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T. Otto · S. Virchow · C. Fuhrmann · F. Steinberg
C. Streffer · M. Goepel · H. Rübben

Detection of vital germ cell tumor cells in short-term cell cultures of primary tumors and of retroperitoneal metastasis – clinical implications

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Abstract By establishing short-term cell cultures derived from retroperitoneal metastasis after neoadjuvant chemotherapy, our aim was to improve the diagnosis and prognosis in patients with advanced testicular germ cell tumors. The histological evaluation of surgically removed metastatic tissue by retroperitoneal lymphadenectomy (RLA) is extremely complicated after previous chemotherapy, but knowledge of persistence of vital tumor cells in residual lesions is of great prognostic value and therapeutic consequence in patients with testicular germ cell tumors. We therefore investigated whether vital tumor tissue could be detected in short-term cell cultures derived from such metastatic lesions by measuring the concentration of the tumor markers beta human chorionic gonadotropin (βHCG) and alpha-1 fetoprotein (AFP) in cell culture supernatants. We initially demonstrated the specificity of the determination in cell cultures of human transitional-cell carcinoma cell lines, human foreskin fibroblasts and normal testicular tissue. In a group of 20 patients with untreated primary testicular germ cell tumors, detection of βHCG and AFP was increased about threefold in cell culture supernatants in comparison to the serum concentration. Finally, we prepared primary cell cultures from surgically removed retroperitoneal metastasis of 12 patients with testicular germ cell tumors after chemotherapy. The serum concentrations of βHCG and AFP of all patients were at normal values when RLA was performed. However, pathologically increased concentrations of βHCG (3/3) and AFP (2/3) in cell culture supernatants were found in 3 of 12 cell cultures. Interestingly, these three patients with a pathological increase in βHCG and AFP as determined in the supernatant of the short-term cell cultures had tumor progression within a mean follow-up of 3 ± 1 months ($P < 0,01$), whereas 9 of 12 patients who had no pathological increase in βHCG and AFP as determined in the supernatant of the short-term cell culture were in complete remission (CR) after a mean follow-up of 40 ± 11.6 months.

Key words Nonseminomatous testicular germ cell tumors · Tumor markers in vitro · Alpha-1 fetoprotein · Beta human chorionic gonadotropin · Retroperitoneal lymphadenectomy · Short-term cell culture

Introduction

Knowledge about the persistence of vital tumor cells in residual lesions after chemotherapy is of great prognostic and therapeutic value in patients with testicular germ cell tumors [6, 9, 14]. The surgical resection of residual lesions is mandatory, but the complete histological investigation of large residual tumor masses in serial sections is not available as a routine procedure. Therefore, we established an additional method to detect residual vital tumor cells using short-term primary cell cultures derived from 20 primary testicular germ cell tumors and from 12 secondary lesions, i.e., retroperitoneal metastasis after chemotherapy, measuring the concentration of tumor markers, i.e., beta human chorionic gonadotropin (βHCG) and alpha-1 fetoprotein (AFP) in cell culture supernatants (4 ± 1 day culturing time).

Material and methods

A total of 32 Caucasian patients with a mean age of 27 years (range 19–46 years) underwent surgery at the Department of Urology, University of Essen. Tissue specimens from primary tumors and residual retroperitoneal lesions with a size of approximately 1 cm^3 were immediately put into minimal essential medium (MEM, Gibco, Germany) supplemented with 20% fetal calf serum (FCS, Gibco, Germany). The tissue specimens were cut into small pieces and transferred to phosphate-buffered saline (PBS) containing 1 mg/ml collagenase/dispase (Boehringer Mannheim, Germany). After

T. Otto (✉) · S. Virchow · M. Goepel · H. Rübben
Department of Urology, University of Essen, Medical School, Hufelandstrasse 55, D-45122 Essen, Germany

C. Fuhrmann · F. Steinberg · C. Streffer
Institute for Radiobiology, University of Essen, Medical School, Hufelandstrasse 55, D-45122 Essen, Germany

30–60 min incubation at room temperature, cells were centrifuged (174 *g*, 5 min) and resuspended in MEM supplemented with 20% FCS, 10 µg/ml transferrin (Boehringer Mannheim, Germany), 5 µg/ml insulin (Sigma, Germany), 0.5 µg/ml hydrocortisone (Sigma, Germany), 5 ng/ml epidermal growth factor (Boehringer Mannheim, Germany) and 10 µl/ml refobacin 80 (Merck, Germany). Cell cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C and the medium was changed once a week. Confluent primary cell cultures were harvested using 0.5% trypsin and resuspended in MEM supplemented with 20% FCS only. The supernatant of 4 ± 1-day-old short-term cultures was collected and frozen at −80°C until determination of the concentration of the tumor markers. The concentration of βHCG and AFP in the cell culture supernatants was determined by solid phase, two-site immunoenzymetric assay (Hybritech, Germany) using monoclonal antibodies against βHCG and AFP (Hybritech, Germany) with a sensitivity of 0.7 mU/ml (βHCG) and a coefficient of correlation of 0.979 and with a sensitivity of 0.2 ng/ml (AFP) and a coefficient of correlation of 0.993. The serum concentrations of βHCG and AFP determined 1 day before orchiectomy or chemotherapy were regarded as normal up to 2 mU/ml and 6 ng/ml, respectively. The concentrations of βHCG and AFP in short-term cell culture supernatants, i.e., 4 ± 1-day-old cell cultures were regarded as normal up to 0.7 mU/ml and 0.2 ng/ml, respectively (values were obtained from short-term cell cultures derived from normal testicular tissue). Cell culture supernatants from short-term cultures of human transitional cell carcinoma cell lines, human foreskin fibroblasts and normal testicular tissue served as negative controls. All data were corrected for unspecific binding and background using culture medium from the appropriate batch (MEM and FCS) used. For statistical analysis, standard univariate and multivariate methods including asymptotic (Pearson-Yates correlation) and exact (Fisher's) tests for differences (two-sided) were performed to analyze marker findings in correlation to clinical follow-up in patients who underwent retroperitoneal lymphadenectomy.

Results

Initially, 20 untreated primary germ cell tumors from 13 patients with seminoma and from 7 patients with non-seminomatous testicular germ cell tumor (NSGCT) were investigated. The serum concentration of βHCG was increased in only 1 of 13 patients with seminoma by 1.3-fold. However, an increased concentration of βHCG in the cell culture supernatants was found in 3 of 13 short-term cell cultures of these patients (Table 1). Two of seven patients with NSGCT had increased serum concentrations of tumor markers, but in six of seven cell culture supernatants of short-term cell cultures of these patients, there were obviously increased concentrations of either βHCG and/or AFP (Table 2). We found an increased sensitivity of 45% for the determination of tumor markers in the cell culture supernatants of short-term cell cultures compared to their determination in the serum of only 15% from patients with primary germ cell tumors. In addition, we were also able to prove the specificity (100%) of this in vitro determination of tumor markers using short-term cell cultures of normal tissues (human foreskin fibroblasts, normal testicular tissue) and transitional cell carcinoma cell lines (data not shown). In a prospective study, we investigated the prognostic value of increased concentrations of tumor markers in cell culture supernatants of short-term cell cultures derived from retroperitoneal metastasis from patients with advanced germ cell tumors (Lugano classification ≥ IIc) after chemotherapy. Twelve patients were included in this study: one patient with seminoma, one patient with burned-out tumor, two patients with teratocarcinoma, three patients with embryonal carcinoma and five patients with mixed tumor (Table 3). Mixed tumors from patients P.J. and K.N. were predominantly teratocarcinomas with a seminomatous part of less than 20%. Mixed tumors from patients D.M., K.T. and K.E. consisted of large seminomatous parts (about 30–50%) with additional parts of embryonal carcinoma (patients D.M. and K.E.). Three of these patients had an initial retroperitoneal tumor mass with a total volume of 125–140 cm^3 (calculated from computerized tomography) and nine patients had palpable retroperitoneal tumor masses (> 140 cm^3) before chemotherapy. All patients were subsequently treated with several courses of polychemotherapy with cisplatin, etoposide and bleomycin according to the PEB regimen and were in partial remission with evidence of residual retroperitoneal lesions before retroperitoneal lymphadenectomy (RLA) was performed (maximum bidimensional diameters 5–15 cm). Furthermore, all patients had normal serum concentrations of βHCG and AFP preoperatively (Tables 3, 4). Residual tumors were not detectable intra- and postoperatively. The postoperative

Table 1 Tumor markers AFP and βHCG in serum and cell culture supernatants from patients with seminoma after surgery alone. Concentrations are given as fold of normal (= 1)

	Serum		Short-term cell culture	
Initials	AFP	βHCG	AFP	βHCG
D. J.	1	1.3	1	1
F. B.	1	1	1	1
G. U.	1	1	1	5.8
K. D.	1	1	1	2.8
S. K.	1	1	1	1
W. H.	1	1	1	3.1
H. W.	1	1	1	1
B. M.	1	1	1	1
J. H.-G.	1	1	1	1
D. K.-J.	1	1	1	1
W. B.	1	1	1	1
Z. J.	1	1	1	1
T. N.	1	1	1	1

Table 2 Tumor markers AFP and βHCG in serum and cell culture supernatants from patients with NSGCT after surgery alone. Concentrations are given as fold of normal (= 1)

		Serum		Short-term cell culture	
Initials	Histological finding	AFP	βHCG	AFP	βHCG
A. T.	Mixed tumor	1	1	6.3	5
B. T.	Mixed tumor	1.5	1	2.8	1
I. C.	Mixed tumor	1	1	35	13.7
K. G.	Burned-out tumor	1	1	8.5	3.1
M. M.	Embryonal carcinoma	1	1	15.5	26.1
R. D.	Mixed tumor	1	1.7	1	1
B. F.	Teratocarcinoma	1	1	1	11

Table 3 Clinical and histological findings from patients with advanced germ cell tumors: diagnosis and treatment until RLA was performed

Initials	Age	Histological finding	Clinical stage[a]	Serum AFP[b]	βHCG[b]	Chemotherapy courses (*n*)[c]
K. R.	37	Seminoma	IIc	1	1	4
M. J.	26	Burned-out tumor	IIc	1	7.1	4
P. J.	24	Mixed tumor	IIIb	1	1 462	8
S. E.	44	Embryonal carcinoma	IIc	1	302	4
K. N.	36	Mixed tumor	IIIb	1	2 503	9
H. R.	24	Teratocarcinoma	IIIb	70	16	3
D. M.	26	Mixed tumor	IIc	85	1	3
B. R.	25	Teratocarcinoma	IIIb	1	23 400	3
K. T.	26	Mixed tumor	IIc	14	1.4	6
K. F.	31	Embryonal carcinoma	IIc	1	2.8	5
G. K.	46	Embryonal carcinoma	IIIa	1.7	1	8
K. E.	23	Mixed tumor	IIc	227	1	4

[a] Lugano classification
[b] Concentration of tumor markers before orchiectomy: fold of normal (= 1)
[c] Polychemotherapy with cisplatin, etoposide and bleomycin according to the PEB regimen

histological findings showed necrosis and fibrosis without evidence for malignancy in 8 of 12 cases, vital tumor was found in 3 of 12 cases and a highly differentiated teratoma was seen in 1 of 12 cases (Table 4). However, the in vitro determination of the tumor markers βHCG and AFP in cell culture supernatants of short-term cell cultures from these patients after chemotherapy showed pathologically increased values for 3 of 12 cultures: 2 of these 3 patients had no evidence histologically of vital tumor tissue and 1 of these 3 patients had a highly differentiated teratoma histologically. After 2, 3 and 4 months, respectively, all three patients with pathologically increased concentrations of tumor markers by in vitro determination in cell culture supernatants had tumor progression ($P < 0.01$, Fisher's test, two-sided). All patients with normal concentrations of tumor markers by in vitro determination in cell culture supernatants were in complete remission after a mean follow-up of 40 ± 11.6 months (Table 4). Additional investigations of cytological parameters including tumor grading, DNA index and karyotype showed no remarkable findings concerning the three tumors with increased concentrations of βHCG and AFP in the cell culture supernatants and early tumor progression (Table 4).

Discussion

The determination of βHCG and AFP is essential for the diagnosis and follow-up of patients with seminomas and NSGCT. After neoadjuvant chemotherapy most of the patients even with advanced disease (Lugano stage IIc, III) will eventually have seroconversion of tumor markers, but mostly there will be residual retro-

Table 4 Histological findings in retroperitoneal lesions after RLA, cytological parameters, tumor markers AFP and βHCG in serum and cell culture supernatants after RLA, clinical course including adjuvant chemotherapy and follow-up in months (*NED* no evidence for disease, *PROG* progression, *ND* not done)

Initials	Histological finding, grading	Serum		Cell culture		Chemotherapy	Follow-up		DNA/karyotype	
		AFP[a]	βHCG[a]	AFP[a]	βHCG[a]	courses (*n*)[b]	NED	PROG	index[c]	
K. R.	Necrosis, fibrosis, ND	1	1	1	1		60		1.0	ND
M. J.	Necrosis, fibrosis, ND	1	1	1	1		52		ND	46 xy
P. J.	Teratoma, well differentiated	1	1	21	7.4			3	0.98	ND
S. E.	Necrosis, fibrosis, ND	1	1	11.5	14.1			2	1.18	ND
K. N.	Necrosis, fibrosis, ND	1	1	1	8.4			4	ND	46 xy, t(3p, 4p)
H. R.	Vital tumor, undifferentiated	1	1	1	1	2	44		1.42	ND
D. M.	Necrosis, fibrosis, ND	1	1	1	1		32		ND	ND
B. R.	Vital tumor, undifferentiated	1	1	1	1	2	42		ND	64 xxy
K. T.	Necrosis, fibrosis, ND	1	1	1	1		26		1.38	ND
K. F.	Necrosis, fibrosis, ND	1	1	1	1		46		ND	46 xy
G. K.	Necrosis, fibrosis, ND	1	1	1	1		29		0.92	ND
K. E.	Vital tumor, undifferentiated	1	1	1	1	2	30		1.58	68 xy

[a] Concentration of tumor markers when RLA was performed: fold of normal (= 1)
[b] Adjuvant polychemotherapy with cisplatin, etoposide and bleomycin according to the PEB regimen
[c] Karyotype and DNA index analysis were performed by Dr. Zhang (Department of Internal Medicine, Cancer Research, University of Essen)

peritoneal lesions. To date, it has not been possible to distinguish nonmalignant residual tissue from residual vital tumor preoperatively [2–5, 9, 11]. Therefore, subsequent RLA is required in all cases with residual retroperitoneal lesions after chemotherapy [3, 6, 8, 12]. The histological investigation of these residual lesions after RLA is of great prognostic and therapeutic importance: if there is histological evidence of vital tumor, adjuvant chemotherapy is absolutely necessary [10]. However, histological investigations sometimes fail to predict accurately the histological stage, and other prognostic parameters investigated in patients with stage I/II NSGCT to distinguish patients at high risk from those at low risk for occult metastasis are not established [1]. Furthermore, the complete histological investigation of large residual tumor masses in serial sections is not available as a routine procedure. For these reasons, we established a method to detect vital germ cell tumor cells using short-term cell cultures derived from these residual retroperitoneal lesions by determination of the tumor markers βHCG and AFP in the cell culture supernatants. Overall, 3 of 13 seminomas of primary germ cell tumors showed an increased concentration of βHCG in the short-term cell culture supernatant and 6 of 7 NSGCT of primary germ cell tumors were βHCG- and/or AFP-positive. However, two patients with pathological increased serum concentrations of tumor markers had normal concentrations of tumor markers in cell culture supernatants. These results may be partially due to a small subpopulation of tumor cells that is able to produce βHCG and/or AFP due to differentiation of omnipotent cells [7, 13, 15] or selection during cell culture establishment. Finally, increased concentrations of βHCG and/or AFP were detected in 3 of 12 short-term cell cultures from residual retroperitoneal lesions after neoadjuvant chemotherapy in a prospective study. These findings may be due to the factors mentioned above. Interestingly, all three patients with positive marker expression detected in the cell culture supernatant developed early tumor progression ($P < 0.01$) although histological investigations found resected material to be fibrotic and necrotic tissue with no evidence of vital tumor for two of these three patients. Furthermore, our results demonstrate a high specificity of 100% and a sensitivity of 50% for this method in the detection of vital tumor cells in retroperitoneal lesions after chemotherapy. However, negative results obtained with the determination of βHCG and AFP in short-term cell culture supernatants do not exclude the presence of vital tumor cells with absolute certainty, as vital tumor tissue was detected in retroperitoneal lesions of three patients by histological investigations without detection of increased tumor markers in the cell culture supernatant. On the other hand, the detection of increased tumor markers in the cell culture supernatant proves the presence of vital tumor tissue, as three of three patients with increased tumor markers in the cell culture supernatant had early tumor progression (positive predictive value of 100%). Therefore, we consider the short-term cell culture assay to be a promising additional technique for the investigation of metastatic lesions of germ cell tumors and for the detection of vital tumor cells. Furthermore and in addition to standard histological investigations, it provides valuable information for the prognosis and therapeutic procedure in patients with advanced germ cell tumors after chemotherapy and RLA.

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