hormonal treatment on the synthesis and secretion of other potential immunomodulators has yet to be defined [8, 9]. In addition, a majority of prostatic tumors dedifferentiate during the development of the disease and thus start to secrete some new gene products while ceasing to produce some of those associated with normal epithelial prostatic cells. A wide spectrum of hormonal variations during therapy as well as other possible alterations in the immunoregulative milieu may influence the regulation of NK activity in treated subjects in a way quite different from the NK cell activity mechanism in untreated subjects. The results of investigations to delineate the relationship between NK activity data and both prostate cancer stage and grade in patients at initial presentation are presented in this preliminary report.

Mean NK activity values recorded in untreated G1 prostate cancer patients were the same as those in patients with G2 and G3 tumors (Table 1). The development of prostate cancer proceeds through a sequence of genotoxic events that are superimposed on the initial genetic mutation [1]. According to our results (Table 1) the activity of NK cells seems to be unaffected by changes in gene product composition. In recent years only a few NK effector cell targets have been recognized [8, 9, 18].

The values for NK cell activity measured in patients with localized prostate cancer (Stage D0) were virtually equal to those in healthy men (Table 2) but were significantly reduced ($P < 0.01$) in patients with stage D prostatic carcinoma. Patients with all cancer differentiation grades (G1–G3) were present in both groups.

A recent report has indicated that there is a positive correlation between autologous tumor-cell-killing (ATK) activity in primary lung tumors and the 5-year survival rate [19]. The advantage of this approach over NK cell activity data in prostate cancer patients has yet to be tested. Our current efforts in this same direction have shown little progress, probably due to the high degree of heterogeneity of prostate cancer.

The strong influence on NK cell activity of many of the chronic diseases that are frequently found in older men, such as diabetes, arthritis and severe rheumatic disorders (Table 2), reduces the grounds for the application of the method in diagnostic and staging protocols.

Serotest PSA and PAP values and NK activity parameters were correlated with tumor grade in an attempt to compare their relative sensitivities as regards tumor spread and stage (Table 1). While NK cell activity was unable to distinguish between lower and higher grade cancers, higher serum PSA and PAP values were recorded in G2+G3 tumors than in well-differentiated cancers. This obervation is probably associated with the more rapid tumor progression and thus generally greater tumor load in less-differentiated cancers [11]. However, only differences in PSA concentrations were found to be statistically significant. The superior sensitivity of the PSA test over PAP concentrations in prostatic neoplasms that retain epithelial characteristics is widely accepted [11, 13].

Correlation between NK activity data and tumor marker serotests in relation to tumor spread (stage D0 vs D1+D2) revealed that both PSA and NK actvity tests (but not PAP values) were reliable for staging purposes (Table 2). Hence, an assay of the lytic capacity of NK cells may be used as a supplement to the routine clinical staging protocols.

Serum prolactin concentrations are lower in untreated G1 patients than in subjects with higher (G2–G3) tumor grades (Table 1). These differences are statistically significant ($P = 0.04$), unlike the testosterone concentrations (higher in subjects with G1 tumor than in G2 and G3 neoplasms) that differ insignificantly ($P > 0.05$). Both findings are in agreement with the results of our previous studies [12]. Blood prolactin values recorded in stage D patients, although often in the upper part of the normal range, did not exceed 1000 mIU/l. Accordingly, these data seem to agree with our previous suggestion of a therapy-provoked (estramustine phosphate and diethylstilbestrol) elevation of plasma prolactin concentration when detected above this limit [14].

There is no consensus on a potential use of the blood cortisol level as a prognosticator of further development of the disease in untreated patients [2, 16]. Data reported here reveal no differences between plasma cortisol levels in untreated patients with prostate cancer stage D0 and stages D1–D2 and thus indicate little use for this parameter for prognostic purposes.

There is no correlation between any of the blood hormone values examined and NK activity data. This result seems to be in line with the observations on hormonally treated prostate cancer patients [4]. In conclusion, preliminary data reported here suggest a sharp drop in NK activity in patients with tumor lesions in lymph nodes, bone and/or soft tissues. Tumor differentiation did not affect the result. Accordingly, the NK activity assay may be a useful addition to staging protocols. Studies are under way to define the role of many factors that may act on NK cell activity and other components of the immune system during the development of prostatic carcinoma. Preliminary unpublished data indicate as especially promising some of the studies on the role of laminin in multifunctional effector-target interactions.

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