Marked increase of the growth factors pleiotrophin and fibroblast growth factor-2 in serum of testicular cancer patients

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Received 9 January 2003; revised 11 June 2003; accepted 17 July 2003

Background: Malignant tumors of the testis are among the most common cancers in men between the ages of 15 and 30 years. The sensitivity of detection of known tumor markers depends upon the tumor histology and stage. In other cancers, increased serum concentrations of various angiogenic growth factors have been described as potential markers for tumor progression and metastasis. One main histological feature of testicular cancer is profound angiogenesis.

Design: In this study, we investigated by sensitive enzyme-linked immunosorbent assays (ELISAs) the levels of various growth and angiogenesis factors in the serum of testicular cancer patients as compared with normal control subjects. For the most profoundly increased growth factors, pleiotrophin (PTN) and fibroblast growth factor-2 (FGF-2), we furthermore analyzed tumor lysates by northern blotting, RT–PCR and ELISA.

Results: We demonstrate a marked elevation of average serum levels of PTN (∼20-fold) and of FGF-2 (∼7-fold) in patients and expression of both growth factors in tumor biopsies. To a lesser extent, vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) serum levels were increased, whereas FGF-4 and transforming growth factor-β levels were similar to those in normal control subjects. Elevation of PTN, FGF-2, EGF and VEGF was detected in seminomatous as well as non-seminatous tumors, and even in early stages.

Conclusions: PTN and FGF-2 may represent promising new diagnostic markers for testicular cancer with high sensitivity even in early-stage testicular cancer. Further studies are warranted to extend our analyses.

Key words: enzyme-linked immunosorbent assay, fibroblast growth factor serum levels, pleiotrophin, testicular cancer

Introduction

Cancer of the testis consists of a morphologically and clinically heterogeneous group of tumors that constitute the majority of malignancies in men between the ages of 15 and 30 years [1], the incidence of which has doubled in the last 30 years. They are classified into two major subgroups, seminomas and non-seminomatous tumors. The majority of patients with testicular cancer present with clinical stage I disease. Measurements of the tumor markers α-fetoprotein (AFP), human chorionic gonadotrophin and its free β-subunit (hCG and hCGβ, respectively) and lactate dehydrogenase (LDH) are important tools for the diagnosis and staging of testicular cancer; however, serum levels are not elevated in all patients, especially at early stages [1]. On the other hand, although growth factors have been investigated in numerous cancers, little is known about their serum levels in tumors of the testis.

In this study, we measured serum levels of various growth factors which: (i) in other tumors exert potent effects on tumor cells and on endothelial cells including stimulation of proliferation, mitogenesis and/or migration; (ii) are often up-regulated in cancer and may correlate with the degree of cancer malignancy; (iii) are often rate-limiting for tumor growth and tumor angiogenesis; and (iv) may play a role in testicular cancer. This has been shown for fibroblast growth factor-2 (FGF-2), which is detected at high concentrations in the serum and urine of various cancer patients [2] and is expressed in the normal testis [3, 4] as well as in teratoma cells where it stimulates cell proliferation and migration [5]. For vascular endothelial growth factor (VEGF), Viglietto et al. [6] have demonstrated a markedly increased expression in 81% of the germ cell tumors investigated, and in situ hybridization has shown a stronger expression in teratoma than in both seminoma and non-seminomatous specimens [7]. FGF-4 (hst-1, K-FGF) has been detected in Sertoli cells of the normal adult mouse testis [8], and its expression has been associated with murine testicular teratogenesis [9]. Transforming growth factor-β1 (TGF-β1) plasma levels predict liver metastasis in colorectal cancer [10] and disease progression in patients undergoing radical prostatectomy [11].
and TGF-β1 shows a stronger expression in tissues of germ cell tumors than in peritumor non-neoplastic tests [12]. Epidermal growth factor (EGF) is frequently overexpressed in cancer, e.g. of the pancreas [13, 14], and is expressed in testicular Leydig cells [15–17]. Pleiotrophin (PTN, HB-GAM) is highly expressed in various tumor cell lines of different origin [18–20]. Elevated PTN serum levels were detected in patients with pancreatic, colon [21] or lung cancer, and in the last case were related to disease stage [22].

In this paper, we demonstrate a marked elevation of FGF-2 and PTN and, to a lesser extent, an increase in VEGF and EGF serum levels. More detailed analysis revealed an increase in seminomas as well as non-seminomas even in early tumor stages. Hence, both PTN and FGF-2, which we show to be expressed in tumor biopsies, might be promising new diagnostic markers for testicular cancer, with high sensitivity even in early stages.

Materials and methods

Samples

Serum samples from testicular cancer patients (age range 20–49 years) were obtained from the Hospital Am Urban, Berlin, Germany, and serum samples from healthy male blood donors (age range 24–61 years) were obtained from the Philipps University Hospital, Marburg, Germany. Serum was isolated from peripheral blood and processed according to standard protocols. Briefly, blood samples were collected into EDTA-coated tubes and stored at 4°C overnight. Subsequently, samples were centrifuged to isolate serum, and supernatants were aliquoted and stored frozen at −20°C. Hemolytic or lipemic samples were excluded. Fresh tumor samples were obtained from the Philipps University Hospital, Marburg, Germany, and were stored at −80°C in Tri Reagent (Sigma, Deisenhofen, Germany) before RNA extraction or were lysed by homogenizing and sonication in TBST [Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) plus 0.5% Tween-20] supplemented with protease inhibitors (complete cocktail; Roche, Mannheim, Germany).

Enzyme-linked immunosorbent assays

The PTN enzyme-linked immunosorbent assays (ELISA) was carried out as described previously [21]. Briefly, a mouse anti-PTN monoclonal antibody (4B7) [21] was diluted to 1 µg/ml in TBS and 100 µl/well of the dilution were incubated in covered 96-well ELISA plates (Life Technologies, Karlsruhe Germany) at 4°C overnight. Wells were washed three times with TBST, and residual free binding sites were blocked with 200 µl TBST containing 1% bovine serum albumin (BSA) for 2 h at 4°C, before wells were washed again. Serum (22 samples from testicular carcinoma patients and 24 healthy controls) was diluted 1:1 with double-concentrated TBST, or supernatants of centrifuged tumor lysates 1:10 to 1:100 in TBST, and 100 µl/well of this dilution was added and incubated at room temperature for 1 h. After washing, 100 µl/well biotinylated affinity-purified goat anti-human-PTN secondary antibody (R&D, Wiesbaden, Germany) was added at a concentration of 500 ng/ml and incubated for 1 h at room temperature. After washing again, the plate was incubated in the dark with 100 µl/well p-nitrophenyl phosphate substrate solution for 2 h. Absorbance was measured in an ELISA reader at 405 nm. Recombinant human PTN (R&D) served as the standard.

ELISAs for FGF-2, FGF-4, VEGF, EGF and TGF-β were from R&D and carried out as described by the supplier. Briefly, samples and standards were diluted appropriately in the respective assay diluent and incubated at room temperature in wells supplied pre-coated with specific capturing antibodies. After washing, the respective detection antibody-conjugate was added and incubated at room temperature before washing again. Addition of substrate solution then resulted in color development and, after addition of a stop solution, color intensity was measured in an ELISA reader at the appropriate wavelength. For correction of optical imperfections in the plate, reading was repeated as recommended by the supplier at a different wavelength, and these results were subtracted.

RNA preparation, RT–PCR, Southern and northern blot analysis

From tumor tissues, total RNA was extracted using the Tri Reagent as described by the supplier (Sigma). RT–PCR was carried out with 2 µg denatured RNA using AMV reverse transcriptase as described by the manufacturer (Roche). The cDNA product (1.5 µl) was amplified in a 50 µl final volume containing 5 µl 10× PCR buffer with MgCl₂, 0.2 mM dNTPs, 10 pmol of each primer and 0.5 µl NeoTherm DNA Polymerase (GeneCraft, Münster, Germany) for 30 cycles under the following conditions: 30 s at 94°C, 45 s at 53°C and 3 min at 72°C. Primer sequences were as follows: PTN sense 5′-TATGTCTCACAGGTGACATC-3′; and PTN antisense 5′-AGAG-GACGTTTCCAACCTCAA-3′. PCR products were loaded on a 1.2% ethidium bromide-stained agarose gel and total RNA was separated in a 1% formaldehyde agarose gel. For Southern or northern blots, DNA or RNA was transferred on to a nylon membrane (HyBond N; Amersham Pharmacia, Little Chalfont, UK) by capillary action and hybridized overnight with 32P-labeled (Rediprime; Amersham Pharmacia) PTN cDNA in 50% formamide, 5x SSC, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA, 0.1% SDS and 100 µg/ml salmon sperm DNA. After twice washing with 2x SSC/0.1% SDS and with 0.1x SSC/0.1 SDS at 42°C, membranes were autoradiographed for 1–5 days.

Statistical analysis

Serum levels of each growth factor were determined in multiple independent experiments for each sample, and mean values were calculated from the data of each patient. To analyze if the samples were normally distributed, the Kolmogorov–Smirnov test was used. Statistical significance was evaluated by Student’s t-test and Mann–Whitney U-test as indicated in the text. Only P values <0.05 were considered statistically significant. Correlation between levels of different growth factors were tested by the Pearson test.

Results

Serum levels in testicular cancer patients versus healthy blood donors

The most profound difference in serum levels between testicular cancer patients and healthy blood donors was observed for PTN (Figure 1A, left). In the serum of healthy control subjects, the mean PTN concentration was 2.55 ± 0.19 ng/ml with all samples being above the limit of detection (<0.1 ng/ml) of the ELISA. Strikingly, in testicular cancer patients, serum levels were 55.95 ± 29.41 ng/ml, which represents a >20-fold, statistically significant (P <0.001, Mann–Whitney U-test) elevation. Very profound differences in serum levels were also observed for FGF-2 (Figure 1A). Serum levels in testicular cancer patients were ~7.3-fold higher as compared with the control group (50.66 ± 6.72 versus 6.96 ± 0.76 pg/ml; P <0.001, Mann–Whitney U-test). Significantly increased serum levels were also found for VEGF (590.0 ± 65.3 versus 284 ± 25.9 pg/ml; P<0.001, Student’s t-test; Figure 1A). Similarly, EGF (Figure 1A, right) mean values in testicular cancer patients were moderately but significantly elevated as compared with the control group (892.5 ± 75.6 versus 645.6 ± 74.4 pg/ml; P = 0.027, Student’s t-test). Finally, for serum levels
of FGF-4 and TGF-β, no significant differences to controls were detected (data not shown).

Analysis of testicular tumor biopsies for PTN and FGF-2, which showed the most profoundly increased serum levels in testicular cancer, revealed expression of both growth factors in the tissue of seminomas as well as of non-seminomatous tumors. While RT-PCR for PTN mRNA showed bands at the expected size (Figure 1B, left), Southern blotting (Figure 1B, center) confirmed the PTN specificity of the PCR and indicated that all samples tested were positive for PTN mRNA. Concomitantly, PTN mRNA was detectable by northern blotting (Figure 1B, right), and in the PTN ELISA all tumor lysates tested were positive for PTN with concentrations being between 0.5 and 17.0 ng PTN/mg total protein (not shown). Likewise, FGF-2 expression was detected in all tumor biopsies at concentrations ranging from 0.08 to 6.5 ng FGF-2/mg total protein (not shown).

**Serum levels in different tumor subtypes and tumor stages**

For PTN (Figure 2A), uniformly high serum levels were found in all four testicular cancer patient groups as compared with normal controls \( (P < 0.001, \text{Mann–Whitney } U\text{-test}) \). Levels did not differ between seminoma and non-seminoma histological groups, and did not increase with tumor size. The median values were as follows: normal serum 1.455 ng/ml, seminoma pT1 16.66 ng/ml, pT2/3 15.31 ng/ml, non-seminoma pT1 16.92 ng/ml, and pT2/3 18.88 ng/ml.

For FGF-2 in seminoma patients, an increase in serum levels with tumor size was noted (Figure 2B). Compared with the control (median 6.5 pg/ml), FGF-2 levels were higher even in patients with stage pT1 tumors (median 31.0 pg/ml; \( P = 0.013, \text{Mann–Whitney } U\text{-test})\), and in stage pT2/3 tumors (median 45.8 pg/ml; \( P < 0.001, \text{Mann–Whitney } U\text{-test})\) a trend towards higher FGF-2 levels as compared with stage pT1 tumors was observed \( (P = 0.078, \text{Mann–Whitney } U\text{-test})\). In non-seminomas (Figure 2B, right), FGF-2 levels were significantly elevated in both stage pT1 \( (P = 0.002)\) and pT2/3 \( (P = 0.001)\) tumors over healthy control serum, again with no statistically significant difference between tumor stages.

VEGF levels in seminoma patients (Figure 2C, left) showed an increase in serum levels between stage pT1 (median 438.0 pg/ml) and stage pT2/3 (median 816.5 pg/ml). VEGF levels of stage pT1 were even significantly elevated compared with the control (median 258 pg/ml; \( P = 0.004, \text{Student’s } t\text{-test})\) as were levels of
stage pT2/pT3 ($P = 0.011$), with differences between seminoma tumor grades not being statistically significant ($P = 0.11$). In non-seminomas (Figure 2C, right) VEGF levels in grade pT1 (median 688.5 pg/ml) were higher, but not significantly different ($P = 0.5$) compared with pT2/pT3 (344.0 pg/ml). Due to the relatively low VEGF serum levels in non-seminoma pT2/pT3 patients, with 80% of the samples being in the range of normal serum, these patients were the only group with serum levels not significantly elevated over normal serum.

**Standard testicular tumor markers**

AFP was elevated in the serum of 66% of the patients with non-seminomatous tumors but in none of the patients with seminoma. hCGβ serum levels were elevated in 23% of the seminoma patients and in 56% of the non-seminoma patients. Increased LDH was observed in the serum of 38% of patients with seminoma and in 20% of patients with a non-seminomatous tumor. No statistically significant correlation was found between the classic tumor markers and any of the growth factors analyzed in this study.

**Discussion**

While in previous studies serum levels of growth factors such as FGF-2, VEGF and EGF have been measured in numerous tumors and correlated with disease stage, course and prognosis (reviewed in [23]), data on testicular cancer are very limited. In this paper, we analyzed sera from testicular cancer patients and healthy control subjects for levels of various growth factors by means of sensitive ELISAs.

The most remarkable findings were highly elevated levels of FGF-2 and PTN in the serum of testicular cancer patients. For PTN, >20-fold increased levels were detected in the serum of testicular cancer patients. Setting a cut-off level at 3.5 ng/ml, which is comparable with that used in earlier studies [22], results in increased serum levels in 100% of the cancer patients examined with no sample having a concentration below ~12.5 ng/ml. Likewise, 100% of the controls were below this cut-off with no serum concentration >3.3 ng/ml. Interestingly, no differences in mean values were seen between different testicular cancer subtypes or the different stages investigated. PTN levels are increased even in early stages of testicular cancer. Analysis of tumor biopsies for mRNA and protein levels confirmed PTN expression in the tumor mass of all samples tested.

For FGF-2, a more than seven-fold increase in mean serum concentrations was determined. Definition of a cut-off level of 8 pg/ml, which is in the range reported by previous papers on other tumors (reviewed in [23]), results in elevated FGF-2 levels in all patients with only one exception consisting of a low-grade (pT1) seminoma. All healthy control subjects were below this cut-off, except for one with an FGF-2 serum level three-fold higher than normal values. Obviously, measurement of FGF-2 serum levels clearly distinguishes between testicular cancer patients and normal individuals and, most importantly, FGF-2 serum levels are elevated even in early stages of testicular cancer. Analysis of tumor biopsies for mRNA and protein levels confirmed FGF-2 expression in the tumor mass of all samples tested.

Elevated levels were also detected for VEGF, revealing a more than two-fold increase over those in healthy control subjects. However, individual values were scattered widely with serum levels from several, including high-grade testicular cancer patients, being in the normal range. For EGF, a statistically significant up-regulation of serum levels in cancer of the testis was seen although the overall differences were comparatively small.

The standard tumor markers AFP, hCG and LDH are of diagnostic and prognostic value and are widely used in testicular cancer patients. However, elevation of serum levels is observed only in a small number of early-stage testicular cancers, and even in patients with advanced stages of testicular cancer increased values are detected in <100%. In contrast, we demonstrate that
serum levels of the growth factors PTN and FGF-2 are even elevated in all (PTN) or all but one (FGF-2) patients with stage I seminoma or non-seminomatous tumors. Our data indicate that these growth factors might be useful novel tumor markers, especially due to their high sensitivity in early-stage testicular cancer. Clearly, to confirm our findings presented in this report and to investigate further the putative diagnostic value of PTN and FGF-2, studies with larger numbers of samples need to be undertaken. Important future directions also include: (i) the exact time-course of changes with regard to later tumor stages; (ii) the determination of post-operative serum levels and their possible association with disease-free survival or the likelihood of tumor recurrence (as seen for example with FGF-2 in women with breast cancer [24]); as well as (iii) analyses of tumor sections by immunohistochemistry and in situ hybridization to correlate these data to serum levels.

Acknowledgements

We thank H. Radler, A. Wüstenhagen and M. Grzelinski for excellent technical performance of the ELISA and RT-PCR experiments, H. Prinz (Department of Medical Biometry and Informatics, Philipps University, Marburg) for expert help with statistical analysis and M. Conrad-Stöppler for critical reading of the manuscript. This work was in part supported by a grant from the Deutsche Forschungsgemeinschaft (AI 24/5-1) to A. A.

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