ErbB-2/HER-2 protein expression, serum tumour necrosis factor-α (TFM-α) and soluble tumour necrosis factor receptor-2 (TNFR-2) concentrations in human carcinoma of the uterine cervix

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Summary

The expression of erbB-2 protein (by immunohistochemistry), serum TNF-α, soluble TNF-receptor 2 (sTNFR-2, ELISA) concentrations and mitogenic (LPS, ConA, PHA) induced TNF-α production of the peripheral blood mononuclear cells (PBMNC) were studied in 91 (UICC Stage 1: 39, Stage 2: 33, Stage 3: 14, Stage 4: 5) patients with carcinoma of the uterine cervix. During a follow-up period of seven years 30 patients died (Stage 4: 5, Stage 3: 12, Stage 2: 11, Stage 1: 2). ErbB-2 protein expression was significantly more frequent in patients with UICC Stages 3-4 (14/19), and in those with fatal outcomes (14/30, $p < 0.0001$, chi-square test). Serum TNF-α (2.70 ± 0.69 pg/ml) and sTNFR-2 (3.85 ± 1.05 ng/ml) concentrations were significantly lower in cancer patients ($p < 0.0001$, Mann-Whitney test) as compared to 64 age-matched control women (TNF-α: 4.32 ± 0.36, TNFR-2: 4.85 ± 0.82). The mitogenic induced TNF-α production of PBMNC was also significantly less with all the three mitogens applied (LPS: 35.24 ± 8.84, ConA: 26.28 ± 7.81, PHA: 20.48 ± 7.04 pg/1 million of cells/24 hours, $p < 0.0001$) as compared to the controls (LPS: 65.33 ± 8.82, ConA: 51.00 ± 8.87, PHA: 41.80 ± 9.01). Serum TNF-α, sTNFR-2 concentrations and the mitogenic induced TNF-α production of PBMNC was significantly decreased in patients with erbB-2 positivity as compared to those with negativity. In conclusion the expression of the oncoprotein and the lower levels of the members of the TNF system seem to be poor prognostic parameters in patients with carcinoma of the uterine cervix.

Key words: TNF-α; TNFR-2; ErbB-2; Cervical cancer.

Introduction

ErbB-2 (also called HER-2/neu) belongs to the tyrosine kinase receptor family and regulates the expression of many genes involved in the cellular proliferation and differentiation leading to the development of different tumours [1, 2]. Recently several ligands for erbB-2 receptor have also been described [3]. The overexpression of erbB-2 correlated with the resistance to chemotherapy and poor prognosis of many cancers [4, 5, 6]. The diagnostic and prognostic significance of erbB-2 expression has also been proposed in some types of gynaecological tumours such as breast and ovarian cancer [4]. The numerous receptors and ligands of the TNF system play an important role in the regulation of the innate and adaptive immune response, metabolic processes, bone differentiation, etc. [7]. One of the most important physiological functions of the TNF-system is the regulation of apoptosis. TNF-α through one of its receptors with a death domain (TNF receptor-1, TNFR-1, 60 kD) can initiate the activation of the caspase intracellular proteolytic system and induce the cellular process of apoptosis. However the stimulation of one of its other receptors, without a death domain, TNFR-2 (80 kD) may inhibit the apoptotic process [8]. The proteolytic cleavage of the cell surface receptors, TNFR-1 and R-2, by the TNF-α cleaving enzyme (TACE, ADAM 17) results in the soluble (s) form of these receptors in the circulation with regulatory influence [9]. ErbB-2 expression may influence the activity of several transcription factors among others NF-kappaB, a potent regulator of TNF-α transcription [10] Recently it has been advanced that the expression of erbB-2 may influence the apoptotic process induced by a ligand of the TNF-system in gynaecological tumours [11]. Therefore in a 5-year follow-up study we investigated the prognostic significance of the expression of erbB-2 protein, serum concentrations of TNF-α, sTNFR-2, and mitogen stimulated TNF-α production of peripheral blood mononuclear cells in patients with carcinoma of the uterine cervix.

Materials and Methods

Ninety-one patients with carcinoma of the uterine cervix diagnosed and observed at the 2nd Department of Obstetrics and Gynaecology of Semmelweis University and the Department of Gynaecological Oncology of St. Stephen Hospital between 1995 and 2001 were included in this study after obtaining their

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informed consent. Tissue samples for immunohistochemical detection of erbB-2 protein expression was gained during the gynaecological examination (tissue samples for immunohistochemistry were obtained by the Volkmann-device in patients with UICC Stage 3-4) and surgery (Wertheim-operation was carried out in patients with UICC Stage 1-2, and tissue samples were taken from the surgically removed specimens). Tumour staging (UICC Stage 1: 39, 2: 33, 3: 14 and 4: 5) was performed by ultrasound and CT scans and histological evaluation of samples removed during the surgical intervention. Serum samples for the determination of TNF-α and sTNFR-2 from the newly diagnosed subjects were taken before the surgical procedure from patients with UICC Stage 3-4 of cervical cancer prior to irradiation. Patients with any clinical and laboratory signs of infection were excluded. Patient mortality was followed up between 1995 and 2001. The clinical data of patients and 64 healthy age-matched women as a control group is summarised in Table 1.

The expression of erbB-2 protein was detected by immunostaining on paraffin-embedded sections using a monoclonal mouse antibody to human erbB-2 (BioGenex, Mainz, Germany) in a working dilution of 1:180 as has already been described [5, 12]. The staining was evaluated semiquantitatively: no staining (score 0); less than 20% of tumour cells with positive staining (score 1); 20-50% of the cells with positive staining (score 2); more then 50% of the cells with positive staining (score 3). Fasting serum TNF-α levels were determined by the ELISA kit (inter- and intra-assay variability: 7.0 and 4.5%, respectively, sensitivity: 0.5 pg/ml, Sigma, St. Louis, USA). Fasting sTNFR-2 concentrations were also measured by the ELISA kit (BenderMedSystem, Austria, inter-assay CV: 2.0%, intra-assay CV: 1.4%, sensitivity: 0.15 ng/ml). Peripheral blood mononuclear cells (PBMNC) were isolated from heparinized venous blood of 34 patients with cervical cancer (16 with and 18 without erbB-2 protein expression) and from 30 matched control subjects on Ficoll-Hypacue density gradient by centrifugation as previously published [13]. Cell viability was checked by a trypan blue exclusion test. One million peripheral blood mononuclear cells in RPMI medium containing 10% FCS were stimulated with bacterial lipopolysaccharide (LPS, E.coli serotype 026:B6, Sigma, St.Louis, USA, 1 μg/ml final concentration), concanavaline A (ConA, Sigma, St. Louis, USA, 1 μg/ml final concentration) and phytohemaglutinine (PHA-P, Sigma, St Louis, USA, 1 mg/ml final concentration) for 24 hours on 24 well Greiner-plates in Forma Sci thermostat at 37°C, 5% CO_2. TNF-α secreted to the medium was detected by L929 cell cytotoxicity bioassay as previously described [14]. Human recombinant TNF-α (Sigma St Louis USA) was used as a standard. Anti-human mouse monoclonal neutralising TNF-α antibodies (Boehringer, Mannheim, Germany) were applied to verify the TNF-α cytotoxicity in the samples. Statistical analysis was performed by the Mann-Whitney test, linear correlation analysis (Spearman) and Chi-square test with Yates' correction. The Prism3 program was used for the analysis and graphical illustration of the results.

Table 1.

	Patients	controls	
n		91	64
age (X+SD)		48 ± 15	45 ± 12
BMI kg/m²		24.1 ± 1.8	24.2 ± 1.6
UICC stages (n)			
(died) [erbB-2+]	1	39 (2) [1]	
	2	33 (11) [2]	
	3	14 (12) [11]	
	4	5 (5) [5]	

Clinical data of patients and controls.

Results

In patients with cancer of the uterine cervix significantly decreased serum TNF-α (X±SD: 2.70 ± 0.69 pg/ml, $p < 0.0001$, Figure 1), sTNFR-2 (3.85 ± 1.05 ng/ml, $p < 0.0001$, Figure 2) concentrations were found as compared to the controls (TNF-α : 4.32 ± 0.36, sTNFR-2: 4.85 ± 0.82).

Significant positive linear correlations (Spearman) were found between serum TNF-α and sTNFR-2 levels both in cancer patients ($r = 0.3974$, $p < 0.0001$) and in the healthy controls ($r = 0.2878$, $p = 0.0211$).

The ratio of the sTNFR-2/TNF-α was significantly ($p < 0.0001$) elevated in the cancer patients (1.49 ± 0.53) as compared to the control group (1.12 ± 0.18). Serum TNF-α and sTNFR-2 concentrations were significantly lower in patients with higher [3-4] UICC stages as compared to those with lower [1-2] ones (Table 2).

Table 2.

	n	TNF-α pg/ml	TNFR-2	TNFR-2/TNF-a ratio
Cancer	91	2.70 ± 0.69*	3.85 ± 1.05*	1.49 ± 0.53*
UICC Stage 1	39	3.29 ± 0.39*	4.60 ± 0.68	1.42 ± 0.30*
Stage 2	33	2.52 ± 0.33*	3.75 ± 0.78*	1.50 ± 0.39*
Stage 3	14	1.81 ± 0.26*	2.68 ± 0.39*	1.47 ± 0.33***
Stage 4	5	1.45 ± 0.22*	1.91 ± 0.18*	1.35 ± 0.27
erbB-2 positivity	16	1.58 ± 0.38**	2.55 ± 0.57**	1.68 ± 0.49
erbB-2 negativity	75	2.82 ± 0.62	4.06 ± 0.87	1.48 ± 0.37
Controls	64	4.32 ± 0.36	4.85 ± 0.82	1.12 ± 0.18

TNFα, TNFR-2 concentrations and TNFR-2/TNF α ratio (X ± SD) in patients and controls

*$p < 0.0001$ as compared to the controls (Mann-Whitney test), **$p < 0.0001$ as compared to patients with erbB-2 negativity, ***$p = 0.0002$ as compared to the controls, TNF-α: UICC Stage 1-2: $p < 0.0001$, Stage 2-3: $p < 0.0001$, Stage 3-4: $p = 0.01$, TNFR-2: Stage 1-2: $p < 0.0001$, Stage 2-3: $p < 0.0001$, Stage 2-4: $p = 0.0002$, Stage 3-4: $p = 0.0026$.

Table 3.

	cancer (n=34)	erbB-2 pos. (n=16)	erbB-2 neg. (n=18)	controls (n=30)
LPS	35.24 ± 8.84*	29.81 ± 6.42**	40.06 ± 7.95	65.33 ± 8.82
ConA	26.26 ± 7.81*	20.00 ± 3.40**	31.21 ± 6.62	51.00 ± 8.87
PHA	20.48 ± 7.04*	14.96 ± 2.12**	25.32 ± 6.18	41.80 ± 9.01

Mitogen-induced TNF α production of PBMNC in patients and controls (X ± SD. pg/1 million cells/24 hours).
*$p < 0.0001$ as compared to the controls. **$p < 0.0001$ as compared to patients with erbB-2 negativity.

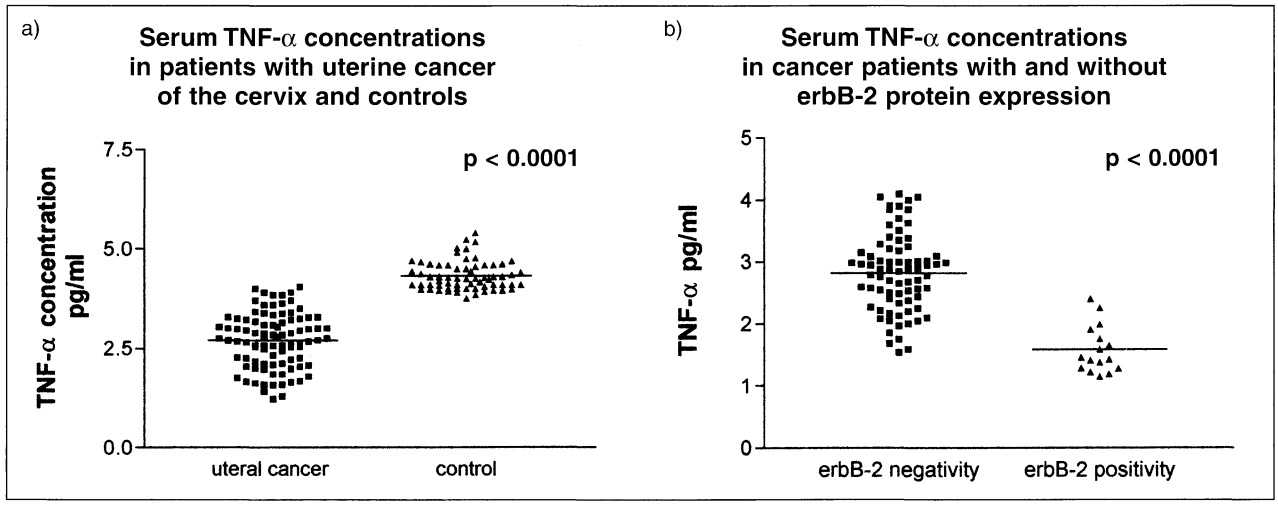

Figure 1. — a) Serum TNF-α concentrations in cancer patients and controls. b) Serum TNF-α concentrations in cancer patients with erbB-2 positivity and negativity.

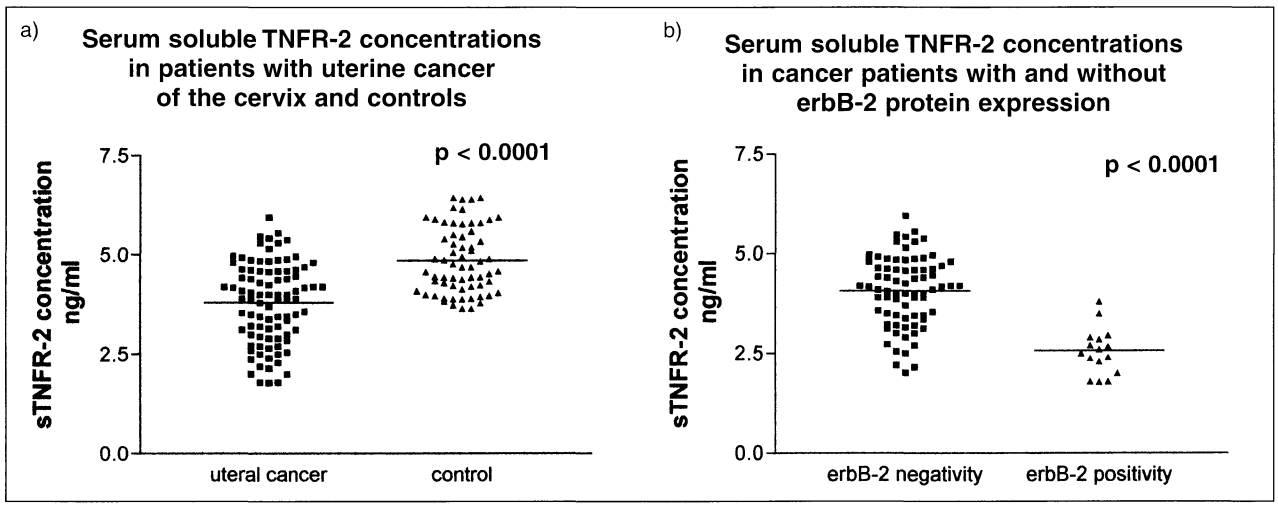

Figure 2. — a) Serum TNFR-2 concentrations in cancer patients and controls. b) Serum TNFR-2 concentrations in cancer patients with erbB-2 positivity and negativity

The mitogenic induced TNF-α production of the peripheral blood mononuclear cells (PBMNC) isolated from cancer patients was also significantly decreased as compared to the healthy controls (Table 3).

The expression of the erbB-2 protein was detected in the tumour samples of 16 patients. In two of them (both UICC Stage 4) erbB-2 protein was also detectable not only in the malignant cells but also in the histologically normal cervical cells. The erbB-2 protein expression was significantly more prominent (score: 2+, 3+) and frequent (chi-square test with Yates' correction, $p < 0.0001$) in patients with UICC Stage 3-4 (n = 14/19) compared to those with Stage 1-2 (2/72, score: 0, 1+). During a follow-up period of seven years 30 patients died. The erbB-2 protein positivity was significantly more frequent (chi-square test with Yates' correction, $p < 0.0001$) among them (14/30) compared to the surviving women (2/61, relative risk: 5.36, Odds ratio: 14.23).

The TNF-α (1.58 ± 0.38, Figure 1, Table 2) and sTNFR-2 (2.55 ± 0.57, Figure 2, Table 2) concentrations were significantly ($p < 0.0001$) lower in patients with erbB-2 positivity compared to those with negativity (TNF-α: 2.82 ± 0.62, TNFR-2: 4.06 ± 0.87).

The mitogenic induced TNF-α production of the erbB-2 positive patients was significantly lower compared to the erbB-2 negative women (Table 3).

Discussion

The expression of erbB-2 may have a prognostic significance in different tumours among others in human breast and ovarian cancer. The overexpression of the erbB-2/HER-2 gene occurs in about 30% of human

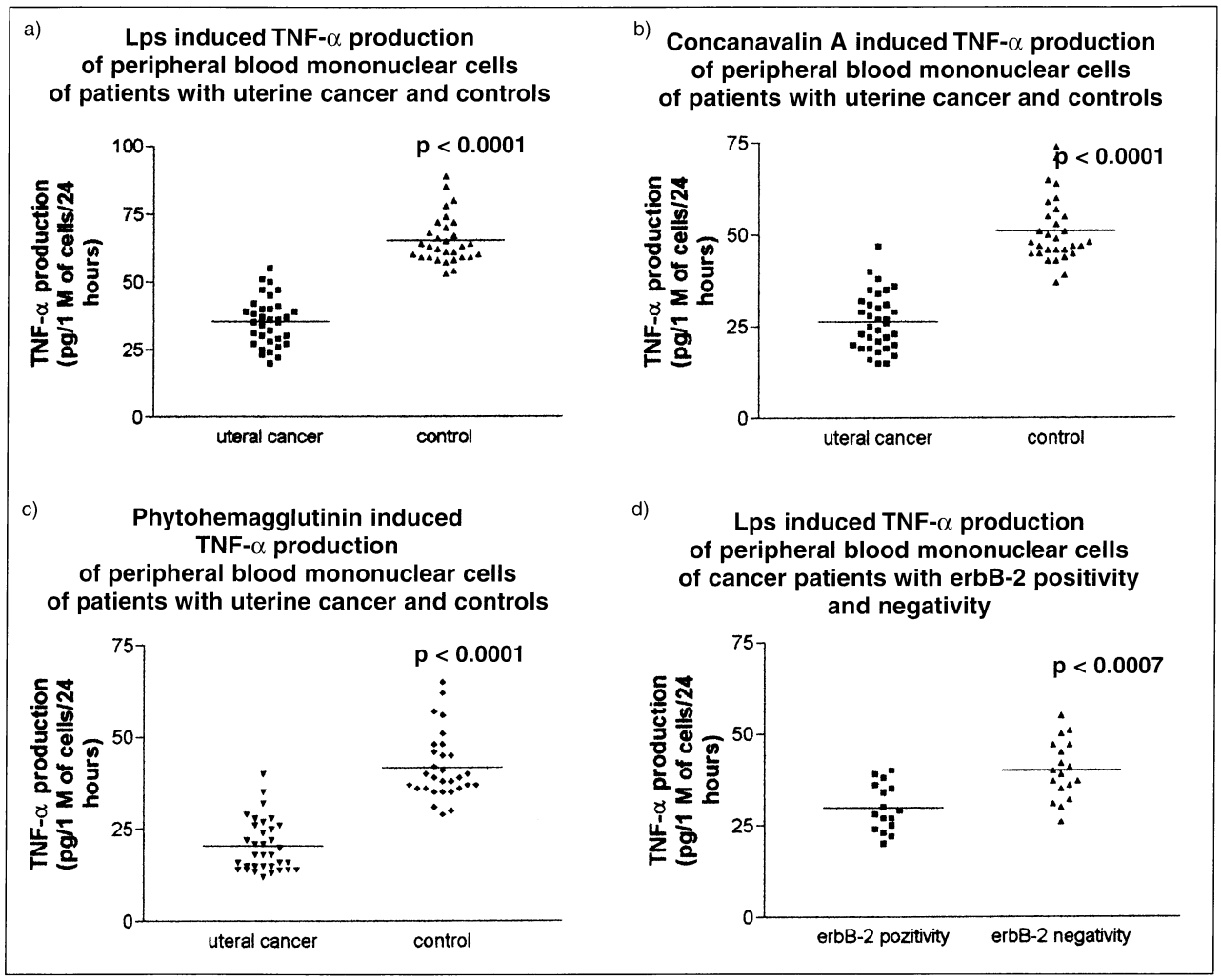

Figure 3. — Mitogenic induced TNF-α production of peripheral blood mononuclear cells isolated from patients with carcinoma of the uterine cervix and controls. a) LPS-induced TNF-α production in patients and controls. b) ConA-induced TNF-α production in patients and controls. c) PHA-induced TNF-α production in patients and controls. d) Lps-induced TNF-α production in cancer patients with erbB-2 positivity and negativity.

mammary carcinomas and ovarian cancer [15, 16]. In the minority of tumour samples the amplification of the erbB-2 gene was detected but in many other cases protein overexpression was observable. Recently immunohistochemistry has been established as a reliable method for the detection of erbB-2 expression level [17]. By applying this method to our patients with cervical cancer we detected the overexpression of erbB-2 protein in 17%, mostly in UICC Stages 3 and 4. The expression of oncoprotein was also significantly more frequent in women with fatal outcomes. The overexpression of erbB-2 protein may also influence the sensitivity of the cancer cells to TNF-α-induced apoptosis and lymphokine-activated killer cell lysis [11, 18]. Decreased cellular immunological response to the malignant cells may have a predisposing component for tumorigenesis. We observed significantly lower serum TNF-α and sTNFR-2 levels in our patients. In women with UICC Stage 1 significantly lower cytokine and receptor concentrations were measured as compared to the healthy controls. A more progressive decrease was detectable in patients with higher UICC stages. The stimulation of different subpopulations (monocytes with LPS, different subsets of T cells with ConA and PHA) of the peripheral blood mononuclear cells also revealed a decreased production of TNF-α with all the mitogens studied. These observations also support the contribution of decreased immunological reactivity in the pathogenesis of cervical cancer. In females with erbB-2 overexpression lower serum TNF-α and sTNFR-2 concentrations and mitogenic-induced TNF-α production were observed. These findings may raise the possibility that the decreased activity of the TNF system can contribute to the increased expression of erbB-2 in patients with advanced stages of cervical cancer. TNF-α mediates the production and shedding of its receptors. This is supported by the observed correlation between serum TNF-α and sTNFR-2 concentrations both in patient and control groups sTNFR-2 receptor may have a

dualistic role in the regulation of TNF-α function in the circulation. On one hand it can neutralise TNF-α in the circulation by competing with its cell surface receptors. On the other hand such binding may increase the half-life of the cytokine in the circulation. The ratio of sTNFR-2/TNF-α was increased in the cancer group suggesting a difference either in the production or in the TNF-α induced shedding of the TNFR-2 in these patients. TNFR-2 does not have an intracellular death domain and does not mediate apoptotic function therefore the relative elevation of the TNFR-2/TNF-α ratio in cancer patients compared to healthy women may also promote tumour development.

Conclusion

In conclusion we suggest a prognostic relevance of the overexpression of erbB-2 protein in connection with the decreased activity of the TNF system in cervical cancer.

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